DOI: 10.1002/jev2.12224

ISEV2022 Abstract Book

About ISEV

The International Society for Extracellular Vesicles is the leading professional society for extracellular vesicle research. ISEV's mission is advancing extracellular vesicle research globally. Our vision is to be the leading advocate and guide of extracellular vesicle research and to advance the understanding of extracellular vesicle biology.

ISEV2022 Annual Meeting

The ISEV annual meeting is the premier international conference of extracellular vesicle research, covering the latest in exosomes, microvesicles and more. With an anticipated 1,000+ attendees, ISEV2022 will feature presentations from the top researchers in the field, as well as providing opportunities for talks from students and early career researchers.

ISEV2022 International Organizing Committee

IOC Chairs: Lorraine O'Driscoll (Ireland), Sophie Rome (France)
IOC Members: Yu Fujita (Japan), Bernd Giebel (Germany), David Greening (Australia), Soazig Le Lay (France), Metka Lenassi (Slovenia), and Ken Witwer (USA)

Journal of Extracellular Vesicles: Editor in Chief

Jan Lötvall (Sweden)

Note from the International Organizing Committee

Names and affiliations of authors are as entered by the submitters. Some names of session chairs may be missing or incorrect because of last-minute declines, acceptances, or other changes.

ACKNOWLEDGMENTS

ISEV gratefully acknowledges the support of the 2022 Annual Conference from our sponsors:

PLATINUM

GOLD

SILVER

FA1: Thursday Featured Abstracts

Chair: Clotilde Théry – Institut Curie, PSL Research University, INSERM U932

Chair: Uta Erdbrügger – University of Virginia

FA1.2 | Human liver stem cell-derived extracellular vesicles interfere with the development of chronic kidney disease in an in vivo experimental model of renal ischemia and reperfusion injury

Elena Ceccotti[1]; Stefania Bruno[2]; Giulia Chiabotto[2]; Massimo Cedrino[3]; Chiara Pasquino[4]; Cristina Grange[5]; Giovanni Camussi[6]

[1]Department of medical science, University of Turin, Torino, Italy; [2]Department of medical science, University of Turin, Turin, Italy; [3]Unicyte, Torino, Italy; [4]Molecular Biotechnology Center, Turin, Turin, Italy; [5]Department of Medical Sciences, University of Turin, Turin, Italy; [6]Department of Medical Sciences, Torino, Italy

Introduction: Renal ischemia reperfusion injury (IRI) is the major cause of acute kidney injury (AKI) which can progress to chronic kidney disease (CKD).
In this study, we investigated the potential therapeutic effect of extracellular vesicles (EVs) derived from human liver stem cells (HLSCs) in an in vivo model of IRI-AKI, which subsequently develops into CKD
Methods: EVs were purified by ultracentrifugation and characterized in accordance with ISEV-guidelines, by transmission electron microscopy, flow cytometry and Western Blot.
BALB-c mice were subjected to 30 minutes IRI and EVs (1x109) were i.v. administered immediately after the surgery and three days after. To evaluate AKI, mice were sacrificed two and three days after the surgery, while to assess the development of CKD, mice were sacrificed two months after.
Renal function and histological analyses were performed using specific kits and microscopic evaluation. By real time PCR analysis, we monitored the gene expression of markers of AKI, vascular damage, cell proliferation, fibrosis development, inflammation, and epithelial to mesenchymal transition (EMT)
Results: In AKI mice, EVs attenuated kidney damage by reducing tubular necrosis, increasing tubular cell proliferation and reducing damage-related gene expression. EV-treatment down-regulated the expression levels of vimentin, TWIST, and fibrosis-related genes, while up-regulated ZO1 expression.
In CKD mice, EVs effectively reduced the development of interstitial fibrosis at the histological level, confirmed at gene expression level by reduced levels of pro-fibrotic and pro-inflammatory genes. Molecular analysis of EMT markers showed increased ZO1 expression and decreased levels of mesenchymal genes in IRI mice treated with EVs
Summary/Conclusion: The administration of HLSC-derived EVs immediately after renal IRI protects the kidney from AKI development and interferes with the development of subsequent interstitial fibrosis
Funding: Unicyte AG (Switzerland)

FA1.5 | Extracellular vesicles: a window into the etiology of Major Depressive Disorder

Pascal Ibrahim[1]; Prakroothi Danthi[2]; Jennie Yang[3]; Corina Nagy[1]; Gustavo Turecki[1]

[1]McGill University, Montreal, Canada; [2]The Hospital for Sick Children, Toronto, Canada; [3]Douglas Hospital Research Institute, Montreal, Canada

Introduction: Major Depressive Disorder (MDD) is a leading cause of disability worldwide. MicroRNA's (miRNA) are disrupted in MDD and can be packaged into extracellular vesicles (EVs), along with other bioactive molecules, such as proteins. Different cell types in the brain have been shown to release EVs, with previous implications in other brain disorders. Therefore, we

hypothesize that EV cargo from the anterior cingulate cortex, a brain region highly implicated in MDD, will have a disease specific profile that could mediate disease development.
Methods: EVs were isolated from post-mortem human brain tissue of 43 MDD subjects and 43 healthy controls. Briefly, tissue was mildly dissociated in the presence of collagenase type III. Tissue debris, large cells, and large vesicles were eliminated by centrifugation. The supernatant was then overlaid on a size exclusion column to separate small EVs according to the manufacturer's protocol. The quality was assessed by western blots, transmission electron microscopy (TEM), and microfluidic resistive pulse sensing. RNA was extracted, and a small-RNA library was constructed and sequenced using the Illumina Platform. Proteins were extracted and profiled using LC-MS/MS. Differential expression analysis was then performed and validated.
Results: Western blots showed no contamination with cellular debris and enrichment of CD9. TEM images showed cup-shaped vesicles with sizes mostly between 30 and 200 nm and labelled with CD81. Preliminary differential analyses revealed sex-specific dysregulation in miR-4485-3p, miR-142-5p, miR-33a-5p, miR-132-5p, and miR-92a-3p, as well as in proteomic profiles of the EVs in MDD.
Summary/Conclusion: This will be the first study to profile brain-derived EV miRNA and protein in the context of depression. This could provide novel mechanistic insights into the pathophysiology of MDD, which could serve as a starting point for the development of targeted therapeutic strategies as well as prevention measures.
Funding: Fonds de Recherche du Québec – Santé

FA2: Friday Featured Abstracts

Chair: Edit I. Buzás – Semmelweis University, Department of Genetics, Cell- and Immunobiology

Chair: Sophie Rome, Lyon University, Lyon, France

FA2.2 | Extracellular vesicles as efficient RIG-I agonist delivery system for anti-cancer immunotherapy

Boya Peng[1]; Trinh Mai Nguyen[2]; Migara Kavishka K. Jayasinghe[1]; Chang Gao[1]; Thach Tuan Pham[1]; Luyen Tien Vu[1]; Eric Yew Meng Yeo[1]; Gracemary Yap[1]; Lingzhi Wang[3]; Boon Cher Goh[3]; Wai Leong Tam[4]; Dahai Luo[2]; Minh T.N Le[1]
[1]*Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, Singapore;* [2]*Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, Singapore, Singapore;* [3]*Cancer Science Institute of Singapore, National University of Singapore, Singapore, Singapore, Singapore;* [4]*Genome Institute of Singapore, A*STAR, Singapore, Singapore, Singapore*

Introduction: With emerging supremacy, immunotherapy has evolved as an exciting modality of anti-cancer treatments. RNA containing 5' triphosphate can trigger cytosolic RIG-I pathway to enhance immune responses. Cancer immunotherapy composes of RIG-I agonists induces vigorous immune responses against cancer. Yet, delivery of 5' triphosphorylated RNA molecules as RIG-I agonists to tumor cells in vivo remains a challenge due to the susceptibility of these molecules to degradation. Here we describe the use of extracellular vesicles (EVs) released from red blood cells (RBCs), which are highly biocompatible and amenable to manipulations such as RNA loading and surface modification, for RIG-I agonist delivery.
Methods: RBCEVs were purified from RBCs of healthy donors by ultracentrifugation and characterized by western blot, nanoparticle tracking analysis, transmission electron microscopy and single-EV flow cytometry. We evaluated the prominent anti-tumor effect of two novel RIG-I agonists, the immunomodulatory RNA (immRNA) with a unique secondary structure for efficient RIG-I activation, and a 5' triphosphorylated antisense oligonucleotide with dual function of RIG-I activation and miR-125b inhibition (3p-125b-ASO), when delivered using RBCEVs.
Results: Delivery of immRNA and 3p-125b-ASO using RBCEVs potently activates the RIG-I pathway, and induces cell death in murine and human breast cancer cells. In the immunogenic breast cancer models, we observe significant tumor suppression upon the treatment of RBCEV-delivered RIG-I agonists. Multiple genes associated with RIG-I activation are significantly up-regulated after treatment, accompanied by increase in the abundance of tumor-infiltrating immune cells. Strikingly, the advantageous anti-tumor efficacy of immRNA is greatly enhanced upon intrapulmonary delivery using RBCEVs functionalized with EGFR-targeting nanobody that resulted in suppression of tumor metastasis and reinvigoration of tumor-specific CD8+ T cell responses in breast cancer metastasis models.
Summary/Conclusion: This study has characterized the immune-potentiating properties of immRNA and 3p-125b-ASO, and provides a new strategy of robust RIG-I agonist delivery using RBCEVs for immunotherapy against cancer and cancer metastasis.
Funding: This project is funded by the National University of Singapore (grant number NUHSRO/2019/076/STARTUP/02), and the Singapore Ministry of Education (NUHSRO/2020/108/T1/Seed-Mar/04). This research is also supported by the

Singapore Ministry of Health's National Medical Research Council under its Open Fund – Individual Research Grant (NMRC/OFIRG/0075/2018 and OFIRG20nov-0049).

FA2.5 | Cardiac progenitor cell-derived extracellular vesicles promote endothelial cell activation through both associated- and co-isolated proteins

Marieke T. Roefs[1]; Jiabin Qin[2]; Julia Bauzá-Martinez[3]; Simonides I. van de Wakker[2]; Pieter Vader[4]; Joost P.G. Sluijter[2]
[1]Department of Experimental Cardiology, University Medical Center Utrecht, The Netherlands, Utrecht, Netherlands; [2]Department of Experimental Cardiology, University Medical Center Utrecht, Utrecht University, The Netherlands, Utrecht, Netherlands; [3]Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands, Utrecht, Netherlands; [4]CDL Research, University Medical Center Utrecht, The Netherlands, Utrecht, Netherlands

Introduction: Cardiac progenitor cell (CPC)-derived EVs have been shown to protect the myocardium against ischemia/reperfusion injury via proangiogenic effects. However, the underlying mechanisms for CPC-EV-mediated angiogenesis remain elusive. Here, we investigated protein-mediated effects of CPC-EVs on the endothelium and explored EV-dependent and –independent recipient cell activation.
Methods: CPCs were stimulated with calcium ionophore (Ca ion-EVs), previously shown to influence EV release, or vehicle (control-EVs) for 24 hours and crude EVs were isolated from serum-free conditioned medium using size exclusion chromatography (SEC). EV concentration and size was assessed using nanoparticle tracking analysis and proteomic composition was profiled using mass spectrometry. Following SEC, iodixanol gradient ultracentrifugation was used to separate EVs from free proteins. CPC-EVs deficient in individual proteins were generated using CRISPR/Cas9 machinery. EV- and protein fractions were functionally characterized based on their potency to activate human microvascular endothelial cells (HMEC-1) and induce wound closure. HMEC-1 activation upon EV-delivery was determined by phosphoproteomics.
Results: HMEC-1 displayed increased wound closure and activation of PI3K-AKT-mTOR and (Insulin/IGF-) MAPK signaling pathways upon stimulation with control-EVs but not with Ca ion-EVs. MS-proteomic analysis identified multiple proteins strongly enriched in control-EVs compared with Ca ion-EVs. GO analysis of these candidate proteins revealed their involvement cell migration and –adhesion. This raised the question whether these identified proteins were truly associated to CPC-EVs, or merely co-isolated. Pure EVs isolated using iodixanol gradients lost part of their ability to activate HMEC-1 compared to crude SEC EV preparations, suggesting a co-stimulatory role of co-isolated proteins in recipient cell activation. When investigating the contribution of individual candidate proteins to CPC-EV functionality, knock-out of NID1 did not affect EV function, while knock-out of PAPP-A yielded in CPC-EVs with reduced functionality. The IGF-receptor inhibitor PPP abrogated CPC-EV-induced HMEC-1 activation, supporting the association of EV-associated PAPP-A with the activation of IGF-MAPK signaling.
Summary/Conclusion: A specific set of EV proteins including PAPP-A is identified that may be functionally responsible for the activation of endothelial cells upon exposure to CPC-EVs. It is important to identify if these proteins are EV-associated or represent co-isolated factors that contribute to endothelial cell activation. This may lead to a better mechanistic understanding of CPC-EV-mediated cell activation and translation of EV-based therapeutics.
Funding: ERC-2016-COG-725229 EVICARE grant

FA3: Saturday Featured Abstracts

Chair: Lorraine O'Driscoll – School of Pharmacy and Pharmaceutical Sciences, Trinity Biomedical Sciences Institute, and Trinity St. James's Cancer Institute, Trinity College Dublin, Dublin 2, Ireland

Chair: Bernd Giebel – Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

FA3.2 | EV-Tag: A novel synthetic reporter system to understand in vivo extracellular vesicle-mediated intercellular communication in health and disease

Marta Garcia-Contreras[1]; Kenneth W. Witwer[2]
[1]Johns Hopkins University, Baltimore, USA; [2]Department of Molecular and Comparative Pathobiology, Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, USA. Johns Hopkins School of Medicine, Johns Hopkins University,

Baltimore, MD, USA. The Richman Family Precision Medicine Center of Excellence in Alzheimer's Disease, Johns Hopkins University School of Medicine, Baltimore, MD, US, Baltimore, USA

Introduction: Extracellular vesicles (EVs) are recognized as new contributors to intercellular communication. Despite significant progress, understanding cell-specific EV distribution and function, especially in vivo, is challenging for reasons including limited abundant, reliable cell-specific markers, engineered tags that hinder interaction or change biogenesis and function, and nonspecific labeling strategies. We developed an "EV-Tag IP" method for rapid (30 min) high-purity/high-yield EV separation.
Methods: We engineered a minimal protein tag "EV-Tag" into multiple locations in surface epitopes of three EV-associated integral membrane proteins. Subcellular localization of EV-Tag was compared with classical EV markers such as Alix. EVs were characterized per MISEV2018 to confirm unperturbed release and EV localization. Single-particle interferometric reflectance imaging sensing (SP-IRIS) queried single-EV localization of key protein markers, and proteomics studies were also done. To determine whether EV-Tag would be feasible for labeling endogenous EVs in mice, we cloned the constructs into adeno-associated virus (AAV) FLEX plasmids.
Results: Based on subcellular localization (e.g., endosomal vs plasma membrane distribution) and release on EVs, we chose an optimal protein tag location for each of three EV surface proteins. Using immunoblotting, we confirmed enrichment for EV markers and depletion of contaminants. Single-particle analysis revealed that EV-Tag did not affect co-release of other EV proteins. We also used mass spectrometry to profile the proteome of isolated EV subpopulations; ~900 proteins were identified.
Summary/Conclusion: Here, we propose an innovative technology that enables exploration of cell type-specific in vitro and in vivo EV circuits in health and disease. Our tag allows: 1) rapid immunoaffinity purification to maintain small molecule content for profiling; 2) cell type-specific expression and tagging; and 3) defining recipient tissues and EV presence in complex biological fluids such as plasma. We are currently further exploring EV-tag IP for cell-type specific expression and EV content mapping in vivo.
Funding: This work was supported by Stanford Bio-X and NIH R33 MH118164.

FA3.5 | Extravesicular TIMP-1 is a non-invasive independent prognostic marker and potential therapeutic target in colorectal liver metastases

Venkatesh Sadananda Rao[1]; Christoph Kahlert[2]; Qianyu Gu[2]
[1]*Department of Visceral, Thoracic and Vascular Surgery, University Hospital Carl Gustav Carus, Technische Universität Dresden., Dresden, Germany;* [2]*Department of Visceral, Thoracic and Vascular Surgery, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany, Dresden, Germany*

Introduction: The molecular reprogramming of stromal microarchitecture by tumour-derived extracellular vesicles (EVs) is proposed to favour pre-metastatic niche formation. TIMP1 overexpression has been implicated in invasion and metastasis, although its role in the remodelling of the liver metastatic milieu remains undefined. Our study aimed to elucidate the role of extravesicular tissue inhibitor of matrix metalloproteinase-1 (TIMP1EV) in pro-invasive extracellular matrix (ECM) remodelling of the liver microenvironment to aid tumour progression in colorectal cancer.
Methods: TIMP1 expression was examined using immunohistochemistry (IHC) on a panel of 81 primary tumours (colorectal cancer; CRC) and 80 colorectal liver metastases resections (CRC liver MET). Using primary fibroblast cultures, we investigated whether CRC-derived extracellular vesicles (CRC-EVs) induced TIMP1 upregulation in the recipient cells. Using our 3D ECM remodelling assay, we evaluated whether TIMP1 upregulation in the recipient fibroblasts promotes ECM remodelling. Inhibition of TIMP1 and its effect on ECM was evaluated by western blotting and 3D ECM-remodelling assays.
Results: Immunohistochemistry revealed a high expression of stromal TIMP1 in the invasion front that was associated with poor progression-free survival in patients with colorectal liver metastases. Molecular analysis identified TIMP1EV enrichment in CRC-EVs as a major factor in the induction of TIMP1 upregulation in recipient fibroblasts. Mechanistically, we proved that EV-mediated TIMP1 upregulation in recipient fibroblasts induced ECM remodelling. This effect was recapitulated by human serum-derived EVs providing strong evidence that CRC release active EVs into the blood circulation of patients for the horizontal transfer of malignant traits to recipient cells. Moreover, EV-associated TIMP1 binds to HSP90AA, a heat-shock protein, and the inhibition of HSP90AA on human-derived serum EVs attenuates TIMP1EV-mediated ECM remodelling, rendering EV-associated TIMP1 a potential therapeutic target. Eventually, in accordance with REMARK guidelines, we demonstrated in three independent cohorts that EV-bound TIMP1 is a robust circulating biomarker for non-invasive, preoperative risk stratification in patients with colorectal liver metastases.

Summary/Conclusion: These results demonstrate the role of CRC-derived TIMP1EV as ECM modulators, hence suggesting the targeting of TIMP1 as a potential avenue for the prevention of liver metastasis in the future.
Funding: This work was funded by the Roland-Ernst-Foundation, Dresden (project no. 5/15) and Deutsche Forschungsgemeinschaft (DFG) (KA 3511/3-1).

Oral Presentations

OT01: EV Biogenesis

Chair: Guillaume van Niel – Institute of Psychiatry and Neurosciences of Paris, INSERM U1266, France

Chair: Rania Ghossoub – Cancer Research of Marseille - Aix-Marseille University

OT01.01 | Selective mechanisms controlling the biogenesis and loading of Rab11a-exosomes and other stress-induced vesicles

Deborah C. Goberdhan[1]; Shih-Jung Fan[1]; John Mason[1]; Adam Wells[1]; Pauline Marie[1]; Claudia Mendes[1]; Chris Cunningham[2]; Adrian Harris[3]; Clive Wilson[1]
[1]Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom; [2]Nuffield Department of Surgical Sciences, University of Oxford, Oxford, United Kingdom; [3]Department of Oncology, University of Oxford, Oxford, United Kingdom

Introduction: We have shown that recycling endosomes marked by Rab11a generate exosome subtypes, which we collectively term Rab11a-exosomes, distinct in cargos and functions from those generated in late endosomes. These exosomes are preferentially released from cancer cells in response to metabolic stress and promote adaptive changes in a xenograft model. Here we investigate mechanisms regulating their selective biogenesis and loading.
Methods: Comparative mass spectrometry and miRNA array analysis of small extracellular vesicle (sEV) preparations from HCT116 colorectal cancer cells, with and without Rab11a-exosome enrichment, were used to identify changes in sEV cargoes. Follow-up studies included knockdowns of candidate regulators in HCT116 cells and also in Drosophila secondary cells, which permit in vivo visualisation of Rab11a-exosome biogenesis using fluorescence microscopy.
Results: Our data in both cancer and fly cell models highlights the selective and conserved role of the accessory ESCRT-III proteins, in generating Rab11a-exosomes, but not in the trafficking of ubiquitinated cargos into late endosomes and lysosomes. Furthermore, our studies suggest that the post-translational modification, SUMOylation, is specifically involved in loading Rab11a-exosome cargoes, like the RNA-binding protein, hnRNPA2B1, which is enriched in Rab11a-exosomes, together with miRNAs that it binds.
Summary/Conclusion: We conclude that stress-regulated, pro-tumorigenic Rab11a-exosome subtypes are formed and loaded via different mechanisms to late endosomal exosomes. Rab11a-exosome cargos, therefore, warrant further investigation in clinical samples as tumour biomarkers. They might also be selectively targeted, for example by anti-SUMOylation therapies, which are currently used in ongoing cancer clinical trials.
Funding: Cancer Research UK (CRUK) Programme Award (C19591/A19076), the CRUK Oxford Centre Development Fund (C38302/A12278), CRUK grant (C2195/A25190) through a CRUK Oxford Centre Clinical Research Fellowship, BBSRC (BB/R004862/1), John Fell Fund, Oxford and the Wellcome Trust (MICRON; #091911, #107457).

OT01.02 | The closed interplay between lysosomes, MVBs and autophagosomes regulate CDDP-chemoresistance through a balance between lysosomal degradation and exosome secretion in ovarian cancer cells

Cristóbal Cerda-Troncoso[1]; Eloisa Arias-Muñoz[1]; Belen Gaete-Ramirez[2]; Viviana A. Cavieres[1]; Francisca Álvarez-Astudillo[2]; Matías Ostrowski[3]; Manuel Varas-Godoy[4]; Patricia V. Burgos[5]
[1]Organelle Phagy Lab, Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Lota 2465, Providencia, Santiago, Chile., Santiago, Chile; [2]Cancer Cell Biology Laboratory, Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Lota 2465, Providencia, Santiago, Chile., Santiago, Chile; [3]INBIRS, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, C1121 ABG, Buenos Aires,

Argentina., Buenos Aires, Argentina; [4]Cancer Cell Biology Laboratory, Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Santiago, Chile., Las Condes, Santiago, Chile; [5]Organelle Phagy Lab, Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Lota 2465, Providencia,Santiago, Chile, Santiago, Chile

Introduction: Ovarian cancer (OvCa) is an aggressive disease treated usually with cisplatin (CDDP) which developed chemoresistance, where exosomes play a central role. Exosomes are formed as intraluminal vesicles (ILVs) inside of multivesicular bodies (MVBs), organelles that fused with the plasma membrane to release exosomes or with lysosomes for degradation, processes regulated by RAB27A or RAB7A respectively. It is known that lysosomal dysfunction and amphisomes formation (MVB/autophagosome hybrid organelle) facilitate exosome secretion. However, the contribution of these organelles to a balance between lysosome degradation and exosome secretion that regulate OvCa CDDP-chemoresistance is unknown.
Methods: We study lysosomes, MVBs, and amphisomes in CDDP-sensitive (A2780) and CDDP-resistant (A2780cis) OvCa cells by several molecular cell biology, and biochemical strategies.
Results: Our data show that A2780cis have a reduced number and activity of lysosomes and a reduction in RAB7A levels. Contrary, we found that A2780cis have an increased number of ILVs/MVBs and amphisomes-like structures. Additionally, A2780cis show high RAB27A and ESCRTs levels, compared to A2780. Together, these findings suggest exosome secretion could be involved in CDDP-resistance. The interruption of exosomes secretion in A2780cis cells with RAB27A silencing rescues lysosomal function and CDDP-sensibility. Moreover, due to the increase in amphisomes-like structures, we performed FIP200 silencing in A2780cis cells to impair its formation. We found that the reduction of amphisomes decreased RAB27A and increased RAB7A levels, promoting a rescue in the lysosomal function.
Summary/Conclusion: We uncovered a relationship between lysosomes, MVBs, and autophagosomes that would stabilize a balance between lysosomal degradation and exosome secretion through RAB7/RAB27A levels and then could be new alternatives to revert CDDP-chemoresistance in OvCa.
Funding: FONDECYT No.1171649/No.1190928, ANID-BASAL No.FB210008/No.ACE210009.

OT01.03 | Role of flotillins in exosome/extracellular vesicle production and perturbation of cellular functions

Cécile GAUTHIER-ROUVIERE[1]; Stéphane BODIN[2]; Franck COMUNALE[3]; Mélanie BURETTE[3]; Himanshu MALHOTRA[4]; Lisa FONTAINE-BODIN[5]; Damien PLANCHON[2]; Sylia CHEHADE[3]
[1]INSERM, MONTPELLIER, France; [2]Montpellier University, MONTPELLIER, France; [3]CNRS, MONTPELLIER, France; [4]Upstate medical university, Syracuse, USA; [5]French government, MONTPELLIER, France

Introduction: Flotillins 1 and 2 are two ubiquitous, highly conserved homologous proteins that assemble to form heterotetramers at the cytoplasmic face of the plasma membrane (PM) in microdomains enriched in cholesterol and sphingolipids. They scaffold membrane microdomains - where different proteins concentrate - thanks to their oligomerization property.
Flotillin 1 and 2 are upregulated in a large number of cancers, which is associated with a poor prognosis (Doi:10.1007/s10555-020-09873-y). Flotillin upregulation is necessary and sufficient for the acquisition of invasive cell properties. The upregulation of flotillins induces the Upregulated Flotillin-Induced Trafficking (UFIT) pathway, an endocytosis pathway that targets cargo proteins in late endosomes (LE) positive for CD63 and LAMP-1, where flotillins accumulate. These flotillin-enriched LE are not degradative and contain numerous intraluminal vesicles, their fusion with the PM allows the recycling of the proteins present in their outer membrane and the release of the exosomes/extracellular vesicles (EVs) they contain in the extracellular medium.
Methods: Two EV purification methods (sequential centrifugation followed by sucrose gradient and SEC), NTA, EM.
Generation and caracterization of Nluc-CD9, -CD63 and -Hsp70-expressing non-tumoral MCF10A cell lines (with endogenous or upregulated flotillin levels)
Functional assays (extracellular matrix degradation, inter-cellular adhesion) with purifed EV
Results: We showed that flotillin upregulation in epithelial mammary (MCF10A) and mesenchymal myoblastic (C2C12) cells increases the number of secreted EVs. To identify the mechanisms, we performed a siRNA-based screen with the Nluc-reporter cell lines descrbed.
These EVs contain cargos of this UFIT pathway such as MT1-MMP and their addition to cells potentiate their degradation activity. They also contain adhesion molecules and they could disrupt intercellular adhesion when added to cells.
Summary/Conclusion: Upregulated-flotillins are involved in the deregulation of endocytosis and vesicular trafficking that impacts on the level of secreted EVs involved in tumoral invasive progression.
Funding: INCa PLBio 2020-101

OT01.04 | The adaptor protein CD2AP controls the biogenesis of endosomal-derived small extracellular vesicles

Kerstin Menck[1]; Barnabas Irmer[2]; Naga Sailaja Imjeti[3]; Guido David[3]; Pascale Zimmermann[4]

[1]Dept. of Medicine A, Haematology, Oncology, and Pneumology, University Hospital Münster, Münster, Germany; [2]Dept. of Medicine A, Haematology, Oncology, and Pneumology, University Hospital Münster, Muenster, Germany; [3]Centre de Recherche en Cancérologie de Marseille, Marseille, France; [4]K. U. Leuven, Leuven, Belgium

Introduction: Extracellular vesicles (EVs) can be divided into several subpopulations that differ in size, cargo, biogenesis, and function. The main EV populations recognized at present are large EVs (diameter 150-1000 nm) and small EVs (sEVs) (diameter 50-150 nm). While large EVs are directly secreted from the plasma membrane, sEVs bud into the lumen of late endosomal compartments and are released into the extracellular space upon fusion of the endosomes with the cell membrane. Recently, the intracellular adaptor protein CD2AP was found to be involved in the early steps of sEV biogenesis by regulating the endocytosis of the exosomal cargo protein GPRC5B. However, whether CD2AP also influences other steps of the sEV biogenesis pathway is currently unknown.

Methods: We modulated CD2AP levels in human MCF-7 breast cancer cells by knockdown/overexpression and analysed the different steps of the endosomal pathway by confocal microscopy. Corresponding sEVs were isolated by differential ultracentrifugation and characterized by nanoparticle tracking analysis and immunoblot.

Results: CD2AP knockdown reduced the expression of two well-known marker proteins, Syntenin and CD63, on sEVs. Consistently, overexpression of CD2AP increased exosomal Syntenin and CD63 levels. In order to understand the molecular mechanism underlying these observations, we analysed the endosomal trafficking of CD63. Confocal microscopy revealed that upon CD2AP knockdown, CD63 showed an increased co-localisation with the late endosomal marker LAMP2 indicating that CD2AP is important for CD63 trafficking through this compartment. Using overexpression of mutant Rab5Q79L, which induces the formation of enlarged endosomes, we showed that CD2AP regulates the budding of CD63 and Syntenin at endosomal membranes. Using RNAi and different inhibitors, we placed the function of CD2AP downstream of SRC, and upstream of ARF6 and its effector PLD2. CD2AP has been described as a direct interactor of ALIX, which could link CD2AP to the Syntenin pathway that is crucial for the biogenesis of sEVs. Indeed, the knockdown of ALIX antagonized the effects of CD2AP on endosomal budding and sEV cargo.

Summary/Conclusion: Taken together, our results identified CD2AP as a novel regulator of trafficking and budding processes at late endosomal compartments and shed further light on the process of sEV biogenesis.

OT01.05 | ER Membrane Contact Sites support endosomal small GTPase conversion required for exosome secretion

Frederik J. Verweij[1]; Maarten Bebelman[2]; Mickael Couty[3]; Anaïs Bécot[4]; Roberta Palmulli[5]; Xavier Heiligenstein[6]; Julia Sires-Campos[6]; Graça Raposo[6]; Dirk M. Pegtel[2]; Guillaume Van Niel[4]

[1]Utrecht University, Utrecht, Netherlands; [2]Amsterdam University Medical Centers, Amsterdam, Netherlands; [3]Université de Paris, Institute of Psychiatry and Neuroscience of Paris (IPNP), INSERM U1266, F-75014 Paris, France, paris, France; [4]Université de Paris, Institute of Psychiatry and Neuroscience of Paris (IPNP), INSERM U1266, F-75014 Paris, France, Paris, France; [5]Department of Pathology, University of Cambridge, Cambridge. CB2 1QP. United Kingdom, Cambridge, United Kingdom; [6]Institut Curie UMR144, PSL Research University, Paris, France

Introduction: Exosomes correspond to Extracellular Vesicles (EV) that are secreted from intracellular compartments. They are generated as intraluminal vesicles within endosomes of multivesicular appearance (so-called Multivesicular Bodies or MVBs) of still elusive identity. Exosomes are then secreted when MVBs fuse with the plasma membrane in a process orchestrated by small GTPases. However, the molecular events regulating the acquisition of fusogenic capacity of these compartments are poorly known.

Methods: We combined various live-imaging approaches including a recently developed CD63-based quantitative dual-color TIRF single cell assay with small molecule inhibitors and biochemical analysis to establish the profile/identity of MVBs fusing with the plasma membrane and interrogate modulators of MVB motility and maturation. In particular, we investigated the role of Membrane Contact Sites between Late Endosomes and the Endoplasmic Reticulum using mutant versions of ORP1L, a key regulator of these contacts.

Results: We identified the compartment of origin of CD63 positive exosomes as a subclass of late endosomes at pre-lysosomal stage that do not display proteolytic activity. These compartments undergo a Rab7a/Arl8b/Rab27a GTPase cascade that allows them to fuse with the plasma membrane. Dynamic Membrane Contact Sites between Late Endosomes and the Endoplasmic

Reticulum via ORP1L have the distinct capacity to modulate this process by affecting Late Endosomes motility but also their maturation state by regulating small GTPase association.

Summary/Conclusion: Thus, CD63 positive exosomes originate from late endosomes that have not yet fused with lysosomes and acquire fusogenic capacity through a multi-step process of GTPase switching regulated by Membrane Contact Sites. All in all, our work highlights the Endoplasmic Reticulum and Membrane Contact Sites as new regulators of exosome-mediated intercellular communication.

Funding: EMBO ALTF 1383-2014 and PJA 20161204808) to F.J.V.
Systems STAR Graduate Program grant 022.005.031 and a Cancer Center Amsterdam travel grant to M.P.B
KWF-5510 and a Cancer Center Amsterdam–VU University Medical Center grant to D.M.P
AJE20160635884 to G.V.N
INCA N°2019-125 PLBIO19-059 to F.J.V. and G.V.N.

OT01.06 | Luminescence-based quantification of CD63-containing extracellular vesicle release reveals a role for PI4KIIIβ in bafilomycin-induced exosome secretion

Maarten P. Bebelman[1]; Caitrin Crudden[2]; Katinka Langedijk[2]; Steven Eleonora[2]; Leontien Bosch[2]; Frederik J. Verweij[3]; Margarida Viola[4]; Jan Paul Bebelman[5]; Guillaume van Niel[6]; Martine J. Smit[7]; Dirk M. Pegtel[8]
[1]Amsterdam UMC, Dresden, Germany; [2]Amsterdam UMC, Amsterdam, Netherlands; [3]Utrecht University, Utrecht, Netherlands; [4]University Medical Center Utrecht, Utrecht, Netherlands; [5]VU University Amsterdam, Uithoorn, Netherlands; [6]Institute of Psychiatry and Neuroscience of Paris (IPNP), INSERM U1266 Université de Paris Paris France, Paris, France; [7]VU University Amsterdam, Amsterdam, Netherlands; [8]Amsterdam University Medical Centers, Amsterdam, Netherlands

Introduction: Dysregulated extracellular vesicle (EV)-mediated signaling is implicated in various pathological conditions. Despite the therapeutic potential of inhibiting EV secretion, little is known about the mechanisms that drive EV biogenesis. Technical difficulties associated with studying EV secretion have precluded large-scale screening for modulators of EV secretion in general and exosome secretion from internal compartments in particular. While CD63-based bioluminescent reporters enable scalable quantification of EV secretion, their forced overexpression influences EV biogenesis and cargo sorting.

Methods: We used CRISPR-Cas9 technology to generate HEK293 cell lines that express the bioluminescent reporter HA-Nanoluc-CD63 at endogenous levels, circumventing disadvantages associated with CD63 overexpression.

Results: Under basal culture conditions, HEK293 cells secrete CD63+ EVs through a mechanism that is independent of the exocytic SNARE protein SNAP23, suggestive of direct budding from the plasma membrane. To induce exosome secretion, we stimulated cells with the vATPase inhibitor bafilomycin and performed a broad-spectrum kinase inhibitor screen. Inhibitors of the lipid kinase PI4KIIIβ were identified as hits and validated by western blot and nanoparticle tracking analysis. siRNA-mediated knockdown and CRISPR knock-out of PI4KIIIβ confirmed its role as a major mediator of bafilomycin-induced exosome secretion. Ongoing studies into the mechanism of PI4KIIIβ-mediated exosome secretion hint at the involvement of non-canonical autophagy.

Summary/Conclusion: This study demonstrates a role for the lipid kinase PI4KIIIβ in exosome secretion and highlights the potential of HA-NanoLuc-CD63 as a versatile screening tool to study exosome biogenesis under basal and pathological conditions.

Funding: This work was supported by grants to D.M. Pegtel by the Dutch Cancer Society (KWF Unique High Risk Project 2017-2; 11308) and an NWO (AIMMS STAR Graduate Program grant 022.005.031) to M.P. Bebelman.

OT02: EV Engineering For Use as Delivery Tools

Chair: Samir El Andaloussi – Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

Chair: Paolo Arosio – Institute for Chemical and Bioengineering, ETH Zurich, Zurich, Switzerland

OT02.01 | Extracellular vesicle delivered RNA directed transcriptional gene silencing

Surya Shrivastava
CITY OF HOPE National Medical Center, Duarte, USA

Introduction: Extracellular vesicles (EV) are excellent delivery vehicle for various genetic therapeutics as they are relatively inert, non-immunogenic, biodegradable, biocompatible and can transit across the blood brain barrier. However, packaging therapeutic modalities into EV has proven challenging. While small interfering RNAs and mRNAs have been shown to be packaged into EV and delivered to target cells, the ability of EV to package and deliver nuclear functional non-coding RNAs that can modulate epigenetic and transcriptional states to target cells has remained unknown.
Methods: HEK293T cells were transfected and EVs were enriched from supernatant of transfected cells using Izon qEV size exclusion columns. Presence of EVs were confirmed using transmission electron microscope and quantitated using Nanosight Tracking Analysis. Real time quantitative PCR was used to confirm the loading of RNA into EV and analyze their functional effect on recipient cells. Acute toxicity and inflammatory response after non-targeting RNA loaded EV were read from C57BL/6 mice using multiplex cytokine panel. We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV210296)
Results: Efficient packaging and delivery of reporter nano-Luciferase (nLuc) mRNA, reporter EGFP targeting antisense EGFP (asEGFP) non-coding RNA and the promoter targeting PTENpg1 asRNA α (Pg1asα) which is a known epigenetic regulator of PTEN expression was achieved. Toxicity and inflammatory response of these engineered EVs were also evaluated in vivo and were found to be insignificant. We report here that nuclear functional lncRNAs can be engineered and re-routed to EVs, delivered to recipient cells, and functionally modulate gene transcription in the recipient cells.
Summary/Conclusion: Here, we generated a method for packaging nuclear functional long non-coding RNAs into extracellular vesicles and show that this system can deliver epigenetic regulatory non-coding RNAs to target cells and transcriptionally control the targeted gene expression. The novel approach presented here represents an effective way to deliver therapeutic long non-coding RNAs to epigenetically modulate gene transcription.
Funding: This work was supported by NIMH R01 113407-01 to Dr Kevin Morris

OT02.02 | Loading proteins into microalgal extracellular vesicles to camouflage allergens for specific immunotherapy

Estella Rao[1]; Angela Paterna[1]; Valeria Longo[2]; Noemi Aloi[2]; Giorgia Adamo[3]; Alessandra Longo[2]; Vincenzo Martorana[4]; Rosina Noto[1]; Sabrina Picciotto[5]; Samuele Raccosta[1]; Daniele Romancino[6]; Valeria Vetri[7]; Paolo Colombo[2]; Antonella Bongiovanni[6]; Mauro Manno[1]

[1]Cell-Tech HUB at Institute of Biophysics (IBF), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy; [2]Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy; [3]Cell-Tech HUB at Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo 90146, Italy, Palermo, Italy; [4]Institute of Biophysics (IBF), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy; [5]Cell-Tech HUB at Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo, Italy; Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy, Palermo, Italy; [6]Cell-Tech HUB at Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy; [7]Department of Physics and Chemistry, University of Palermo, Palermo, Italy, Palermo, Italy

Introduction: Extracellular vesicles (EVs) have a high potential as drug delivery systems due to their intrinsic capability to vehicle biological materials and information. Beyond the transport of small drugs and therapeutics, a specific need consists to load nanoparticles and macromolecules with large size, including antibodies and other proteins. Here, we address this still challenging perspective and we set up a procedure to load proteins into EVs. In particular, we use nanoalgosomes, that are biocompatible, sustainable and green EVs derived from microalgae and thoroughly characterized in our previous work, and a recombinant calcium-binding protein (CBP), a minor allergen from Parietaria Judaica (Pj).
Methods: Nanoalgosomes are isolated from the marine microalgae Tetraselmis chuii by tangential flow filtration and characterised in accord to MISEV2018 guidelines by different physicochemical techniques. Proteins are loaded by extrusion and loaded EVs are then purified by affinity and size exclusion chromatography. Loaded proteins are detected by fluorescent labeling and immunoblotting.
Results: We implement an efficient method to load CBP into nanoalgosomes and to purify loaded EVs. Fluorescence microscopy shows proteins and EVs colocalisation; specific immunoblotting demonstrates that loaded proteins are detectable only after EVs lysis but are masked by intact nanoalgosomes.
Summary/Conclusion: We developed a biotechnological approach to load proteins into EVs, which is a not trivial outcome due to protein large size. Further, we showed that nanoalgosomes may be efficiently exploited also to carry large macromolecules. The camouflage of an allergen demonstrated in the present study addresses an unmet need in the treatment of allergies, that is the possibility to present allergens in a controlled manner and without side effects.
Funding: VES4US and BOW projects funded from the H2020-EU programmes under grant agreements N 801338 and N 952183.

OT02.03 | Extracellular-vesicle mediated delivery of CRISPR/Cas9 by targeted modular aptamer-based loading and UV-activated cargo release

Omnia M. Elsharkasy[1]; Olaf L. Cotugno[1]; Ingmar Y. de Groot[1]; Willemijn S. De Voogt[1]; Jerney J. J.J.M. Gitz-François[1]; Raymond M. Schiffelers[2]; Pieter Vader[3]; Olivier G. de Jong[4]
[1]CDL Research, University Medical Center Utrecht, The Netherlands, Utrecht, Netherlands; [2]University Medical Center Utrecht, Utrecht, The Netherlands, Utrecht, Netherlands; [3]CDL research, University Medical Center Utrecht, Utrecht, The Netherlands, Utrecht, Netherlands; [4]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands., Utrecht, Netherlands

Introduction: CRISPR/Cas9 is a prokaryotic endonuclease capable of targeting and editing genomic sequences with high specificity and efficiency. As such, CRISPR/Cas9 holds tremendous therapeutic potential for the treatment of genetic pathologies. One of the major hurdles for the development of CRISPR/Cas9-based therapeutics is the intracellular delivery of the Cas9-sgRNA ribonucleoprotein (RNP) complex because of its large size, negative charge, and immunogenicity. Extracellular vesicles (EVs) hold the potential to overcome this hurdle due to their biocompatibility and intrinsic capability of highly efficient intercellular transfer of RNA molecules and proteins.
Methods: To facilitate targeted loading of the RNP complex, sgRNAs with high-affinity MS2 coat protein-interacting aptamers were generated and expressed alongside Cas9 and EV-enriched proteins fused to the MS2 coat protein. The MS2 coat protein, lacking the Fg loop to prevent capsid formation, was cloned in tandem on the N-terminus of CD9, CD63, CD81 and ARRDC1 or the C-terminus of Δ687-PTGFRN or a myristoylation sequence, linked by a UV-sensitive photocleavable protein (PhoCl). Cas9 loading and UV-mediated PhoCl cleavage were measured by Western Blot analysis. To study Cas9 delivery, we used a previously published fluorescent stoplight reporter system which is activated by Cas9 activity (De Jong et al, Nat Commun. 2020).
Results: EV loading of Cas9, as well as UV-mediated cleavage of the PhoCl fusion proteins, was confirmed by Western Blot analysis. Using EVs with MS2-PhoCl-CD63 fusion proteins we observed efficient Cas9 delivery (14.5%), but only after UV-treatment of EVs and co-expression of the VSV-G glycoprotein. Comparing RNP delivery efficiency using various EV-targeted fusion proteins revealed that CD9 and the myristoylation sequence showed notably high delivery of Cas9, followed by CD63, Δ687-PTGFRN, CD81, and lastly ARRDC1. Western Blot analysis revealed that these results strongly correlated to Cas9 loading in EVs.
Summary/Conclusion: Here, we describe a novel modular platform for EV-mediated loading and delivery of Cas9 RNPs. Our results demonstrate that EVs are indeed capable of functional Cas9-RNP delivery and that Cas9 loading and delivery was strongly dependent on the targeted loading protein that was employed. Moreover, these data indicate that additional modifications for regulated cargo release and endosomal escape strongly increase Cas9-RNP delivery.
Funding: This work was supported by the Dutch Research Council (NWO) VI.Veni.192.174.

OT02.04 | EV-encapsulation of CRISPR-Cas9 modulated by N-myristoylation

Joseph Whitley; Houjian Cai
University of Georgia, Athens, USA

Introduction: Extracellular vesicles (EVs) have recently been co-opted as vehicles for the delivery of therapeutics, including the gene editing tool CRISPR-Cas9 (Cas9), and are now being modified for greater efficacy. Efficacy of EV-based delivery is determined, in part, by the loading of cargo into EVs, an area of particular concern for the 160 kD protein Cas9. N-myristoylation is a co- and post-translational modification known to translocate Src kinase to cell membranes. We reasoned that fusion of the N-terminus of Src to Cas9 may increase membrane localization where endogenous EV-loading occurs, subsequently enriching Cas9 in EVs and increasing gene editing in EV-treated recipient cells.
Methods: An octapeptide derived from the N-terminus of Src was fused to spCas9, creating modified spCas9 (mCas9). mCas9/sgRNA DNA constructs with EGFP-targeting sgRNA (mCas9/sgRNA-EGFP) were transfected into 293T cells to produce EVs encapsulating mCas9/sgRNA-EGFP. EVs were concentrated from conditioned media via differential centrifugation and filtration. Various characteristics of our EVs were described in accordance with the MISEV2018 guidelines, including density, size distribution, morphology, and protein/RNA content. We used flow cytometry analysis and T7 endonuclease I assay to evaluate gene editing. We determined off target effects with RNA sequencing.
Results: Fusion of the Src octapeptide to Cas9 induced N-myristoylation, which did not significantly alter Cas9 function. Encapsulation of mCas9 and sgRNA-EGFP into EVs (small EVs according to fractions obtained by iodixanol gradient) was confirmed

with proteinase K and RNase A protection assays, respectively. We estimated that mCas9 accounted for 0.7% of total EV protein. Levels of Cas9 protein in recipient cells increased up to 8 hours post-EV-treatment. Up to 45% EGFP knockout occurred, based on fluorescent microscopy, flow cytometry, and T7 endonuclease I assay. RNA sequencing suggested minimal off target effects.

Summary/Conclusion: Our modified Cas9 is enriched in EVs and functional in recipient cells while causing no significant changes in off target RNA expression. These results provide a proof of concept for the utilization of endogenous N-myristoylation as a process to increase the efficacy of EV-delivered therapeutics.

OT02.05 | Fluorescent-labelled EVs to ease their quantification and tracking as a tool for manufacturing bioprocess development

Maria José Sánchez[1]; Pau Leivar[2]; Salvador Borrós[1]; Cristina Fornaguera[3]; Martí Lecina i Veciana[4]

[1]GEMAT- Grup Enginyeria de Materials. Institut Químic de Sarrià. Universitat Ramon Llull, Barcelona, Spain; [2]GQBB- Grup de Química Biològica i Biotecnològica. Institut Químic de Sarrià, Universitat Ramon Llull, Barcelona, Spain; [3]GEMAT- Grup Enginyeria de Materials. Institut Químic de Sarrià. Universitat Ramon Llull; Cancer Drug Resistance Group, IPATIMUP—Institute of Molecular Pathology and Immunology and i3S—Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Barcelona, Spain; [4]GEMAT- Grup d'Enginyeria de Materials, Institut Químic de Sarrià, Universitat Ramon Llull, Barcelona, Spain

Introduction: EVs have recently emerged as promising therapeutic delivery systems due to their cellular origin, nanoscale size and their intrinsic function in intercellular communication. However, challenges like EVs detection and quantification, with minimal disturbance of their properties remains as a key issue. Thus, EVs bioprocessing development and tracking are costly and time consuming.

Methods: A549 and HEK293 cells were modified to express fused CD63-eGFP and CD9-mCherry. Bicistronic and tricistronic vectors were constructed to generate a library of plasmids to stably express one/both fluorescent transpanins. Fused proteins expression was analysed by flow cytometry (FC), and fluorescent and confocal microscopy. Labelled EVs were isolated by sequential centrifugation, quantified, and analysed by western blot, nanoparticle tracking analysis (NTA), FC and fluorometry.

Results: Fluorescent EVs expressing cells were analyzed under fluorescent and confocal microscopy, showing a dot pattern into the cytoplasm and in the cellular membrane. After cells selection, almost 100% of cells were positive by FC. Labelled-EVs were measured after isolation and directly form supernatant from high cell density cultures, by NTA, FC and fluorometry. Linear correlation was observed when plotting NTA fluorescent analysis versus fluorometry measurements, allowing a straight quantification method. Additionally, labelled-EVs uptake by recipient cells was measurable by FC, reaching over 80% of fluorescent wild type cells after 4h of incubation with 9·108 labelled-EVs/ml.

Summary/Conclusion: Easy and cheap quantification methods can be used for labelled EVs quantification. EVs in vitro tracking and uptake is possible without the need of additional reagents and EVs treatment. Fluorescently labelled EVs are useful for accelerating manufacturing bioprocess development.

Funding: Secretaria de Recerca i Universitats de la Generalitat de Catalunya and Universitat Ramon Llull, ref. 2021-URL-Proj-21

OT02.06 | Targeting of Extracellular Vesicles via de novo Antigen Binding CD81

Madhusudhan Bobbili[1]; Stefan Vogt[2]; Gerhard Stadlmayr[3]; Katharina Stadlbauer[3]; Jørgen Kjems[4]; Florian Rüker[5]; Gordana Wozniak-Knopp[5]; Johannes Grillari[6]

[1]Ludwig Boltzmann Institute for Traumatology, Vienna, Austria; [2]acib GmbH (Austrian Centre of Industrial Biotechnology), Petersgasse 14, A-8010 Graz, Austria, Vienna, Austria; [3]Christian Doppler Laboratory for Innovative Immunotherapeutics, Department of Biotechnology, University of Natural Resources and Life Sciences (BOKU), Vienna, Muthgasse 18, A-1190 Vienna, Austria, Vienna, Austria; [4]Interdisciplinary Nanoscience Center, Aarhus University, Aarhus, Denmark; [5]Institute of Molecular Biotechnology, Department of Biotechnology, University of Natural Resources and Life Sciences (BOKU), Vienna, Muthgasse 18, A-1190 Vienna, Austria, Vienna, Austria; [6]Ludwig Boltzmann Institute of Traumatology in Cooperation With AUVA, Vienna, Austria., Vienna, Austria

Introduction: The research of extracellular vesicles (EVs) has boomed in the last decade, with the promise of them functioning as target-directed drug delivery vehicles, able to modulate proliferation, migration, differentiation, and other properties of the recipient cell that are vital for health of the host organism. To enhance the ability of their targeted delivery, we employed an intrinsically overrepresented protein, CD81, to serve for recognition of the desired target antigen.

Methods: Yeast libraries displaying mutant variants of the large extracellular loop of CD81 have been selected for binders to human placental laminin, EGFR and Her2 as an example target. Their specific interaction with laminin, EGFR and HER2 was confirmed in a mammalian display system. Derived sequences were reformatted to full-length CD81 and expressed in EVs produced by HeLa cells for laminin binders; by HEK293 cells for EGFR and HER2.
Results: To assess the novel functionality of antigen-binding CD81 LEL variants, internalization of such EVs into laminin-secreting cell lines Huh-7 and NCI-N87 was compared with EVs derived from wild-type CD81-transfected production cell line, and further, their reactivity with several recombinant laminin isoforms was examined. The specificity of binding of laminin targeting EVs to their cognate antigen was tested under competitive conditions. Finally, the ability of laminin-targeting EVs to transfer cel-miR-39 to Huh-7 cell line was examined.
CD81 LEL variants for EGFR and HER2 were accessed in respective cancer cells with high expression levels for EGFR and HER2. Additionally, EGFR and HER2 targeting EVs loaded with doxorubicin induce apoptosis in recipient cells more effectively compared to wild-type CD81 EVs.
Summary/Conclusion: To our knowledge, this is the first example of harnessing an EV membrane protein as mediator of de novo target antigen recognition via in vitro molecular evolution, opening horizons to a broad range of applications in various therapeutic settings. The advantage of the method presented here is that it can rapidly deliver binders to any antigen of choice, which can simply be 'clicked' into the full-length CD81, recombinantly expressed on the EV surface, enabling specific EV-mediated delivery to a large variety of cells and tissues.
Funding: This project has been supported by the Austrian FFG-COMET- Funding Program (grant to S.V.) and Evercyte GmbH. S.V. is a fellow of the international PhD programme "BioToP-Biomolecular Technology of Proteins", funded by the Austrian Science Fund (FWF) (W1224)

OT03: Cardiac Repair and Cardiac Protection

Chair: Susmita Sahoo – Cardiovascular Research Center Icahn School of Medicine at Mount Sinai

Chair: Hargita Hegyesi – Semmelweis University Department of Genetics, Cell- and Immunobiology

OT03.02 | Adult cardiomyocytes-derived EVs for the treatment of cardiac fibrosis

Marta Prieto-Vila[1]; Yusuke Yoshioka[2]; Asao Muranaka[1]; Takahiro Ochiya[3]
[1]Tokyo Medical University, Tokyo, Japan; [2]Department of Molecular and Cellular Medicine, Institute of Medical science, Tokyo Medical University, Shinjuku-ku, Japan; [3]Department of Molecular and Cellular Medicine, Institute of Medical science, Tokyo Medical University, Shimjuku-ku, Japan

Introduction: Cardiac fibrosis, provoked by many diseases, results from an erroneous hyperactivation of fibroblasts. This dysfunction evokes an excessive accumulation of extracellular matrix (ECM), decreasing cardiac function and eventually leading to death. It is known that extracellular vesicles (EVs) are deeply involved in the communication of cardiomyocytes (CM) with surrounding cells, including fibroblasts. Therefore, CM-derived EVs may be a promising cell-free system for fibrosis treatment.
Methods: We used a defined conjunct of chemicals by which mature CM culture was highly improved to ensure a high collection of EVs. Terminal differentiation, as well as senesce markers apparition, were delayed in comparison to the predetermined culture medium without apparent malignant alteration. EVs were isolated by ultracentrifugation, and their characteristics (particle number, membrane markers, and internalization) were analyzed. Finally, their effect on fibrosis was tested.
Results: Chemically-treated CM secreted a relatively large amount of EVs, which expressed the common EV membrane markers. Interestingly, CM-derived EVs were significantly more internalized by cardiac fibroblasts in comparison to other corporal fibroblasts. Treatment of EVs on TGF-β-activated cardiac fibroblasts showed a decrease of fibroblast activation markers both at mRNA and protein levels. Furthermore, ECM secretion was also reduced. Next-generation sequencing (NGS) also showed a rescued phenotype by EV treatment at the signaling pathway level. In accordance, the EV content was comprised of multiple myocyte-specific and antifibrotic-related microRNAs, although their effect individually was not as effective as the EV treatment. In addition, intracardiac injection of EVs improved the cardiac function in an animal model of cardiac fibrosis.
Summary/Conclusion: Our findings indicate that, due to the antifibrotic effects and the specificity of the EV cargo, the use of EVs derived from adult-CM is a promising treatment for cardiac fibrosis.

OT03.03 | Size matters: functional differences of progenitor cell-derived small extracellular vesicle subpopulations

Simonides I. van de Wakker[1]; Julia Bauzá-Martinez[2]; Carla Rios Arceo[3]; Eduard F Willms[4]; Olivier G de Jong[5]; Wei Wu[6]; Joost P.G. Sluijter[1]; Pieter Vader[7]

[1]Department of Experimental Cardiology, University Medical Center Utrecht, Utrecht University, The Netherlands, Utrecht, Netherlands; [2]Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands, Utrecht, Netherlands; [3]UMC Utrecht, Utrecht, Netherlands; [4]La Trobe University, Melbourne, Australia; [5]Utrecht University, Utrecht, The Netherlands, Utrecht, Netherlands; [6]Utrecht University, Utrecht, Netherlands; [7]CDL research, University Medical Center Utrecht, Utrecht, The Netherlands, Utrecht, Netherlands

Introduction: The use of cardiac progenitor cell (CPC)-derived small Extracellular Vesicles (sEVs) has shown great potential to stimulate cardiac repair. Increasing evidence indicates that sEVs present a heterogeneous population with distinct functions and this represents a major challenge in the field. Studying sEV heterogeneity could provide new insights into contributing therapeutic mechanisms underlying sEV-mediated cardiac repair.
Methods: CPC-derived sEVs were purified by flow through chromatography followed by size-exclusion chromatography (SEC) or asymmetric flow field flow fractionation (A4F) for fractionation of different sEV-subfractions. sEVs were characterized using western blot, nanoparticle tracking analysis, bicinchoninic acid protein assay, transmission electron microscopy and mass spectrometry (MS). Functional differences were studied using different cellular assays to determine AKT phosphorylation, wound healing, angiogenesis and proteasome activity.
Results: SEC or A4F were used to separate distinct subpopulations of CPC-derived sEVs which were identified based on differential expression of common sEV markers. These sEV subpopulations differed in size, appearance, proteomic composition and function. MS analysis confirmed the differences in expression levels of classical sEV marker proteins, as well as annexins, rab proteins, integrins, histones and proteasomal proteins. Furthermore, differences in cellular components linked to their cellular origin were found, as larger sEVs were linked to lysosomes and the plasma membrane and smaller sEVs had more markers linked to the centrosome and cytosol. Exposure of recipient endothelial cells to sEV subpopulations demonstrated clear functional differences. Additionally, differences in proteolytic activity of the sEV subpopulations were identified.
Summary/Conclusion: SEC and AF4 allow for isolation and in-depth study of the functional heterogeneity of sEVs. In our study, we observed the existence of different subpopulations based on size, with a different composition and biological function. Increased knowledge of sEV heterogeneity will contribute to a better understanding of the mechanisms of action of sEVs and will improve translation to the clinic and potentially an off-the-shelf approach to stimulate cardiac repair.
Funding: This work is financially supported by the Van Herk Foundation and the ERC EVICARE (725229)

OT03.04 | Cardiomyocytes (CM) derived small Extracellular Vesicles (sEV) plays an important role in heart development

Carolina Balbi[1]; Davide Ceresa[2]; Concetta Schiano[1]; Paolo Malatesta[2]; Lucio Barile[1]; Giuseppe Vassalli[1]

[1]ICCT-Ente Ospedaliero Cantonale, Bellinzona, Switzerland; [2]IRCCS Ospedale Policlinico San Martino., Genova, Italy

Introduction: Neonatal rats have the capacity to regenerate their hearts in response to injury, but this potential is lost after the first week of life. Cardiac maturation lays the foundation for postnatal heart development and disease, yet little is known about the contributions of the microenvironment to cardiomyocyte maturation.
Extracellular vesicles (EV) are bilayer-membrane nanoparticles released by all cell types, carrying proteins, lipids, and nucleic acids, which reflect the activation state of parental cells. Secreted small extracellular vesicles (sEV), prominently figure among extracellular signals that regulate cell function.
Purpose: We aim to determine whether cardiomyocyte (CM) derived sEV carried miRNA that has a role in heart development with specific regards to cardiomyocyte maturation.
Methods: sEV were isolated from rat cardiomyocyte at day 0 and 7 after birth by Size Exclusion Chromatography and serial ultracentrifugation. sEV were characterized by NTA and analyzed by Western blot for the presence of classical EV markers (TSG101, Syntenin-1). The role of sEV in cardiomyocyte proliferation was assesed by analysis of EdU incorporation on neoantal rat CM treated with sEV_p0 or sEV_p7. miRNA content on sEV was assesed using a rat-miRNome MicroRNA Profiling Kit and the identified miRNA's targets confirmed by RealTime-PCR and Western Blot.
Results: NTA and Western Blot analysis confirmed the presence of sEV in both the extracellular vesicles preparation. sEV_p0 showed to be able to sligtly increase EdU incorporation in treated cardiomyocyte (1.15-Fold) while sEV_p7 significantly inihibit CM proliferation (0.78-Fold) toghether with a change in cardiomyocyte citoscheletal architecture. Data from miRNome analysis showed in sEV_p7 a significan increase in miRNA with cyclines as tagets. Downregulation of Cdk1 ; Cdk4 ; Cdk2 ; CcnB1 and

CcnD1 was confirmed on CM trated with sEV_p7 compared to Ctrl. Downregulation of CCND1 was aslo confrmed at the protein level by Western Blot analysis.

Summary/Conclusion: These preliminary resultes showed an important role in heart develompent of cardiomyocyte derived-sEV. A deeper investigation of the pathaway activated by sEV may have a potential interest for the identification of possible regulators for stimulating heart regeneration.

Funding: Schweizersche Herzstiftung - FF21017
Fondazione Leonardo Lugano

OT03.05 | Cardiac progenitor cell-derived EVs skew human macrophages towards a pro-inflammatory phenotype

Margarida Viola[1]; Pieter Vader[2]; Saskia C.A. de Jager[3]; Joost P.G. Sluijter[4]
[1]University Medical Center Utrecht, Utrecht, Netherlands; [2]CDL research, University Medical Center Utrecht, Utrecht, The Netherlands, Utrecht, Netherlands; [3]Laboratory of Experimental Cardiology, University Medical Center Utrecht, The Netherlands, Utrecht, Netherlands; [4]Department of Experimental Cardiology, University Medical Center Utrecht, Utrecht University, The Netherlands, Utrecht, Netherlands

Introduction: Repair of damaged heart tissue upon myocardial infarction remains a major challenge. Transplantation of cardiac progenitor cells (CPCs) has been studied as a potential regenerative therapy, but recent studies have shown that the cardioprotective effect of CPCs is mediated by the release of extracellular vesicles (EVs). The benefits of CPC-derived EVs have mostly been associated with stimulation of angiogenesis and inhibition of cell death. Although macrophages have been suggested to be key for cardiac repair, the effect of CPC-EVs on macrophage polarization is poorly explored. Here, we hypothesized that CPC-derived EVs can modulate the inflammatory response after myocardial infarction by interacting with macrophages.

Methods: EVs were isolated from serum-starved CPCs by ultrafiltration followed by size exclusion chromatography. Macrophages were stimulated with LPS + IFNy or with IL4 in order to induce an inflammatory M1 and reparative M2 phenotype, respectively. A third group of macrophages were cultured in media without LPS, IFNy or IL4 to resemble naive M0 macrophages. The obtained macrophages were exposed to CPC-EVs and analyzed by flow cytometry, bulk RNA sequencing and confocal microscopy to assess macrophage phenotype changes. Gene candidates were selected based on the p-value and fold-change, and validated by RT-PCR. Small molecule inhibitors were used to further investigate the mechanism by which CPC-EVs are taken up by macrophages and the pathways involved in macrophage response.

Results: Stimulation of macrophages with CPC-EVs enhanced the expression of the pro-inflammatory marker CD80, while slightly decreasing the anti-inflammatory marker CD206, in M0 and M2 macrophages. CPC-EV-stimulated macrophages also adopted a morphology that reflects the inflammatory macrophage. In line with these findings, bulk RNA sequencing on M0 and M2 polarized macrophages revealed upregulation of genes involved in inflammatory response, including cytokine and both type I and II interferon signaling. EV exposure did not significantly affect gene expression in M1 polarized macrophages. Ongoing investigations will provide in-depth insight into the mechanism by which CPC-EVs induce this response in macrophages.

Summary/Conclusion: Our data suggests that CPC-EVs are able to induce macrophage polarization towards an inflammatory phenotype, which might have implications for CPC-EV treatment after myocardial infarction. This underlines an urgent need to understand the molecular mechanisms underlying the immunomodulatory effect of CPC-derived EVs before moving into a clinical setting.

Funding: This research is supported by the European Research Council (ERC) consolidator grant EVICARE (ERC-2016-COG-725229).

OT03.06 | Integrated omics for plasma extracellular vesicle diagnostics is influenced by the isolation method in healthy volunteers and myocardial infarction patients

Daan Paget[1]; Antonio Checa[2]; Raphael Heilig[3]; Mayooran Shanmuganathan[4]; Raman Dhaliwal[5]; Errin Johnson[5]; Maléne Møller Jørgensen[6]; Oxford Acute Myocardial Infarction Study[4]; Rikke Baek[7]; Craig E. Wheelock[2]; Keith M. Channon[4]; Roman Fischer[3]; Daniel C. Anthony[8]; Robin P. Choudhury[4]; Naveed Akbar[4]
[1]University of Oxford, Oxford, United Kingdom; [2]Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden, Stockholm, Sweden; [3]Target Discovery Institute, Centre for Medicines Discovery, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom, Oxford, United Kingdom; [4]Division of Cardiovascular Medicine, Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom, Oxford, United Kingdom; [5]Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom, Oxford, United Kingdom; [6]Department of Clinical Immunology/Department of Clinical Medicine, Aalborg University Hospital, Aalborg, Denmark, Aalborg, Denmark; [7]Department of Clinical Immunology, Aalborg

University Hospital, Aalborg, Denmark, Aalborg, Denmark; [8]Department of Pharmacology, University of Oxford, Oxford, United Kingdom, Oxford, United Kingdom

Introduction: Plasma extracellular vesicle (EV) number and composition are altered following myocardial infarction (MI) and offer potential as biomarkers. However, there remains limited information on how plasma EV isolation methods influence plasma EV-acquired omics in patients. Here, we compared five different plasma EV isolation methods using single and multi-integrated omics.

Methods: Platelet-poor plasma EV were isolated from healthy donors (N=4) and MI patients (N=6) at presentation to hospital and 1-month post-AMI using: ultracentrifugation (UC), precipitation, acoustic trapping, size-exclusion chromatography (SEC) and immunoaffinity capture using EV markers CD9, CD63, CD81 versus an IgG control. Isolated EV were characterised by Nanoparticle Tracking Analysis, transmission electron microscopy, an EV-protein array, proteomics and sphingolipidomics (LC-MS/MS).

Results: The isolation method influenced plasma EV number, size, protein concentration, EV-subpopulations, and proteomic and sphingolipid profiles. An integrated analysis using hierarchical clustering showed that plasma EV isolated by UC, precipitation, and SEC form distinct clusters, whereas acoustic trapping and immunoaffinity capture cluster together. IgG beads were indistinguishable from CD9, CD63, and CD81 immunoaffinity capture beads. MI patient plasma derived EV were significantly higher at time of presentation versus 1-month post-MI when isolated with UC, precipitation and acoustic trapping (1.4-fold p=0.04, 2.1-fold p=0.04 and 2.7-fold p=0.02, respectively) but not with SEC and immunoaffinity capture. The plasma EV concentration at time of presentation was significantly associated with infarct size determined by MRI obtained at 6 months post-MI when isolated with UC ($R2 = 0.89$, p=0.02) but not with the other techniques.

Summary/Conclusion: Our data show that the choice of plasma EV isolation method influences the omics-acquired signature of plasma EV and impacts the ability to distinguish clinical changes.

Funding: NA acknowledge support by research grants from the British Heart Foundation (BHF) Centre of Research Excellence, Oxford (NA: RE/13/1/30181 and RE/18/3/34214) and British Heart Foundation Project Grant (NA: PG/18/53/33895). DP is funded by the Department of Pharmacology, the Alison Brading Memorial Fund Lady Margaret Hall, the Clarendon Fund provided by the University of Oxford and the Medical Research Council (MR/N013468/1).

All human investigations were conducted in accordance with the Declaration of Helsinki. The Oxfordshire Research Ethics Committee (Ref: 08/H0603/41 and 11/SC/0397) approved human clinical protocols. All healthy volunteers and AMI patients provided informed written consent.

OT04: Optimization and Standardization of EV Analysis

Chair: Kenneth W. Witwer – Johns Hopkins University School of Medicine, Department of Molecular and Comparative Pathobiology

Chair: Edwin van der Pol, Jr. – Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands

OT04.01 | Efficient quantification of human cytomegalovirus and extracellular vesicles in cell culture samples using flow nanoanalysis

Vladimir Bokun[1]; Blair Strang[2]; Yan Liu[1]; Beth Holder[1]
[1]Imperial College London, London, United Kingdom; [2]University of London, London, United Kingdom

Introduction: Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus with ability to cause congenital infection and disease in the immunocompromised. HCMV infection is accompanied by production of virally induced extracellular vesicles (EVs). These particles are challenging to separate and quantify in cell supernatants. Therefore, we designed a novel, flow nano-analysis (FNA)-based protocol for quantification of HCMV virions and other non-virion particles in cell culture media.

Methods: We utilized uninfected or HCMV-infected human foreskin fibroblast cells to generate conditioned culture media. Media samples were either clarified by a 10-min 1,000xG centrifugation; concentrated by ultracentrifugation at 150,000xG onto 50% iodixanol; or purified by size exclusion chromatography. We used fluorogenic nucleic acid dyes SYTO13 or DRAQ5 to label nucleic acid in particles and examined them using nanoFCM, a specialized nanoscale flow instrument designed for nanoparticle research. Gating on populations of interest allowed determination of the particle concentration. FNA data was compared to transmission electron microscopy (TEM) analysis of virion size and viral copy number qPCR.

Results: Labelling particles with SYTO13 and DRAQ5 (separately) yielded a distinct viral population which was gated for enumeration and sizing using concentration and size standards. The highest reported virion concentration was 1.56x10^9 particles/ml, while the highest reported non-virion particle concentration was 1.88x10^10 particles/ml. Enumeration of virions and non-virion particles by FNA was consistent with qPCR analysis of viral genome number. FNA enabled sizing with a higher throughput than TEM; however, virion sizes were smaller compared to TEM (168.04nm vs 179.74nm).
Summary/Conclusion: Our results highlight FNA as a rapid and accurate tool for enumeration and sizing of HCMV and EVs. This technique has potential to be used in analysis of other viruses and their relationships with EVs.
Funding: This project is supported by Imperial College President's PhD scholarship awarded to Vladimir Bokun; acquisition of nanoFCM instrument was supported by a Wellcome Trust multi-user equipment grant awarded to Dr Beth Holder and Dr Richard Kelwick.

OT04.02 | Adapter dimers contamination in extracellular vesicles sRNA-sequencing datasets result in sequencing failure and batch effects

Joaquín J. Maqueda[1]; Alberta Giovanazzi[2]; Mafalda Rocha[3]; Sara Rocha[3]; Joana Carvalho[3]; Marca H.M Wauben[4]; Carla Oliveira[5]
[1]Bioinf2Bio, i3S, Sevilla, Spain; [2]Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands; [3]i3S; IPATIMUP, Porto, Portugal; [4]Division of Cell Biology, Metabolism & Cancer, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands, Utrecht, Netherlands; [5]i3S; IPATIMUP; Department of Pathology, Faculty of Medicine, University of Porto., Porto, Portugal

Introduction: Small RNAs (sRNA) carried by extracellular vesicles (EVs) modulate function of recipient cells and are promising biomarkers in several contexts. EV-sRNA profiling with Next-Generation Sequencing (NGS) often fails or delivers poor sequencing outcomes, independently of reagents, platforms or pipelines used. Besides the financial loss, this may be a major source of lack of reproducibility across similar studies. Herein, we explored the output of pre-sequencing quality control steps, in order to unveil key points for quality control assessment that may predict poor sequencing outcomes and batch effects, thus biasing biological results.
Methods: RNA isolation, library preparation and sequencing protocols used in three independent EV-sRNA experiments from human and mouse body fluids, respective generated sequencing datasets and quality control outputs were analysed and compared. The level of adapter dimer contamination and batch effects were analysed. Manatee algorithm, Linux software, Bioanalyzer graphs, FastQC and in-house R scripts were used for data analysis. Peer-reviewed EV-sRNA published articles available in Gene Expression Omnibus (GEO) were mined to identify successful EV-sRNA sequencing workflows.
Results: In three independent sRNA-seq experiments, the presence and height of a constant peak in cDNA electropherograms from library preparation was correlated with the level of adapter dimer contamination after sequencing (as high as 90% of the sequenced reads in some instances), and confirmed by NGS FastQC analysis. The sRNA expression quantification pipeline further exposed a tight correlation between adapter dimers contamination and batch effects, which strongly biased sequencing results. A literature review confirmed that there are EV transcriptomics experiments available which by preventing adapter dimers contamination ensure the delivery of a large number of useful reads, with clear benefit for sequencing data interpretation and reproducibility.
Summary/Conclusion: The current analysis identified the quality control steps that are mandatory to improve EV-sRNA sequencing experiments performance. Further, this workflow allows monitoring quality parameters in a step-by-step manner, correct for deviations in library preparations, and proceed to sequencing with a greater likelihood of success. Additionally we provide compelling evidence that a major source of batch effects in EV-sRNA sequencing is adapter dimers contamination.
Funding: Funding: European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 722148 and GenomePT (POCI-01-0145-FEDER-022184), supported by COMPETE 2020/Portugal2020/ERDF/FCT

OT04.03 | Adherence to minimal experimental requirements for defining extracellular vesicles and their functions: what's new in 2022?

Rodolphe W. Poupardin[1]; Liliia Paniushkina[2]; Martin Wolf[1]; Kenneth W. Witwer[2]; Dirk Strunk[1]
[1]Cell Therapy Institute, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI - TReCS), Paracelsus Medical University (PMU), Salzburg, Austria, Salzburg, Austria; [2]Johns Hopkins University School of Medicine, Department of Molecular and Comparative Pathobiology, Baltimore, USA

Introduction: The fast growing extracellular vesicle (EV) research field was accompanied by the evolution of guidelines regarding 'Minimal Information for Studies of Extracellular Vesicles' (MISEV) in 2014, which were revised in 2018. Efforts to enhance interpretability and comparability of results are of increasing importance for assuring and improving EV research quality.
Methods: In this context, we conducted a systematic review using a text mining approach to assess adherence to MISEV criteria by analyzing the methodology used for EV isolation and characterization in 5,093 open-access publications during 2012-2020. This review was now updated covering the latest 2021 manuscripts and non-open access papers.
Results: Our analysis showed that the EV characterization improved significantly using a higher number of methods and EV markers to assess both, quantity and quality of EVs, in more recent years. Papers mentioning the MISEV criteria applied a higher number of characterization methods. Interestingly, increased adherence to MISEV requirements led to more frequent citations. These results will be compared with the latest 2021 data including current insights into most recent EV isolation and characterization trends.
Summary/Conclusion: The analysis confirmed the positive impact of MISEV guidelines on the EV research quality. Additional efforts will be required to improve proper reporting of the methods used based on the new discoveries made in the field.
Funding: This work was supported in part by funding from the European Union's Horizon 2020 research and innovation program (grant agreement no. 733006 to Dirk Strunk), by Land Salzburg IWB/EFRE 2014 - 2020 P1812596 and WISS 2025 20102-F1900731-KZP EV-TT (to Dirk Strunk).

OT04.04 | A new strategy to count and sort neutrophil-derived extracellular vesicles: validation in infectious disorders

Amandine Bonifay[1]; Stéphane Robert[1]; Bélinda Champagne[1]; Paul-Rémi Petit[2]; Aude Eugène[2]; Corinne Chareyre[1]; Anne-Claire Duchez[1]; Mélanie Vélier[1]; Shirley Fritz[2]; Loris Vallier[1]; Romaric Lacroix[1]; Françoise Dignat-George[1]
[1]Aix-Marseille University, C2VN, INSERM 1263, INRA 1260, Marseille, France, Marseille, France; [2]Department of Hematology and Vascular Biology, CHU La Conception, APHM, Marseille, France, Marseille, France

Introduction: Newly recognized polymorphonuclear neutrophil (PMNs) functions include the ability to release subcellular mediators such as neutrophil-derived extracellular vesicles (NDEVs) involved in immune and thrombo-inflammatory responses. Elevation of their plasmatic level has been reported in a variety of infectious and cardiovascular disorders, but the clinical use of this potential biomarker is hampered by methodological issues. Although flow cytometry (FCM) is currently used to detect NDEVs in the plasma of patients, an extensive characterization of NDEVs has never been done. Moreover, their detection remains challenging because of their small size and low antigen density. Therefore, the objective of the present study was first to establish a surface antigenic signature of NDEVs detectable by FCM and therefore to improve their detection in biological fluids by developing a strategy allowing to overcome their low fluorescent signal and reduce the background noise.
Methods: A large panel of monoclonal antibodies (54 specificities) was tested on purified NDEVs generated from human circulating PMNs by reference to monocyte and lymphocyte EV. Size exclusion chromatography (SEC), antibody combination and fluo-sensitive FCM were used to increase separation index (SI) in FCM between background noise and specific labeling. The new combined strategy was used for Jet-in-Air NDEV selective sorting from plasma of sepsis patients.
Results: Among the 54 antibody specificities already reported to be positive on PMNs, we identified a profile of 15 membrane protein markers, including 4 (CD157, CD24, CD65 and CD66c) never described on NDEVs. Among them, CD15, CD66b and CD66c were identified as the most sensitive and specific markers to detect NDEVs by FCM. Using this antigenic signature, we developed a new strategy combining the three best antibodies in a cocktail and reducing the background noise by size exclusion chromatography. This strategy allowed a significant improvement in NDEVs enumeration in plasma from sepsis patients and made it feasible to efficiently sort NDEVs from COVID-19 patients.
Summary/Conclusion: Altogether, this work opens the door to a more valuable measurement of NDEVs as a potential biomarker in clinical practice. A similar strategy could also be applied to improve detection by FCM of other rare subpopulations of EVs generated by tissues with limited access, such as vascular endothelium, cancer cells or placenta.

OT04.05 | Off-the-shelf, stable biological test samples to validate calibration procedures for extracellular vesicle measurements

Britta A. Bettin[1]; Chi Hau[2]; Edwin van der Pol[1]; Rienk Nieuwland[3]
[1]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Amsterdam, Netherlands; [2]Department of Clinical Chemistry; Vesicle Observation Center, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands; [3]Department

of Clinical Chemistry; Vesicle Observation Center; Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands

Introduction: Concentrations of extracellular vesicles (EVs) in body fluids are being explored as disease biomarkers. To measure EV concentrations, most laboratories use flow cytometers (FCMs), but concentrations are incomparable between FCMs. To improve comparability, the METVES II consortium develops reference materials and methods to calibrate FCMs, which require validation by test samples containing EVs. To minimize variation introduced by the test samples itself, we developed off-the-shelf, stable and flow cytometry compatible plasma-derived EV test samples (PEVTES).
Methods: Plasma was collected and prepared from healthy donors and pooled. EVs were double-labeled with CD61-APC and CD235a-PE or lactadherin-FITC, isolated by size-exclusion chromatography to reduce swarm detection, diluted in cryopreservation agent (trehalose), frozen in liquid nitrogen, and stored at 80°C. After thawing the EV concentrations were measured directly, and after 1, 3, 6, and 12 months, on a calibrated FCM (Apogee A60-Micro).
Results: Compared to the fresh starting material, the concentration of CD61+ (platelet) and CD235a+ (erythrocyte) EVs decreased 10% (p=0.74, p=0.34), and 30% for lactadherin+ EVs (p=0.01) after 1 month. After 12 months of storage, the concentration of CD61+ EVs decreased 36% (p=0.07) compared to 1 month of storage, but the concentration of CD235a+ and lactadherin+ EVs were stable (p=0.76, p=0.95).
Summary/Conclusion: PEVTES can be stably stored in trehalose for up to 12 months at -80°C. The PEVTES will be key to validate newly developed reference materials and methods in a global comparison study involving 25 laboratories. Furthermore, the PEVTES can be used as a quality control sample for EV flow cytometry measurements.

OT04.06 | Presence and removal of platelets from human blood plasma to improve the quality of extracellular vesicle research

Britta A. Bettin[1]; Aleksandra Gasecka[2]; Bo Li[3]; Bert Dhondt[4]; An Hendrix[5]; Edwin van der Pol[1]; Rienk Nieuwland[6]
[1]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Amsterdam, Netherlands; [2]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands; 1st Chair and Department of Cardiology, Medical University of Warsaw, Warsaw, Poland, Warsaw, Poland; [3]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands; Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, China, Guangzhou, China (People's Republic); [4]Department of Urology, Ghent University Hospital, Ghent, Belgium; Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Ghent University, Ghent, Belgium; Cancer Research Institute, Ghent, Belgium, Ghent, Belgium; [5]Department of Urology, Ghent University Hospital, Ghent, Belgium; Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Ghent University, Ghent, Belgium; Cancer Research Institute, Ghent, Belgium, Ghent, Belgium; [6]Department of Clinical Chemistry; Vesicle Observation Center; Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands

Introduction: Human blood plasma is the most widely studied body fluid for extracellular vesicle (EV) research. To prepare plasma as a starting material for EV research, blood is centrifuged to remove cells, including platelets. Removing all platelets by centrifugation is impossible, and because platelets cannot be separated from EVs during isolation, platelets are a confounder of plasma EV analysis. Our goal was to investigate how effectively platelets are removed by centrifugation, and whether truly platelet-free plasma can be prepared.
Methods: Plasma was prepared in three laboratories according to the International Society on Thrombosis and Haemostasis protocol (2x 15 minutes, 2,500 g, 20°C). Platelets and platelet-derived EVs were stained with CD61 and measured with a calibrated flow cytometer (Apogee A60-Micro / BD FACS Canto II) directly (one study) or after freeze-thawing (two studies). A polycarbonate filter with a pore size of 0.8 μm was tested to remove platelets.
Results: The mean platelet concentration differed over 60-fold between laboratories. The concentration of platelets and platelet-derived EVs showed a linear correlation (R2=0.56). Moreover, filtration reduced the platelet concentration 146-fold compared to double centrifugation (p=0.0006), without affecting platelet-derived EVs.
Summary/Conclusion: All plasma samples contained platelets, but the platelet concentration differed strongly between laboratories. The correlation between concentration of platelets and platelet-derived EVs suggests platelet fragmentation during a freeze-thaw cycle, thus producing fragments likely to be indistinguishable from real platelet-derived EVs. To improve the reproducibility of plasma EV research, we recommend to remove platelets by centrifugation and filtration, and/or to report the concentration of platelets in plasma samples.

OT05: EVs in Immunity and Inflammation

Chair: Steffi Bosch – IECM, Oniris, INRAE, USC1383, Nantes, France

OT05.01 | Acute joint inflammation induces a sharp increase in EV release and modifies the phospholipid profile of synovial fluid-derived EVs

Laura Varela[1]; Marca H.M Wauben[2]; Chris van de Lest[1]; René van Weeren[1]; Ger Arkesteijn[3]; Janneke Boere[4]
[1]Division of Equine Sciences, Department of Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands, Utrecht, Netherlands; [2]Division of Cell Biology, Metabolism & Cancer, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands, Utrecht, Netherlands; [3]Division of Infectious Diseases and Immunology, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands, Utrecht, Netherlands; [4]Lygature, Utrecht, Netherlands, Utrecht, Netherlands

Introduction: Inflammation is the trademark of most joint disorders. How the induction, perpetuation, and resolution of joint inflammation is regulated is not fully clear. Since extracellular vesicles (EVs) are critical for intercellular communication, we aim to unveil their role in these processes. Here, we investigated EVs' kinetics and phospholipidome profile from synovial fluid (SF) of healthy equine joints and horses with lipopolysaccharide (LPS) induced synovitis. This disease model has an acute inflammation phase at 5-8h post-induction followed by a resolution stage starting at approximately 24h. We analyzed the amount of EVs and the biomembrane-derived non-neutral lipids (glycerophospholipids and sphingolipids).
Methods: SF was collected at 0h, followed by synovitis induction with an injection of LPS. Subsequently, SF was harvested at 5, 24, and 48h. EVs were isolated by differential ultracentrifugation followed by density gradient-ultracentrifugation. Single EV analysis was performed with flow cytometry. Lipids were analyzed by high-resolution mass spectrometry. Data were processed using R packages and GraphPad.
Results: LPS exposure triggers a rapid increase of SF-EVs at 5-8h, which starts to return to healthy baseline levels at 24h. We reproducibly detected over 150 lipid species within 9 lipid classes in purified SF-EVs. Importantly, we identified major changes in the lipid profile of EVs during the inflammatory stage (5 and 24h). Compared to healthy SF-EVs (0h), SF-EVs collected at 5, 24, and 48h showed an increase in hexosylceramides (HexCer), which are associated with inflammation in rheumatoid arthritis. In contrast, the lipid composition of 5h and 24h SF-EVs was depleted in phosphatidylserine, phosphatidylcholine, and sphingomyelin. Based on identified specific lipid markers, we composed lipid profiles associated with healthy and inflammatory state-derived SF-EVs.
Summary/Conclusion: An inflammatory stimulus leading to acute synovitis results in a sharp increase in SF-EVs with an altered phospholipid bilayer composition compared to steady-state SF-EVs. The correlation of specific lipids with either healthy or inflammatory state-derived SF-EVs is of potential interest for biomarker profiling and unveiling the role of lipids in EV function.
Funding: EU's H2020 research and innovation program, Marie S. Curie COFUND RESCUE grant agreement № 801540.

OT05.03 | Serial immunocapture of P2Y12+/TMEM119+ microglia derived extracellular vesicles reveals distinct activation states in normal and inflamed neonatal mouse brain

George Biouss; Lina Antounians; Agostino Pierro; Augusto Zani
The Hospital for Sick Children, Toronto, Canada

Introduction: Microglia are brain resident macrophages that once activated by neuroinflammatory signals undergo changes in morphology and surface marker expression. Little is known about microglia-derived extracellular vesicles (M-EVs) in the neonatal brain during sepsis. Herein, we separated and characterized M-EVs in a neonatal sepsis model and evaluated their neuroinflammatory cargo.
Methods: Animals: Brains of 9-day-old C57BL/6 mice were harvested from normal pups or pups with sepsis and brain neuroinflammation confirmed by high expression of inflammatory markers IL1β and NLRP3 (WB), and high density of ameboid IBA1+ microglia (immunofluorescence).
M-EVs: Brains were minced, digested with dispase, differentially centrifuged, and filtered (300g/1200g/100,000g). M-EVs were enriched by serial immunocapture of canonical microglia receptors with a biotin-streptavidin platform:
- EVs were incubated with P2Y12 antibody and biotin-tagged secondary antibody
- Streptavidin coated agarose beads were added
- After washes, P2Y12+EVs were released by acid catalysis
- Incubation steps were repeated using TMEM119 antibody, and P2Y12+/TMEM119+ EVs were separated.

M-EVs were assessed for size (NTA), morphology (TEM), and CD63, TSG101, Histone-H3, IL1β and NLRP3 protein expression (WB). Immunogold labelling was performed to confirm capture of P2Y12+/ TMEM119+ M-EVs (TEM).
Results: M-EVs from control and inflamed brains had similar size distribution and were CD63+/TSG101+ and Histone-H3-. Immunogold labeling showed P2Y12 and TMEM119 localization on the M-EV extracellular domain. M-EVs from inflamed brains had higher P2Y12 (p=0.03), NLRP3 (p=0.002) and IL1β (p=0.03), and lower TMEM119 (p=0.03) protein levels compared to control M-EVs.
Summary/Conclusion: Our method was effective in separating M-EVs from normal and inflamed neonatal brains. In an experimental model of neonatal sepsis, M-EVs have a different signature during neuroinflammation.
Funding: SickKids Foundation

OT05.04 | Extracellular vesicles from antigen-presenting cells stimulate activated T cells in an antigen-dependent manner in vivo

Jan Kranich[1]; Lisa Rausch[1]; Ashretha Latha[1]; Christine Ried[1]; Anne Trefzer[2]; Veit Buchholz[3]; Reinhard Obst[1]; Tobias Straub[4]; Thomas Brocker[1]
[1]Institute for Immunology, LMU Munich, Planegg-Martinsried, Germany; [2]Roche, Zurich, Zurich, Switzerland; [3]TU Munich, München, Germany; [4]BioMedical Center, LMU Munich, Planegg-Martinsried, Germany

Introduction: To facilitate the analysis of naturally occurring extracellular vesicles (EVs) and their interactions partners, we have developed Ca2+-independent phosphatidylserine binding reagents based on MFG-E8. These reagents allow us to analyze PS+ EVs and their target cells in vitro and in vivo using imaging flow cytometry and deep learning-assisted image interpretation. Using this method, we showed that APC-derived exosomes interact with activated cytotoxic T cells during acute LCMV infections of mice (Kranich, JEV, 2020). Furthermore, the binding of PS+ platelet-derived microparticles to activated T cells strongly correlates with disease severity in COVID-19 patients (Rausch JEV, 2021). Next, we wanted to investigate if and how EVs influence T cells.
Methods: To investigate the influence of PS+ EVs on T cell activation and differentiation, we stained T cells carrying PS+ EVs in vivo by injecting MFG-E8-eGFP into LCMV-infected mice. We then FACS-sorted activated EV- and EV+ CD8 T cells and performed RNAseq analysis.
Furthermore, we transferred EVs purified by gradient ultracentrifugation produced by ovalbumin-pulsed activated bone-marrow-derived dendritic cells (BMDCs) into mice that had received T cell receptor (TCR)-transgenic ovalbumin-specific and control T cells. To exclude the possibility that dendritic cells take up EVs and present their peptides to cognate T cells, we used H2-Kbm1mice as hosts that cannot present the specific ovalbumin peptide. We then monitored translocation of nuclear factor of activated T cells (NFATc1) to the nucleus as a readout for TCR stimulation.
Results: EV-binding of T cells in LCMV-infected mice was highest during the effector phase of the T cell response and then rapidly declined. RNAseq analysis of EV+ T cells from LCMV-infected mice revealed increased expression of effector cell associated genes.
After transfer of BMDC-derived EVs, we found that transferred ovalbumin-specific but not unspecific EV+ T cells showed significantly increased nuclear factor of activated T cells (NFATc1) translocation to the nucleus.
Summary/Conclusion: NFAT-translocation to the nucleus is downstream of the T cell receptor–calcium-calcineurin signaling pathway and a prerequisite for expressing a panel of genes required for T cell activation. Therefore, our results demonstrate that activated T cells receive antigen-specific stimulatory signals from EVs, which play an essential role during viral T cell responses by increasing T cell effector function.
Funding: Deutsche Forschungsgemeinschaft CRC 1054 (TP B03, grant no. 210592381)

OT05.05 | Extracellular environment determines the effect of the neutrophil granulocytes derived extracellular vesicles

Viktória Szeifert[1]; Ferenc Kolonics[2]; Delaram Khamari[3]; Pál Vági[4]; László Barna[4]; Erzsébet Ligeti[2]; Ákos M. Lőrincz[5]
[1]Semmelweis University, Department of Physiology, Budapest, Hungary; [2]Department of Physiology, Semmelweis University, Budapest, Hungary; [3]Department of Genetics, Cell- and Immunbiology, Semmelweis University, Budapest, Hungary; [4]Nikon Center of Excellence, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary; [5]Department of Physiology, Semmelweis University; Second Department of Internal Medicine, Szent György Hospital, Budapest, Hungary

Introduction: Previously, our group characterized different type of extracellular vesicle (EV) released from neutrophilic granulocytes (PMN). The three characterized populations are the EVs formed spontaneously (spEV), during apoptosis (apoEV) and upon activation with opsonized particles (aEV). The aEVs show rather pro-inflammatory effect on resting neutrophils and

antibacterial effect on bacteria and fungi, while the sEVs tend to mediate anti-infammatory effects. We also found that the Mac-1 integrin receptor is crucial for the aEV formation. We aimed to examine how the selective activation of the Mac-1 and the calcium signal affect the production of neutrophils EV.

Methods: We isolated medium sized PMN EVs from peripheral human blood neutrophils by two-step centrifugation and gravitational filtration validated by size exclusion chromatography. We characterized the EVs by flow cytometry, Bradford protein assay, NTA and TEM. We tested the effect of Ca-ionophore and examined the EV production on C3bi and fibrinogen surface and in soluble form. We examined their effect on bacterial survival, and we measured the ROS and cytokine production of PMNs and monocytes after 3 hours co-incubation with the EVs. We also followed the cluster formation of Mac-1 by TIRF microscopy.

Results: EV yield was higher with differential centrifugation than with SEC. On C3bi coated surface, we observed an increased EV production, these EVs possessed antibacterial capacity. However, in soluble condition C3bi did not induce EV production. We found that Ca-ionophore initiated EV formation, but these EVs were ineffective in functional tests. We observed EV production increase after Ca-ionofore treatment both in the presence and in the absence of extracellular Ca. SpEV and apoEV treatment inhibited the ROS and IL-8 production of phagocytes, however aEVs increased the ROS and IL-8 production.

Summary/Conclusion: The Ca-signal is crucial, but not sufficient alone in the generation of aEVs. The selective Mac-1 activation and receptor clustering is not just crucial, but sufficient in initiation of the aEV biogenesis that show a completely different biological activity on other cells than spEV or apoEV. This observation suggests that neutrophils are able to change their EV production according to the environmental conditions detected by their receptors.

Funding: EFOP-3.6.3-VEKOP-16-2017-00009, NKFIH FK 137770, MOLORKIV TKP2021-EGA-24, János Bolyai Research Scholarship of the Hungarian Academy of Sciences, and the ÚNKP-21-3 New National Excellence Program of the Ministry for Innovation and Technology from the source of the National Research, Development and Innovation Fund.

OT05.06 | Extracellular vesicles from M1 and M2 primary macrophages have distinct non-coding RNA cargo; functional implications

Paschalia Pantazi[1]; Toby Clements[1]; Morten Venø[2]; Vikki Abrahams[3]; Beth Holder[1]
[1]Imperial College London, London, United Kingdom; [2]Omiics, Aarhus, Denmark; [3]Yale University School of Medicine, New Heaven, USA

Introduction: Macrophages are professional antigen-presenting cells involved in defence against pathogens, wound healing, and regulation of other immune cells. They are often classified into M1 'classical' and M2 'alternatively-activated' macrophages. M1 macrophages have been linked with inflammation-associated pathologies, whereas a switch towards an M2 phenotype indicates resolution of inflammation and tissue regeneration. Given the limited number and inaccessibility of extracellular vesicles (EVs) from healthy tissues, and the requirement for large numbers of cells to generate sufficient EVs in vitro, comprehensive unbiased information on primary macrophage EV cargo is lacking. Here, we provide a first insight into the complete small RNA cargo of human macrophage M1/M2 EVs.

Methods: Monocyte-derived macrophages were polarised into M1 (GM-CSF+LPS+IFNγ) or M2 (M-CSF+IL-4+IL-13) and EVs isolated by size exclusion chromatography. EVs were characterised by nanoparticle tracking analysis, electron microscopy, and ELISA. EV RNA samples were prepared for small RNA sequencing using Qiagen's GIAseq small RNA Library Prep kit and sequenced on an Illumina NextSeq500. Functional enrichment analysis was performed using MIENTURNET, based on validated miR-target interactions from miRTarBase.

Results: Many types of small non-coding RNAs were found in EVs from M1/M2 macrophages including miRNAs, isomiRs, tRNA fragments, piRNA, snRNA, snoRNA and yRNA fragments. Distinct differences were observed between M1 and M2 EVs, with higher relative abundance of miRNAs, and lower abundance of tRNA fragments in M1 EVs compared to M2 EVs. 66 tRNAs were significantly different between M1 and M2 EVs. 72 miRNAs were significantly different between EVs from M1 and M2 cells, including the miR-99b/let-7e/mir-125a cluster in M1 EVs, which is involved in regulating cellular proliferation, invasion, and migration. miRNA-target enrichment analysis identified several gene targets involved in gene expression and metabolic processes.

Summary/Conclusion: M1 and M2 cells release EVs with distinct tRNA and miRNA cargo, which have the potential to contribute to the unique effect of these cell subsets on their microenvironment.

Funding: Research reported in this publication was supported by the National Institute for Child Health (NICHD) of the National Institutes of Health under award number R01HD093801.

OT06: Mitochondrial Dysfunction or EV Transportation Mitochondria Components

Chair: Juan M. Falcon-Perez – Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Exosomes Laboratory, 48160 Derio, Spain

Chair: Efrat Levy – Nathan S. Kline Institute for Psychiatric Research

OT06.01 | Cocaine stimulates brain mitovesicle secretion and alters mitovesicle cargo in a sex-dependent fashion

Efrat Levy; Pasquale D'Acunzo
Nathan S. Kline Institute for Psychiatric Research, New York, USA

Introduction: Cocaine effects on the brain are complex and include alterations of mitochondrial activity and buildup of reactive oxygen species (ROS). We previously demonstrated that mitochondrial ROS enhance the release of a newly identified type of extracellular vesicles (EVs) of mitochondrial origin that we named mitovesicles. Thus, we hypothesized that cocaine addiction perturbs mitovesicle homeostasis, including in the brain of chronically cocaine-administered mice.
Methods: 2.5-month-old C57BL/6J mice were injected daily for two weeks with either cocaine or saline. EVs were isolated from the right hemibrains using a high-resolution iodixanol gradient. Mitovesicles, enriched in the densest fraction of the gradient, were analyzed by electron microscopy, NTA, total protein content, and western blotting. Protein expression was studied in the left hemibrains by western blotting. Male and female mice were included in the study as separated cohorts.
Results: The number of mitovesicles, demonstrated by NTA and protein content, was higher in the brain of cocaine treated mice when compared to saline-injected controls, regardless of the sex. Consistently, mitochondrial fragmentation was higher in cocaine-injected mice compared to controls in both sexes, as revealed by Western blot analysis of Drp1, master regulator of mitochondrial fission, in brain homogenates. However, while the amount of COX-IV was also higher in mitovesicle of cocaine-treated mice of both sexes, the level of the dopamine-degrading enzyme MAO was lower in mitovesicles from male but not female brains.
Summary/Conclusion: Mitovesicles are a previously unidentified player altered by cocaine in the brain. Sex-specific perturbations in mitovesicle cargo may be responsible, at least in part, for the sex-specific behavioral differences described in addicted human subjects.
Funding: Supported by NIH (DA044489).

OT06.02 | High mitochondrial DNA (mtDNA) diversity in plasma extracellular vesicles from colorectal cancer patients

Tonje Bjørnetrø[1]; Paula A Bousquet[1]; Kathrine Røe Redalen[2]; Anne-Marie Siebke Trøseid[3]; Torben Lüders[4]; Christin Johansen[1]; Espen Stang[5]; Anniken Jørlo Fuglestad[6]; Christian Kersten[1]; Sebastian Meltzer[1]; Anne Hansen Ree[1]
[1]*Department of Oncology, Akershus University Hospital, Lørenskog, Norway, Lørenskog, Norway;* [2]*Department of Physics, Norwegian University of Science and Technology, Trondheim, Norway, Trondheim, Norway;* [3]*The Blood Cell Research Group, Department of Biochemistry, Oslo University Hospital Ullevaal, Oslo, Norway, Oslo, Norway;* [4]*Department of Clinical Molecular Biology, Akershus University Hospital, Lørenskog, Norway, Lørenskog, Norway;* [5]*Department of Pathology, Oslo University Hospital Rikshospitalet, Oslo, Norway, Oslo, Norway;* [6]*Department of Research, Southern Hospital Trust, Kristiansand, Norway, Kristiansand, Norway*

Introduction: Extracellular vesicles (EVs) serve as a communication system between various cell types but also expel cellular waste. Recent reports have demonstrated that the entire mitochondrial genome can be encapsulated and secreted in EVs. The biological attributes of secreted cell-free mtDNA still remain insufficiently understood. We compared mtDNA from various blood compartments, including EVs, and tumor tissue from colorectal cancer (CRC) patients by next-generation sequencing.
Methods: Plasma EVs were isolated by size exclusion chromatography and characterized with transmission electron microcopy, nanoparticle-tracking analysis, and western blot (expression of CD9, CD63, ALIX, GM130, and APOA1). We compared the mtDNA in EVs with that in whole blood (WB), peripheral blood mononuclear cells (PBMCs), and formalin-fixed paraffin-embedded (FFPE) tumor tissue from eight rectal cancer patients. mtDNA was enriched by applying two different PCR approaches with either multiple (for FFPE tumor) or only two (for EVs, WB, and PBMCs) primer sets prior to sequencing. WB- and fresh-frozen (FF) tumor mtDNA from eight colon cancer patients was sequenced as controls for the two PCR primer

approaches. mtDNA sequence mapping and variant analyses were done using the revised Cambridge Reference Sequence and the HaploGrep2- and Variant Effect Predictor softwares.

Results: EV mtDNA presented twice as many variants with significantly more heteroplasmy (mutant mtDNA copies mixed with wild-type copies) than mtDNA from WB and PBMCs. The proportion of EV mtDNA variants that were non-synonymous (i.e., estimated to affect the mitochondrial function) was significantly higher than in WB. FFPE tumors showed a manyfold increase in detected mtDNA variants, which was not observed for FF tumors.

Summary/Conclusion: The EV mtDNA exhibited higher diversity than in the other blood compartments, with a higher degree of heteroplasmy and more variants with impact on the mitochondrial function.

OT06.03 | Can extracellular vesicles detect oxidative stress and mitochondrial dysfunction in Parkinson's disease?

Adityas Purnianto[1]; Leah C. Beauchamp[1]; Eleanor C. Saunders[2]; David P. De Souza[3]; Catriona A. McLean[4]; Ashley I. Bush[5]; David I. Finkelstein[6]; Kevin J. Barnham[6]; Laura J. Vella[6]

[1]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, VIC, Australia, Parkville, Australia; [2]The Department of Biochemistry and Molecular Biology, The University of Melbourne, Parkville, VIC, Australia, Parkville, Australia; [3]Metabolomics Australia, Parkville, VIC, Australia, Parkville, Australia; [4]Department of Anatomical Pathology, Alfred Hospital, Melbourne, VIC, Australia, Melbourne, Australia; [5]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Australia, Parkville, Australia; [6]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Australia, Melbourne, Australia

Introduction: Oxidative stress and mitochondrial dysfunction occur in the prodromal stage of Parkinson's disease (PD) which precedes motor symptoms manifestation by 10-20 years. Evidence in other diseases, such as inflammation and cancer, suggests that oxidative stress alters the composition and function of EVs. Hence, we investigated EVs as potential source of biomarkers of oxidative stress and mitochondrial dysfunction in PD.

Methods: SH-SY5Y cells were treated with low dose rotenone (mitochondrial complex I inhibitor) to induce oxidative stress and mitochondrial dysfunction while maintaining cell viability, then small EVs (sEVs) were isolated using size exclusion chromatography. Density gradient ultracentrifugation was used to isolate sEVs from human frontal cortex. Immunoblot and electron micrograph were used for validation. Oxidative stress-related contents were measured with immunoblot, proteomics and untargeted metabolomics.

Results: Subtle mitochondrial dysfunction and oxidative stress caused by low dose rotenone treatment significantly increased the level of a mitochondrial outer membrane protein and protein post-translational modifications in sEVs. These changes were more pronounced in sEVs than in the parental cells and were not observed in non-mitochondrial-related oxidative stress inducers, implying a phenomenon that is highly sensitive and specific to oxidative stress with a mitochondrial origin. Similar trends were observed in the sEVs from frontal cortex of human subjects with PD. To verify these changes, we are examining these components in sEVs from murine models of PD and human caudate and putamen brain tissue.

Summary/Conclusion: This study shows that sEVs are a sensitive reporter of oxidative stress and mitochondrial dysfunction in PD. Hence, sEVs, which are present in plasma, are candidate biomarkers of PD, particularly for early stage disease where biomarkers are currently lacking and can be used as a tool to study the early neuropathological changes that occur in the prodromal stages of the disease.

OT06.04 | Extracellular vesicles express unique characteristics in sickle cell disease, according to the mode of vesiculation : A phenotypical and functional analysis

Salwa Abid[1]; Dominique Charue[1]; Marie Lê Hoang[1]; Jean-Michel Guigner[2]; Florent Dingli[3]; Damarys Loew[3]; Dominique Helley[4]; Jean-Benoît Arlet[5]; Francois Lionnet[6]; Sylvain Le Jeune[7]; Olivier P. BLANC-BRUDE[8]

[1]Paris Center for Cardiovascular Research, Institut National de la Santé et de la Recherche Médicale U970, Université de Paris, France, Paris, France; [2]Institut de Minéralogie, de Physique des Matériaux et de Cosmochimie, IRD - CNRS UMR 7590 - MNHN, Sorbonne Université, Paris, France, P, France; [3]Institut Curie, PSL Research University, LSMP - Curie CoreTech Mass Spectrometry Proteomics, Paris, France; [4]Service d'Hématologie Biologique, AP-HP, Hôpital Européen Georges Pompidou, Paris, France, Paris, France; [5]Service de Médecine Interne, AP-HP, Hôpital Européen Georges Pompidou, Paris, France, Paris, France; [6]Service de Médecine Interne, AP-HP, Hôpital Tenon, Paris, France, Paris, France; [7]Service de Médecine Interne et d'Hypertension Artérielle, AP-HP, Hôpital Avicenne, Bobigny, France, Paris, France; [8]Paris Center for Cardiovascular Research, Institut National de la Santé et de la Recherche Médicale U970, Université de Paris Cité, France, Paris, France

Introduction: Chronic hemolytic anemia like Sickle cell disease (SCD) is associated to extracellular vesicle (EV) shedding into the circulation. EV from red blood cells (RBC) can interact with the endothelium and alter vascular function, or activate inflammatory cells, fostering vaso-occlusions. Vesiculation occurs during intravascular hemolysis, with RBC remodeling and phospholipid shuffling. EV express phosphatidylserine (PS) at their surface and carry hemoglobin and heme, which are toxic for blood vessels. However, we ignore whether EV differ only in quantity in SCD, or if EV display specificities. We compared EV shed by healthy and SCD RBC in vitro, to plasma EV.
Methods: We collected blood from SCD patients (stables) and controls. We purified RBC and saved plasmas. We stimulated RBC vesiculation in vitro by Ca2+ influx (EV-Ca2+), or shear stress (EV-shear). We characterized the phenotypes and functions of the EV produced, by cryogenic transmission electron microscopy, nanoparticle tracking (NTA), proteomic mass spectrometry, FACS, absorbance spectra, or with cardiolipin probes. We measured their activity in a test based on charged phospholipid availability (PS, cardiolipin): Thrombin activation in a modified CAT assay.
Results: In SCD, circulating EV displayed a reduced size, submembrane deposits and secondary membrane inclusions like organelle fragments. We noted the presence of cardiolipin, a charged phospholipid of mitochondriae. In vitro, SCD RBC stressed by shear or Ca2+ influx shed more EV than control RBC SCD RBC EV were similar to circulating EV : Reduced size. EV-Ca2+ were rich in submembrane deposits, and EV-shear had more secondary inclusions. Hence, the EV phenotype depended on stimulus as much as parent RBC. Hb leaked out of stressed RBC, but mainly out of EV, with either type of stimulus. Only a fraction remained bound to EV, as shown by absorbance spectra. SCD EV carried PS and cardiolipin, with an increased ability to activate thrombin, versus control EV. Recombinant Annexine-A5, a charged PL inhibitor, could abrogate the effects of all EV.
Summary/Conclusion: SCD was associated to the shedding of RBC EV with unique phenotypes into the circulation, even during steady state. SCD EV had smaller diameters, increased surface/volume ratios and expressed charged PL. They also carried deposits, probably degraded Hb, and mitochondria fragments rich in cardiolipin. Each stimulus favored EV loading with one of these features. Massive EV shedding, higher surface/volume ratio and cardiolipine suggest that circulating EV could be a major target in SCD. Cardiolipin-targeting therapeutics may thus represent a novel approach to protect hemorheology and tissue perfusion.
Funding: Inserm transfert, Fondation pour la Recherche Médicale, AddMedica.

OT06.05 | Exosome-like vesicles from Virus Mimetic-primed Human Airway Epithelium Carry Viral Material and Induce Inflammatory Response in Microglia

Aiste Jekabsone; Deimante Narauskaite; Dovydas Gecys; Aiste Masaityte; Juozas Bute; Zbigniev Balion
Lithuanian University of Health Sciences, Kaunas, Lithuania

Introduction: Viral respiratory infections induce airway cell exosomes containing viral genetic material and inflammation-related inflammatory factors. Exosomes easily cross the blood-brain barrier and can transmit the inflammatory signal to the brain. Our previous study demonstrated that rodent poly I:C-primed airway ELV enter microglial cells in the brain within an hour after intranasal delivery and induce inflammatory changes. This study aimed to investigate the effect of poly I:C-primed human bronchial epithelial cell (HBEC) exosomes on human microglial cells.
Methods: Exosome-like vesicles (ELV) were isolated from poly I:C-primed HBEC culture medium by polymer precipitation and characterized by particle number/size (Zetasizer), structure (TEM) and specific markers (ELISA). Poly I:C in ELV was monitored by DHR-poly I:C. The internalization of fluorescently-labelled ELV by human microglia (HM) was identified by fluorescent microscopy. Mitochondria and glycolysis of HM were assessed by Seahorse XFp analyzer. Intracellular ROS were determined by DCFDH, mitochondrial superoxide – by MitoSOX™ Red. Inflammasome activation was detected by caspase-1 luminescent assay. Inflammatory gene expression was assessed by RT-PCR, protein expression - by Western Blot. Statistical analysis was performed by SigmaPlot 13.0.
Results: Over 95% of the EVs produced by poly I:C-primed HBEC were 40 - 140 nm in diameter, surrounded by a lipid bilayer and contained CD9, CD63, CD81, syntenin-1, Tsg101, but not calnexin and Hsp70. ELV carried poly I:C molecules and were internalized by HM cells within 2 hours of the treatment. Poly I:C-primed HBEC ELV induced suppression of mitochondrial respiration and activation of glycolysis, generation of intracellular and mitochondrial ROS and activation of NLRP3 inflammasome-related caspase-1 followed by IL-1beta and IL-18 secretion, and a significant increase in expression of IL-6, TNF-alfa and PTGS2 genes and proteins. None of the effects was observed after treating HM with not poly I:C-primed HBEC exosomes. Moreover, untreated HBEC exosomes significantly reduced the expression of the inflammatory genes.
Summary/Conclusion: In HM, poly I:C-primed HBEC exosomes induce the transition of energy production phenotype from mitochondrial to glycolytic and stimulate inflammatory activation manifested as ROS generation, inflammasome activation and production of pro-inflammatory cytokines. On the contrary, exosomes from the untreated HBEC have an anti-inflammatory effect on HM.

Funding: The study is a part of the EXOTARGET project funded by grant No. 01.2.2-LMT-K-718-01-0036 from the Research Council of Lithuania.

OT07: EV Production and Separation

Chair: Amanda Brun-Silva – Université Sorbonne Paris Cité, Laboratoire Matière et Systèmes Complexes, CNRS UMR 7047 Université Paris Diderot, France

Chair: Lorena Martin-Jaular – Institut Curie, PSL Research University, INSERM U932

OT07.01 | Gentle method for the purification of extracellular vesicles using liquid polymer droplets

Carolina Paganini[1]; Umberto Capasso Palmiero[1]; Mauro Manno[2]; Antonella Bongiovanni[3]; Paolo Arosio[1]
[1]Institute for Chemical and Bioengineering, ETH Zurich, Zurich, Switzerland, Zurich, Switzerland; [2]Cell-Tech HUB at Institute of Biophysics (IBF), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy; [3]Cell-Tech HUB at Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy

Introduction: Successful application of extracellular vesicles (EVs) as biotherapeutics relies on the ability to produce large amounts of EVs (Paganini et al, Biotech. J., 1800528, 2019). Current techniques are either not scalable, or yield EVs with low purity, or require multiple concentration and diafiltration steps that lower the final EV yield. There is a need for scalable high-throughput purification techniques that yield high quantities of pure EVs in short time. Here, we present a novel method to purify EVs based on polymers that undergo liquid-liquid phase separation and form liquid droplets which can be designed to selectively recruit the species of interest and exclude impurities.
Methods: Liquid droplets of responsive polymers recruit 293F-EVs at low salt concentrations and release them at high salt concentrations. The process steps, the polymer composition and the salt concentration are adjusted to separate the EVs from DNA and protein impurities.
Results: The polymer droplets allow to uptake and concentrate EVs from a solution after only one minute of incubation. We demonstrate that EVs are released intact from the droplets which prevent the formation of solid EV precipitates and make this a gentle purification method. The separation of EVs by ion exchange with the droplets allows to simplify the setup with respect to current technique while guaranteeing comparable purity.
Summary/Conclusion: We present a novel method to purify EVs which exhibits a series of advantages with respect to conventional approached in terms of scalability, purity and simplicity of operation. This method is a promising addition to the existing tools for EV isolation, in particular for high-throughput purification of EVs.
Funding: H2020-EU.1.2.1-FET Open programme via the VES4US Grant agreement 801338
H2020-EU.1.2.2-FET Proactive programme via the BOW Grant agreement 952183

OT07.02 | Pre-processing of bovine milk prior to EV isolation is essential for purity, but various protocols affect colloidal and functional properties of milk EVs

Martijn van van Herwijnen[1]; Marije Kleinjan[1]; Francesca Loria[2]; Andres Lõhmes[3]; Natasa Zarovni[3]; Anna Carnerup[4]; Tommy Nylander[4]; Andrea Ridolfi[5]; Marco Brucale[5]; Francesco Valle[5]; Lucrezia Caselli[6]; Lucia Paolini[7]; Paolo Bergese[7]; Marca H.M Wauben[8]
[1]Utrecht University, Utrecht, Netherlands; [2]HansaBioMed Life Sciences Ltd, Tallinn, Estonia; [3]HansaBioMed Life Sciences Ltd,, Tallinn, Estonia; [4]Lund University, Lund, Sweden; [5]Italian National Research Council (CNR), Institute for the Study of Nanostructured Materials (ISMN), Bologna, Italy, Bologna, Italy; [6]Università degli Studi di Firenze, Firenze, Italy; [7]Università degli studi di Brescia, Brescia, Italy; [8]Division of Cell Biology, Metabolism & Cancer, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands, Utrecht, Netherlands

Introduction: Although bovine milk is rich in extracellular vesicles (EVs), other abundant components like casein micelles hamper the isolation of pure EVs. Pre-clearing milk by precipitation or disruption of caseins overcomes this issue. However, the impact of pre-clearing on EV integrity and function has not been investigated. We compared three different protocols used to reduce casein micelles to untouched milk and analyzed the effects of pre-processing on subsequently isolated EVs.
Methods: Raw bovine milk was subjected to differential centrifugation after which milk remained untouched, or caseins were cleared by precipitation due to acidification with hydrochloric acid (HCl), or caseins were disruption by addition of EDTA or

sodium citrate. EVs were further purified using density gradient floatation and Size Exclusion Chromatography (SEC). EV purity was assessed by Augmented COlorimetric NANoplasmonic (CONAN), total protein, total RNA, phospholipid and cholesterol; presence of EV markers by Western blotting (WB); morphology and size by Cryo-Electron Microscopy, Atomic Force Microscopy (AFM), Nano Tracking Analysis (NTA) and Dynamic Light Scattering (DLS). Additionally, colloidal properties including Zeta potential, membrane stiffness and adhesion to lipid surfaces were determined. Functionality of EVs was analyzed in a Xanthine Oxidase activity assay and an in vitro T cell modulation assay.

Results: Clearing casein micelles from bovine milk prior to density gradient separation and SEC greatly enhanced the purity of the milk EV samples in all tested methods, with acidification giving the purest samples. However, precipitation of caseins by acidification influenced size, lipid surface interactions and gave a loss in vitro functionality. EDTA treatment also affected EVs in their size, but also membrane stiffness and T cell modulation. Only sodium citrate treatment did not influence most of the evaluated EV properties.

Summary/Conclusion: Using a comprehensive analysis, we were able to show that pre-processing of bovine milk is needed and greatly enhances purity of milk EV samples, but can also influence the functionality and colloidal properties of milk EVs. For instance, acidification has detrimental effects on milk EVs. Therefore, for isolation of bovine milk EVs, we advise to carefully consider which pre-processing to apply. Based on our results we recommend the use of sodium citrate for the clearance of caseins in bovine milk.

Funding: This work was supported by the European Union's Horizon 2020 Framework Programme under the grant FETOPEN-801367 evFOUNDRY

OT07.03 | Isolation of plasma EVs by dual-mode chromatography: a head-to-head comparison between Sepharose CL-2B and Sepharose CL-4B

Jillian Bracht[1]; Mandy Los[1]; Edwin van der Pol[2]; Rienk Nieuwland[3]
[1]Department of Clinical Chemistry, Vesicle Observation Center, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Amsterdam, Netherlands; [2]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Amsterdam, Netherlands; [3]Department of Clinical Chemistry; Vesicle Observation Center; Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands

Introduction: Plasma contains extracellular vesicles (EVs) and non-EV particles, platelets, lipoproteins and chylomicrons, that overlap in size and density with EVs, and which hamper isolation of EVs. Recently, dual-mode chromatography (DMC) was introduced, in which Sepharose CL-4B was combined with Fractogel. We investigated the effect of each resin separately on plasma fractionation by measuring the presence of platelet (CD61+), leukocyte (CD45+) and erythrocyte (CD235a+) EVs, (very) low density lipoproteins ((V)LDL), and proteins.

Methods: Human platelet-free plasma was fractionated by size exclusion chromatography (SEC) using sepharose CL-4B and SEC CL-2B. EV-containing fractions were collected, pooled, and run over Fractogel (type EMD SO3- (M)) columns. The EV concentrations were measured by Apogee A-60 flow cytometry (150-1,000 nm), and (V)LDL and protein concentrations were determined using a human Apolipoprotein B ELISA and Bradford protein assay, respectively.

Results: Compared to SEC CL-2B, EV isolation by SEC CL-4B resulted in a 2-fold higher EV yield, also in a 28-fold higher (V)LDL concentration, and a 14-fold higher protein concentration. Fractogel efficiently removed >90% of the (V)LDL, but also reduced the EV yield about 2-fold.

Summary/Conclusion: Although the EV yield is higher when using SEC CL-4B, the purity of EVs is better when using SEC CL-2B. Fractogel efficiently removes (V)LDL, but in our experiments the highest purify of EVs was obtained by combining SEC CL-2B followed by Fractogel.

Funding: This study is funded by Health Holland (AQrate project)

OT07.04 | Plant suspension culture derived extracellular vesicles

Batuhan T. Bozkurt; Oğuz K. Kırbaş; Pakize N. Taşlı; Bahar Soğutmaz Özdemir; Fikrettin Şahin
Yeditepe University, Istanbul, Turkey

Introduction: Despite their ubiquitous presence across life, studies on extracellular vesicles (EVs) remain mostly limited to the study of mammalian EVs. Plant derived EVs appear to be a particularly promising area for further research - both as therapeutic agents against human diseases, and as an undiscovered element of plant physiology. However, the lack of standardized isolation and characterization methods is a major obstacle for the study of plant EVs. Some of the earlier studies that form the majority

of plant EV research isolate vesicles from whole-plant tissues using harsh isolation procedures and do not provide adequate characterization of the vesicles used in their studies. A method similar to the in vitro production of mammalian vesicles using cell cultures is necessary for further developments in plant EV research.

Methods: We developed a workflow that uses plant cell suspension cultures as the source of plant EVs. Using these cultures, we isolated large and small EVs of Nicotiana tabacum, Stevia rebaudiana, and Vitis vinifera using density cushion ultracentrifugation. Isolated vesicles were physically characterized using electron microscopy and nanoparticle tracking analysis. Proteomic analysis of cells and sEVs of the model plant N. tabacum were performed to determine potential plant EV biomarkers.

Results: A total of 135 proteins were identified in N. tabacum sEVs, which exhibited similar gene ontology enrichment profiles to human EVs. Comparative proteomic analyses were made between N. tabacum sEVs with N. tabacum cell proteome, as well as with six previously published plant proteomes. By comparing the protein sequences of each proteome with top 300 human EV proteins, we identified 24 potential plant EV biomarkers that were present in at least five of the plants, revealing a degree of homology in the EV secretion mechanisms between plant species.

Summary/Conclusion: Here, we demonstrated the potential of plant cell suspension cultures as a promising alternative to plant lysates for the isolation of plant EVs. Our proteomics studies suggests a number of potential plant EV biomarkers, which show a degree of conservation between different plant species. Finally, the high degree of homology observed between human and plant EV proteins suggests that they may take part in cross-kingdom interactions, with potential therapeutic effects.

OT07.05 | Biocompatible photonic hydrogels for the isolation and detection of extracellular vesicles

Raquel S. Vaz; Nayara Freitas; M. Goreti G. F. Sales; Manuela F. Frasco
BioMark@UC, Faculty of Sciences and Technology, University of Coimbra, Coimbra, Portugal, Coimbra, Portugal

Introduction: Extracellular vesicles (EVs) are crucial in intercellular communication at physiological and pathological processes, but their complexity and heterogeneity have delayed clinical translation. Biosensors try to overcome current limitations in the isolation and detection of EVs, enabling sensitive, simple, and low-cost technologies. Molecularly imprinted polymers (MIPs) are highly selective for a given analyte, thus being able to mimic natural recognition molecules. MIPs have physical and chemical stability and the polymeric matrix can be developed with biocompatible materials. Furthermore, colorimetric photonic-based assays are promising label-free detection methods in biosensing.

Methods: Herein, the biosensor composed of biocompatible materials is constructed by infiltration of a pre-polymer mixture within the photonic crystal (PC). The PC is first developed by dip-coating deposition of colloidal spheres of silica. Also, liposomes as mimics of EVs are used as templates to generate the imprinted polymer. After removal of the liposomes, specific recognition cavities assure the selective recognition of the biomarker.

Results: The combination of biocompatible materials and PCs conducted to an easy-to-use biosensor with high intensity colours. The MIP hydrogel could be differentiated from the control at naked eye, due to colour differences. The biosensor performance when the target is present is followed by changes in the reflectance spectra.

Summary/Conclusion: The alliance of highly selective MIPs with the label-free PC-based detection is expected to offer great EVs recognition ability. The aim is to obtain a biosensor with a low detection limit, fast response, high selectivity, repeatability and reproducibility.

Funding: The authors acknowledge the financial support from the European Commission/H2020, through MindGAP/FET-Open/GA829040 project. The author RV also acknowledges Fundação para a Ciência e a Tecnologia for the PhD grant (2020.09673.BD).

OT07.06 | ExoArc: A scalable microfluidic technology for label-free EVs isolation from whole blood

Hui Min Tay[1]; Hong Boon Ong[2]; Sheng Yuan Leong[3]; Wan Wei Lok[2]; Rinkoo Dalan[4]; Han Wei Hou[5]
[1]School of Mechanical and Aerospace Engineering, Nanyang Technological University, singapore, Singapore; [2]School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore, Singapore; [3]School of Mechanical and Aerospace Engineering, Nanyang technological University, Singapore, Singapore; [4]Endocrine and Diabetes, Tan Tock Seng Hospital, Singapore, Singapore; [5]School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore 639798, Singapore, Singapore, Singapore

Introduction: Circulating extracellular vesicles (EVs) in blood may contain disease-specific biomolecular cargo, making them ideal disease diagnostics biomarkers. Current gold-standard for EVs isolation is differential ultracentrifugation (UC), which is

labour intensive, time-consuming, and prone to losses. Herein, we report a novel microfluidic technology (ExoArc) for high-throughput EVs separation from blood and culture media directly. ExoArc is label-free, scalable, and offers significantly higher EVs yield and reproducibility with its single-step operation.

Methods: The ExoArc chip has a 2-inlet, 2-outlet half-loop channel design. Blood, plasma or culture media sample is introduced at channel outer wall and pinched by a sheath flow. Small EVs (50-200nm) migrate faster laterally toward the channel inner wall under the influence of Dean vortices and are sorted into EV outlet, while medium-sized EVs (200-1000nm), cell debris (>1μm), and blood cells (~2 to 20mm) experience stronger Stoke's Drag and are removed via the waste outlet.

Results: We performed EVs isolation from culture media using ExoArc in a single step (no centrifugation) to achieve superior EV yield and ~8x shorter processing time than UC (~1hr for 10mL media). Particle count by nanoparticle tracking analysis was less heterogenous as compared to UC, suggesting better reproducibility with the single-step operation. For blood EVs isolation, ExoArc-isolated particle yield was ~10x higher than UC, and western blot showed thicker bands for EV markers (TSG101, CD9, CD81) per unit volume, implying higher EV concentration. To demonstrate scalability, a dual ExoArc device (2 ExoArc subunits) was developed to achieve a throughput of 0.2 mL/min of blood processing (fastest to-date for microfluidics EV processing).

Summary/Conclusion: We have developed a rapid, scalable and microfluidic-based approach for direct isolation of small EVs from blood and culture media. With future automation, ExoArc can further advance exosome biology research and development of exosome diagnostic tools.

Funding: SMART Innovation Centre (ING-001058 BIO IGN) and NTU Startup grant

OT08: Cell to Neuron Communication

Chair: Ursula S. Sandau – Oregon Health & Science University

OT08.01 | Extracellular vesicle-mediated crosstalk between pancreatic cancer and Schwann cells

Fang Cheng Wong[1]; Christoph Kahlert[2]

[1]*University Hospital Dresden, Dresden, Germany;* [2]*Department of Visceral, Thoracic and Vascular Surgery, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany, Dresden, Germany*

Introduction: Neural invasion (NI) involves the growth and invasion of cancer cells around and into nerves, a process that has been known as a hallmark of pancreatic adenocarcinoma (PDAC) due to its extremely high incidence rate (>90%). NI has been reported to occur at the early stage of PDAC development and glial cells of peripheral nerves, i.e. Schwann cells (SCs) have been shown to be attracted to cancer cells before the onset of cancer invasion. However, the mechanisms leading to NI have not been completely understood. We hypothesized that PDAC-derived extracellular vesicles (EVs) mediate the migration of SCs towards the cancer site and the reciprocal interactions between cancer and SCs eventually lead to neural invasion.

Methods: A 3D coculture assay with primary SCs confronted to cancer/normal epithelial cells was first conducted to evaluate the chemoattraction affinity of SCs towards cancer cells. To investigate the role of EVs in mediating migration of SCs, EVs were isolated from cancer cell lines and tissues by differential ultracentrifugation and characterised according to the MISEV2018 guidelines. A novel 3D migration assay incorporating SCs and EVs in a single Matrigel drop was employed to evaluate the migratory ability of SCs in response to EVs, which was further confirmed using EV uptake inhibitors. Protein alterations in SCs treated with tissue-derived EVs was examined by mass spectrometry.

Results: The 3D coculture assay revealed that human SCs (hSCs) migrated towards PDAC cell lines, but not to the normal cells. It was further confirmed that murine SCs (mSCs) exhibited higher migratory ability when confronted to murine neuroinvasive PDAC cells than to non-neuroinvasive PDAC cells, which could be reversed by heparin. The 3D migration assay demonstrated significantly stronger migratory ability of hSCs/mSCs after EV treatment from human PDAC/murine neuroinvasive cancer cells than that of the human normal cell-/murine non-neuroinvasive cancer cells, which could be reversed by heparin. At a translational aspect, the increased migration of hSCs could also be observed after treatment of tumor tissue-derived EVs from PDAC patients with neural invasion (Pn1) compared to those diagnosed with no neural invasion (Pn0) pathologically, indicating the relevance of neuroinvasive cancer derived-EVs in mediating SC migration. Proteomic analyses indicated that hSCs treated with tumor-derived EVs displayed significant changes in the expression levels of cytoskeleton and phosphoproteins.

Summary/Conclusion: PDAC-derived EVs may be responsible for the migration of SCs towards cancer cells, underscoring the mechanisms underlying the SC carcinotropism in PDAC-associated NI.

Funding: Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), Grant Numbers: DE 2428/10-1, KA 3511/5-1.

OT08.02 | "Changes in human astrocyte-enriched extracellular vesicle miRNAs after an in vivo lipopolysaccharide challenge"

Leandra K. Figueroa-Hall[1]; Kaiping Burrows[1]; Ahlam M. Alarbi[2]; Chibing Tan[2]; T. Kent Teague[2]; Brett A. McKinney[3]; Rajagopal Ramesh[4]; Yoon-Hee Cha[5]; Salvador Guinjoan[6]; Sahib S. Khalsa[1]; Victoria B. Risbrough[7]; Martin P. Paulus[1]; Jonathan B. Savitz[1]

[1]Laureate Institute for Brain Research, TULSA, USA; [2]University of Oklahoma, TULSA, USA; [3]University of Tulsa, TULSA, USA; [4]University of Oklahoma, Oklahoma City, USA; [5]University of Minnesota, Minneapolis, USA; [6]Laureate Institute for Brain Research, Tulsa, USA; [7]University of California, San Diego, La Jolla, USA

Introduction: A subset of individuals with major depressive disorder (MDD) show altered inflammatory responses but the precise mechanisms are not understood. Low-dose lipopolysaccharide (LPS) is a useful experimental model of several inflammatory diseases, including MDD. Identifying the pattern of microRNAs (miR) produced in the brain in response to an inflammatory challenge may help elucidate mechanistic pathways that are putatively dysregulated in inflammation-associated depression. In this pilot study, we leveraged a low-dose lipopolysaccharide (LPS) challenge (0.8 ng/kg) in adults with MDD and healthy comparisons (HC) to investigate the temporal changes in inflammatory-responsive miR in astrocyte-enriched (AE) extracellular vesicles (EV) at baseline (T0), 2 (T2), and 24 (T24)-hours post LPS infusion.

Methods: This study was approved by the Western Institutional Review Board, participants' written informed consent obtained, and carried out in accordance with the principles expressed in the Declaration of Helsinki. Combined results from MDD (n=8) and HC (n=5) receiving low-dose LPS are presented here. Human serum was used to isolate EV with a polymer-based kit and for enrichment of AEEV by a streptavidin magnetic bead immunocapture kit with a (GLAST)-biotinylated antibody. Characterization included western blot and flow cytometry to confirm AEEV populations, and nanoparticle tracking analysis for size estimation and concentrations. For sequencing, miRNA was extracted from AEEV and sample concentration and small RNA quantification were determined. Raw sequencing data files were analyzed with Bowtie Alignment and miRNAs normalized to counts per million and log(e) transformed. EV-Track # EV220001

Results: Relative to T0, lower levels of miR-let-7f-5p (Cohen's d= 0.78) and miR-374a-5p (d= 0.80) were observed at T2. Higher concentrations of miR-let-7f-5p (d= 1.29), miR-122b-3p (d= 1.13), and miR-374a-5p (d= 1.00), and lower concentrations of miR-101-3p (d= 0.83), miR-15b-5p (d= 1.22), miR-29c-3p (d= 0.55), miR-451a (d= 0.98), and miR-486-5p (d= 0.86) were observed at T24 vs. T2. There were no changes in miRNA expression at T0 versus T24.

Summary/Conclusion: Our initial findings suggest that peripheral LPS administration is associated with altered regulation of astrocyte-associated pathways. Next steps include investigating a larger sample to determine whether these miRNAs are differentially expressed in MDD relative to HC.

Funding: This work has been supported in part by The William K. Warren Foundation, the National Institute of Mental Health (R21MH11387; R01MH123652), and the National Institute of General Medical Sciences (P20GM121312).

OT08.03 | Addressing the role of extracellular vesicles and nanotube processes in neuronal communication; lessons from photoreceptor transplantations

Aikaterini A. Kalargyrou; Mark Basche; Emma West; Alexander Smith; Robin Ali; Rachael Pearson

Kings College London, London, United Kingdom

Introduction: Neuronal cells typically communicate via synapses and gap junctions. Recently, we and others have shown that transplantation of donor photoreceptors into partially dystrophic retinae can restore aspects of visual function, but that this occurs via a mechanism of molecular exchange, so-called "material transfer", with remaining host photoreceptors. Here, we examined the potential role of extracellular vesicles (EVs) and nanotube (NTs) processes in vivo during host-graft interactions in the retina.

Methods: Cre recombination, Electron Microscopy, Confocal live imaging, Fluorescence recovery after photobleaching, Photoreceptor transplantations, Ocular administration of extracellular vesicles, Chimeric retinae, Genetic manipulation of actin dynamics.

Results: Photoreceptors release active EVs that are specifically taken up by Muller glia, but form NTs that mediate material transfer with other photoreceptors, in vivo. NTs permit the transfer of cytoplasmic and membrane-bound molecules and, rarely, lysosomes and mitochondria and can bring about gain-of-function in the acceptor cells. Strikingly, chimeric retinae revealed that cytoplasmic transfer can occur between photoreceptors in the intact adult retina.

Summary/Conclusion: Photoreceptor nanotubes mediate material transfer during photoreceptor transplantation in an actin dependent manner. These neurons also release EVs that are preferentially up-taken by Muller glia. This study provides the first evidence of nanotube processes formation and function between sensory neurons in vivo.

Funding: This work was supported by Fight for Sight UK (1566/1567), Moorfields Eye Charity (E170004A; R180005A); Medical Research Council UK (MR/J004553/1 and MR/T002735/1).

OT08.04 | The role of EVs in septic encephalopathy immunometabolism: a novel therapeutic target

Ioannis Koutroulis[1]; Panagiotis Kratimenos[1]; Claire Hoptay[2]; Evan Goldstein[1]; Wade O'Brien[1]; Beata Jablonska[1]; Vittorio Gallo[1]; Robert J. Freishtat[1]
[1]Children's National Hospital, Washington, USA; [2]Children's National Research Institute, Washington, USA

Introduction: Metabolic encephalopathy affects one in three intensive care unit (ICU) patients, with the most serious subtype occurring in the context of sepsis [septic encephalopathy (SE)], which is associated with up to 70% mortality. The cerebellum is particularly sensitive to injury early in the SE process. Here we utilized mesenchymal stem cell-derived extracellular vesicles (MSC EVs) to examine immunometabolic dysfunction in metabolic encephalopathy using a murine model of SE.
Methods: We administered cecal contents by an intraperitoneal injection to 7-10-week-old, C57BL/6J mice to induce sepsis/SE. Encephalopathic and control mice received either MSC EVs or EV-depleted media intravenously 6 hours post-sepsis induction. Mice were scored every 2 hours until euthanasia (score equal or greater than 15) using a sepsis scoring system that also evaluates neurological status. We obtained cerebellar samples (n=8/group) for Hematoxylin-Eosin (H&E) staining. Total RNA was isolated from cerebellar samples (n=3/group) and was sequenced to identify differentially expressed genes (DEGs).
Results: Average scores at sacrifice were lower in the EV-treated group compared to non-treated mice with SE, indicating improved disease severity. H&E staining on septic cerebella revealed significant neuropathological alterations compared to controls, including shrunken Purkinje and granule cells with irregular nucleus and absent nucleolus, acidophilic (pink) cytoplasm, apoptotic profiles, and disrupted neuropil. Additionally, when comparing the DEGs in RNA seq, 103 downregulated and 119 upregulated genes were identified in the cerebella of EV-treated mice with SE, as compared to the non-treated group. Using the Ingenuity Pathway Analysis software, we showed that neuroinflammatory pathways remained activated in treated mice, while the IL-6 signaling pathway was inhibited. In addition to immune pathways, metabolic signaling pathways that promote ATP generation including PI3K/AKT, IGF-1, AMPK and PPARa were also activated, suggesting an SE-related decreased intracellular energy production.
Summary/Conclusion: We demonstrated that EVs alter cerebellar immune and metabolic pathways that may be responsible for the SE-induced neuropathological changes and poor outcomes. Targeting specific immunometabolic signaling pathways that promote energy generation and alleviating immune dysregulation present an opportunity to successfully treat septic and other metabolic encephalopathies.
Funding: K12-HD-001339 (NICHD)

OT08.05 | An altered extracellular vesicle protein cargo drives the (mis)communication between astrocytes and neurons in ALS

Manuela Basso[1]; Alice Migazzi[2]; Paolo Vincenzo Fioretti[2]; Giulia Canarutto[3]; Laura Pasetto[4]; Fabio Fiordaliso[4]; Daniele Peroni[5]; Romina Belli[2]; Silvano Piazza[3]; Toma Tebaldi[2]; Vito Giuseppe D'Agostino[6]; Maria Pennuto[7]; Valentina Bonetto[4]
[1]Department of Cellular, Computational and Integrative Biology – CIBIO, University of Trento; Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano (Italy)., Trento, Italy; [2]Department of Cellular, Computational and Integrative Biology – CIBIO, University of Trento, Trento, Italy; [3]International Centre for Genetic Engineering and Biotechnology, Trieste, Italy; [4]Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano, Italy; [5]Department of Cellular, Computational and Integrative Biology – CIBIO, University of Trento, Milano, Italy; [6]Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy; [7]Department of Biomedical Sciences (DBS), University of Padova, Padova, Italy

Introduction: Amyotrophic Lateral Sclerosis (ALS) is a progressive and devastating motor neuron disease that affects upper and lower motor neurons. Among several factors, the progression of the disease is mediated by altered intercellular communication in the spinal cord between neurons and glial cells. One of the possible ways in which the cell-to-cell communication occurs is through extracellular vesicles (EVs) that transport proteins, lipids and nucleotides from one cell to the other.
Methods: To characterize EVs in ALS, we used a charged-based methodology, called nickel-based isolation (NBI), allowing rapid and efficient isolation of EVs. By this approach, it is possible recover not only a high and pure amount of astrocyte derived EVs, but also to preserve the integrity and the stability of vesicles and analyze their cargo content. We tested the effect of glial EVs on receiving neurons and motor neurons by using Operetta® and we analyzed the effect of EVs on receiving neurons by RNA sequencing and immunofluorescence. We validated our results on purified astrocyte and microglia populations derived from mice carrying the mutation in TDP-43Q331K.

Results: Our preliminary results suggest that EVs derived from astrocytes of a transgenic mouse model of ALS, overexpressing TDP-43Q331K, transmit toxicity to wild type neurons by activation of cell death pathways. We further focused on which component of the EVs would be responsible for propagation of toxicity. We performed an unbiased characterization of the protein and RNA cargos through a proteomic and small RNA sequencing analysis. Small RNA sequencing did not reveal notable differences between samples. Interestingly, the protein cargos differ significantly between control and disease condition, suggesting that the change of protein messages delivered to receiving neurons by EVs is sufficient to start neuronal damage.
Summary/Conclusion: Changes in the protein loading of glia EVs in ALS contributes to motoneuronal toxicity.
Funding: Supported by the MSCA-IF ExItALS (752470) and the Italian Ministry of Health (GR-2016-02361552).

OT08.06 | Cocaine modulates the neuronal endosomal system and extracellular vesicles in a sex-dependent manner

Efrat Levy; Pasquale D'Acunzo
Nathan S. Kline Institute for Psychiatric Research, New York, USA

Introduction: It was previously demonstrated that repeated administration of cocaine causes alterations in endocytic and lysosomal compartments. We previously found inter-relationship between neuronal endosomal abnormalities and brain exosomes in neurodevelopmental and neurodegenerative disorders, including Down syndrome and spinocerebellar ataxia. Therefore, we conducted a comprehensive analysis of extracellular vesicles (EVs) in the brain of a mouse model of cocaine addiction.
Methods: C57BL/6J mice received either cocaine or saline injections for two weeks starting at 2.5 months of age and the effect on locomotor activity investigated. EVs were isolated from the brain using a high-resolution iodixanol gradient that separates EV fractions enriched in microvesicles and exosomes. Morphology, number, and content of EVs were analyzed by electron microscopy, NTA and western blotting. Size and number of neuronal endosomes were studied by immunohistochemistry using antibodies to early (Rab5) and late (Rab7) endosome markers. The role of female hormones in the response to cocaine was studied in ovariectomized females as compared to sham-surgery controls.
Results: Drug-induced sex differences were found with higher locomotion activity in female mice when compared to males. Cocaine caused a decrease in the number of both neuronal endosomes and exosomes (but not microvesicles) in the brains of male but not female mice. Cocaine increased the amount of α-synuclein per exosome in the brain of females but did not affect exosomal α-synuclein content in the brains of males. The response to cocaine in ovariectomized females mirrored males.
Summary/Conclusion: The reduction of exosome levels in the brain of males and enhanced packaging of α-synuclein into female brain exosomes suggest a mechanism for the higher addictive response of females to cocaine compared to males.
Funding: Supported by NIH (DA044489).

OT09: Various Approaches for Therapeutic Applications of EVs

Chair: Rodolphe W. Poupardin – Cell Therapy Institute, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI - TReCS), Paracelsus Medical University (PMU), Salzburg, Austria

Chair: Yanis Mouloud – Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

OT09.01 | Extracellular Vesicles from Hair Follicle-derived Mesenchymal Stromal Cells: Isolation, Characterization and Therapeutic Potential for Chronic Wound Healing

Kevin Las Heras[1]; Felix Royo[2]; Clara Garcia-Vallicrosa[2]; Manoli Igartua[1]; Edorta Santos-Vizcaino[1]; Rosa Maria Hernandez[1]; Juan M. Falcon-Perez[3]
[1]NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy (UPV/EHU)., Vitoria-Gasteiz, Spain; [2]3Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Exosomes Laboratory, 48160 Derio, Spain., Derio, Spain; [3]Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Exosomes Laboratory, 48160 Derio, Spain., Derio, Spain

Introduction: There has been an increasing interest in the use of hair follicle-derived mesenchymal stromal cells (HF-MSCs) for chronic wounds healing due to their demonstrated regenerative potential and their numerous advantages over other sources of MSCs — abundant availability of HFs and an eas collection not affected by gender or age —. In this regard, the paracrine mediators of HF-MSCs have been considered as the main responsible of these effects. However, the properties and bioactivity of the extracellular vesicles (EVs) secreted by HF-MSCs (HF-EVs) remain unknown. We reported here a complete isolation, characterization and functional comparison of HF-EVs against EVs secreted by adipose tissue-derived MSCs (AT-EVs).

Methods: HF-EVs and AT-EVs were isolated by diferential ultracentirfugation (EV-TRACK ID: EV210337). EVs were analyzed by nanotracking analysis and cryo-electron microscopy. Marker analysis was performed by western blotting and immunoafinity beads-based flow cytometry. Cell uptake, proliferation and migration studies were performed in adult human dermal fibroblasts (HDFs). The angiogenic potential of EVs was tested on the tube formation assay using human umbilical vein endothelial cells (HUVECs). Finally, the protective activity of EVs against hyperglicaemia induced cytotoxicity was assayed in HDFs.
Results: HF-EVs as well as AT-EVs showed the typical EV-markers CD9, CD63, CD81 and LAMP-1 among other interesting markers — CD44, CD29, SSEA-4 —. Moreover, we found that AT-MSCs produced larger quantities of EVs than HF-MSCs compared to the final cell count. We also found that both EVs, equally, were able to increase the proliferation and migration of HDFs, as well as increase the tube formation of HUVECs. Finally all EVs were able to protect HDFs against hyperglicaemia induced cytotoxicity to the same extent.
Summary/Conclusion: EVs derived from HF-MSCs may be promising candidates for the treatment of chronic wounds, as they enhance proliferation, migration and protection against hyperglicaemia in HDFs and also induce angiogenesis in endothelial cells.
Funding: Basque Government: PRE_2018_1_0412 and Consolidated Groups, IT 907-16.

OT09.02 | A phase I clinical trial for safety determination of the intra-articular administration of umbilical cord mesenchymal stromal cells-derived small extracellular vesicles in patients with knee osteoarthrosis

José Matas[1]; Eliseo Parra[2]; Aliosha I. Figueroa-Valdés[2]; Jimena Cuenca[3]; Yessia Hidalgo[4]; María Ignacia Cádiz[5]; Celeny Figuera[5]; Dario Donoso Meneses[3]; Fernando Figueroa[3]; Maroun Khoury[4]; Francisca Alcayaga-Miranda[3]; Francisco Espinoza[1]
[1]Clínica Universidad de los Andes, Santiago, Chile; [2]Laboratory of Nano-Regenerative Medicine, Centro de Investigación e Innovación Biomédica (CiiB), Universidad de los Andes, Santiago, Chile, Santiago, Chile; [3]Universidad de los Andes, Santiago, Chile; [4]IMPACT, Center of Interventional Medicine for Precision and Advanced Cellular Therapy, Santiago, Chile, Santiago, Chile; [5]Consorcio Regenero S.A., Santiago, Chile

Introduction: Osteoarthrosis (OA) is a disease characterized by articular cartilage loss associated with degenerative joint and subchondral bone changes. Currently, no therapeutic strategy has proven to be effective for OA treatment. Our previous results showed that small extracellular vesicles (sEV) derived from umbilical cord mesenchymal stromal cells (UC-MSC; CelliStem®OA-sEV) application in preclinical OA models evidenced a decrease in OA progression. Following the completition of the pre-clinical package, including safety and biodistribution, we initiated an evaluation in patients with knee OA to clarify the safety of using this emerging acellular therapy. A feasibility and security study was proposed.
Methods: Patients were recruited following informed written consent according to the declaration of Helsinki and the study was approved by the institution ethics committee. sEV were produced and isolated under Good Manufacturing Practice (GMP) conditions by differential centrifugation and then characterized following the MISEV 2018 guidelines by nanoparticle tracking analysis, flow cytometry, western blot and transmission electron microscopy.
Results: GMP-grade sEV showed a size of 140 nm and classical cup-shape morphology; their identity was confirmed by the presence of CD63, CD9, CD81, Flotillin-1, Syntenin-1 and their purity were verified by the absence of Calnexin and TOMM20. UC-MSC-derived sEV treated patients (n = 10) will be followed to determine the percentage with any post-infiltration adverse reaction, synovitis or pain (days 7 and 30), the percentage with an adverse event away of the intra-articular infiltration (days 30, 90 and 180), WOMAC score changes (days 30, 90 and 180) and VAS pain score changes (days 30, 90 and 180).
Summary/Conclusion: The findings will be of translational relevance for a cell-free sEV-based OA therapy. To our knowledge, this is the first-in-joint clinical trial for the use of sEV derived from allogeneic MSC for the treatment of OA.
Funding: Cells for Cells S.A., Chile; Consorcio Regenero S.A., Chile; FONDECYT #1211749, ANID, Chile; IMPACT #FB210024, ANID, Chile.

OT09.03 | Scalable mass production of Gram-negative bacterial outer membrane vesicles for the next-generation cancer immunotherapeutic agents

Yong Song Gho[1]; Jaemin Lee[1]; Changjin Lee[2]; Solchan Won[3]; Sunghyun Song[2]; Seoyoon Bae[1]; Jaewook Lee[4]; Tae Ryong Lee[2]; Dong-Sup Lee[3]
[1]POSTECH, Pohang, Republic of Korea; [2]Rosetta Exosome Co. Ltd, Seongnam, Republic of Korea; [3]Seoul National University College of Medicine, Seoul, Republic of Korea; [4]Rosetta Exosome Co. Ltd, Pohang, Republic of Korea

Introduction: For intercellular and interkingdom communication, both Gram-negative and Gram-positive bacteria actively released extracellular vesicles (EVs) into extracellular milieu. Recently, our group discovered that Gram-negative and Gram-

positive bacterial EVs have potent anti-tumor activities by inducing a sustainable anti-tumor immune response in several mouse tumor models (Kim et al. 2017). However, it is difficult to produce large quantities of bacterial EVs for pre-clinical studies and clinical trials. This study aims to develop manufacturing processes for scalable mass production of Escherichia coli EVs, known as outer membrane vesicles (OMVs) and to investigate the in vivo anti-tumor activities of E. coli OMVs in mouse tumor model.
Methods: E. coli was grown in bioreactors and OMVs were isolated by the combination of tangential flow filtration and size exclusion chromatography. Purified E. coli OMVs were characterized by protein amount, number, size, morphology, and outer membrane protein A (Omp A). For in vivo anti-tumor activities, E. coli OMVs were injected twice a week in tumor-bearing mice for 2 weeks, and tumor volume was measured every 3-4 days. To evaluate adverse effects, E. coli OMVs were injected and monitored the body weight and survival rate.
Results: Using a 200-liter scale bioreactor, we isolate large quantities of E. coli OMVs with high purity: 2.2 mg in total protein amounts and 2.5 ×1012 particles of OMVs were isolated from one litter of the conditioned media. The purified OMVs were spherical morphology limited by lipid bilayer, 20 nm in a diameter, and enriched with Omp A (a well-known OMV marker protein). Furthermore, the purified E. coli OMVs showed a dose-dependent anti-tumor activities in mouse bladder cancer model. Treatment of 0.5 μg E. coli OMVs/head caused not only complete regression of tumor growth but also completely block the tumor growth of re-challenged bladder cancer cells by inducing long-term anti-tumor memory effects. We also observed that no mice were dead after administration of 10 μg and 30 μg of E. coli OMVs: the mice administered with OMVs showed temporal loss of body weights, but they gradually gained body weights from a week after OMV treatment.
Summary/Conclusion: This study shows that Gram-negative bacterial OMVs can be produced in a large amount with high purity, and have potent anti-tumor activities with a wide therapeutic window, suggesting that Gram-negative bacterial OMVs are novel candidates for the development of next-generation cancer immunotherapeutic agents.
Funding: This work was supported by National Research Foundation of Korea (2018R1A2A1A05079510 and 2021R1A2C3005275).

OT09.06 | Human induced pluripotent stem cells as inexhaustible source of immunomodulatory extracellular vesicles

Valeria Peli[1]; Mario Barilani[1]; Clelia Pistoni[1]; Paolo Manzini[2]; Francesca Pischiutta[3]; Enrico Caruso[3]; Francesco Rusconi[1]; Alessandro Cherubini[1]; Valentina Bollati[4]; Luisa Fiandra[5]; Ricardo Malvicini[6]; Gustavo Yannarelli[7]; Giada De Lazzari[8]; Michela Pozzobon[8]; Giovanna Damia[3]; Elisa Zanier[3]; Lorenza Lazzari[1]
[1]Laboratory of Regenerative Medicine – Cell Factory, Department of Transfusion Medicine and Hematology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, Italy; [2]Laboratory of Regenerative Medicine – Cell Factory, Department of Transfusion Medicine and Hematology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Reggio Emilia, Italy; [3]Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy, Milano, Italy; [4]Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy, Milano, Italy; [5]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy, Milano, Italy; [6]Instituto de medicina traslacional, trasplante y bioingeniería (IMeTTyB-CONICET), buenos Aires, Argentina; [7]IMeTTyB-CONICET, Instituto de medicina traslacional, trasplante y bioingenieria, Buenos Aires, Argentina, Buenos Aires, Argentina; [8]Department of Women's and Children's Health, University of Padova, Padua, Italy. Fondazione Istituto di Ricerca Pediatrica Città della Speranza, Padua, Italy, Padova, Italy

Introduction: Extracellular vesicles (EV) hold promise as cell-free therapeutics to overcome the clinical limitations of their parental cells. Mesenchymal stromal cells (MSC) are the most studied therapeutic EV source. Yet, clinical application of EV entails large-scale production via cGMP-compliant processes that must meet strict quality requirements. In this framework, primary MSC have two main drawbacks: limited life-span; intra- and inter-donor heterogeneity. Thus, we investigated whether human induced pluripotent stem cells (hiPSC), generated from clinical-grade HLA-typed cord blood MSC, may act as an unlimited source of therapeutic EV.
Methods: EV were isolated by serial centrifugations and purified by size-exclusion chromatography and sucrose density gradient. MISEV2018-guided characterization was performed by nanoparticle tracking analysis, western blot, flow cytometry and electron microscopy. Kinetics of EV release by hiPSC and uptake by H19-7 rat neural cells were explored in vitro. Therapeutic potential was tested on activated T-cell and in an ex vivo murine model of brain ischemia by oxygen and glucose deprivation (OGD).
Results: Biophysical features were compatible with small EV identity, and indicated pluripotent origin and absence of contaminants. Interestingly, these EV did not express HLA class I and II. EV production was robust and uptake by recipient cells was dose-dependent. In the OGD model hiPSC-EV induced significant brain protection and downregulation of inflammatory key drivers TNFa and IFNg. Immunomodulation was validated on activated T-cell. EV safety was successfully tested.
Summary/Conclusion: These data point at hiPSC as a source of EV with immunomodulatory properties. Further efforts are needed to confirm these results in vivo and to set up a translatable manufacturing process.
Funding: Fondazione Regionale per la Ricerca Biomedica, Regione Lombardia, Project nr.CP_10/2018. ERP-2020-EuronanoMed3-NANO4STROKE, Project nr.ERP-2020-23680649.

OT10: Predictive EV Biomarkers in Cancer

Chair: Dolores Di Vizio – Department of Surgery, Division of Cancer Biology and Therapeutics, Cedars-Sinai Medical Center, Los Angeles, CA, USA

OT10.01 | Tumor-derived extracellular vesicles predict clinical outcomes in oligometastatic prostate cancer treated with stereotactic ablative radiotherapy

Fabrice Lucien[1]; Yohan Kim[1]; Jing Qian[1]; Henan Zhang[1]; Ali Arafa[1]; Feven Abraha[1]; Ishwor Thapa[2]; Erik Tryggestad[1]; William Harmsen[1]; Jorgena kosti[1]; Hesham Ali[2]; Jacob Orme[1]; Val Lowe[1]; Goeffrey Johnson[1]; Eugene Kwon[1]; Haidong Dong[1]; Sean Park[1]
[1]Mayo Clinic, Rochester, USA; [2]University of Nebraska in Omaha, Omaha, USA

Introduction: Stereotactic ablative radiotherapy (SABR) has demonstrated clinical benefit in oligometastatic prostate cancer (omPC) patients. However, the risk of developing new distant metastatic lesions remains high and only a minority of patients experience durable progression-free response. There is a critical need to identify which subset of oligometastatic patients will benefit from SABR alone versus combination SABR and systemic agents. Here, we provide the first proof-of-concept for the clinical value of circulating PCEVs as non-invasive predictor of oncological outcomes in omPC patients treated with SABR.
Methods: Platelet-free plasma of 79 oCRPC patients collected at baseline and days 1, 7, and 14 post-SABR (NCT02816983) was analyzed by nanoscale flow cytometry (Apogee A60MP) in a blinded manner. Particle detection was calibrated with Rosetta beads (Exometry Inc) and MESF beads (Spherotech). Prostate cancer-derived extracellular vesicles (PCEVs) were defined as EVs positive for one of the following prostate-specific markers PSMA or STEAP1. Kaplan-Meier estimates were used to estimate survival curves. Association of PCEV levels with clinical features was determined using two-sided Mann-Whitney U tests. Linear regression analysis of PCEV levels with imaging features or levels of peripheral CD8 T cells was used to determine Spearman's r values and associated p values.
Results: PCEVs were detected in 100% of patients. Positive correlation was observed between PSMA+ and STEAP1+ EVs ($r=0.599$, $p< 0.0001$). High PCEV levels at baseline were predictive of shorter median time to distant recurrence (3.5 vs 6.6 months, $p=0.0087$). High PCEV levels were also associated with lower levels of circulating effector CD8 T cells. In contrast, radiomic features of 11C-choline PET/CT before and after SABR were not predictive of clinical outcomes. Following SABR, PCEV levels reached a peak at day 7 and median overall survival was significantly longer in patients with higher PCEV levels (32.7 vs 27.6 months, $p=0.003$).
Summary/Conclusion: Our study shows that pre-treatment PCEV levels can be a biomarker of tumor burden while early changes post-treatment can predict response to SABR. Combination of PET imaging with PCEV measurement has the potential to improve the selection of responders to SABR. Further studies are ongoing to investigate the underpinnings of metastatic progression including the role of PCEVs in response to SABR treatment.
Funding: This research was supported by NCI R01 CA200551 (SSP, VJL, EDK, and HD), departmental grant (FL) and generous benefactors (FL). FL was recipient of a postdoctoral fellowship from the Fonds de Recherche du Quebec-Sante (FRQS).

OT10.02 | Assessment of levels and clinical utility of prostate-derived extracellular vesicles (EVs) for the management of prostate cancer using nanoscale flow cytometry

Yohan Kim; Ali Arafa; Cameron Britton; Mohamed Ahmed; Vidhu Joshi; Robert Karnes; Eugene Kwon; Fabrice Lucien
Mayo Clinic, Rochester, USA

Introduction: There is a need to develop tools to accurately measure the levels of EVs in biofluids and interrogate their clinical utility as disease biomarkers. We conducted a comprehensive analysis of prostate-derived EV (PEVs) levels from the blood of healthy individuals and prostate cancer patients with different tumor burdens using standardized and calibrated nanoscale flow cytometry (nFC).
Methods: Blood and urine were prospectively collected from 4 healthy donors (HD), 24 patients with localized (nmPC), 123 oligometastatic (omPC) and 20 metastatic prostate cancer (mPC). Particle and PEV detection was calibrated with Rosetta beads (Exometry) and MESF beads (Spherotech). PEV levels were measured by Apogee A60-MicroPlus nanoscale flow cytometer using fluorescent antibodies (Anti-PSMA and STEAP1).
Results: Median levels of plasma PSMA+ and STEAP1+ EVs were higher in mPC (2.7-fold, $p=0.006$ and 9.0-fold, $p\leq0.0001$ respectively) compared to nmPC. Median levels of plasma PSMA+ and STEAP1+ EVs were higher in nmPC (7.6-fold, $p< 0.0001$, and 2.2-fold, $p=0.006$ respectively) compared to HD. Moderate correlation between PSMA+ and STEAP1+ EVs was observed in nmPC ($r=0.48$, $p=0.02$) and omPC ($r=0.45$, $p< 0.0001$). Weak correlation was seen in mPC ($r=0.23$, $p=0.34$). For nmPC, 42% and 33% of pts showed detectable levels of PSMA+ and STEAP1+ EVs respectively. Radical prostatectomy was associated

to a decrease in PSMA+ (3.9-fold, p≤0.01) and STEAP1+ EVs (2.6-fold, p≤0.01). Higher concentrations of PSMA+ (1.8-fold, p=0.014) and STEAP1+ EVs (4.8-fold, p=0.004) were observed in plasma compared to patient-matched urine.
Summary/Conclusion: Our study demonstrates the performance of standardized and calibrated nFC for the quantification of PEV levels from biofluids. A positive association found between PEV levels and tumor burden supports their potential as liquid cancer biomarkers. Finally, this is a transparent and reproducible framework for future prospective studies evaluating the clinical utility of EVs for the management of PC using nFC.
Funding: This research was supported by a departmental grant and generous benefactors. FL was recipient of a postdoctoral fellowship from the Fonds de Recherche du Quebec-Sante (FRQS).

OT10.03 | The capture of tumor-derived extracellular vesicles spontaneously released by xenotransplanted tumors induces a pro-inflammatory reaction in the pre-metastatic niche

Yves A. DeClerck[1]; Laurence Blavier[2]; Rie Nakata[2]; Paolo Neviani[2]; Aitor Benedicto[3]; Irina Matei[4]; Lyden David[4]
[1]Children's Hospital Los Angeles University of Southern California, Los Angeles, USA; [2]Children's Hospital Los Angeles, Los Angeles, USA; [3]University of the Basque Country, Leioa, Spain; [4]Weill Cornell Medical College, New York, USA

Introduction: Primary tumor-derived Extracellular Vesicles (TEV) captured in the pre-metastatic niche (PMN) promote metastasis. However, studies have relied on the administration of exogenous TEV in animal models. Here we describe a novel approach to monitor the spontaneous release of TEV from orthotopic models and their capture at metastatic sites, that allowed us to dissect the mechanisms through which TEV contribute to the PMN formation and its transition to the metastatic niche (MN).
Methods: Human neuroblastoma (NB) and melanoma (MEL) cells engineered to release green fluorescent labeled TEV (GFTEV) were orthotopically implanted into mice to track the capture of GFTEV in the PMN. An analysis of these GFTEV purified by differential ultra centrifugation followed by density gradient (MISEV2018) revealed the presence of GF protein in all EV including exosomes. Immunofluorescence imaging and flow analysis identified GFTEV-capturing cells, which were then isolated and analyzed for gene expression profiling by TaqMan low density array.
Results: GFTEV revealed a specific organotropism to the liver (macrophages and stellate cells) in the NB model and to the lung (alveolar macrophages) in the MEL model prior to homing of tumor cells. A gene expression analysis of GFTEV capturing macrophages (NB model) revealed the upregulation of anti-apoptotic (Bcl2), pro-tumorigenic (Cox-2, Il-6), angiogenic (Vegfa), and inflammatory (Ccl2, Cxcl-10, Il-18, Il-1alpha/beta) genes, and alterations in TGF-beta pathway. Changes in stellate cells suggested a contribution to monocyte recruitment (Ccl2) and PMN matrix (Fibronectin). Longitudinal studies in the MEL model, revealed a decrease in pro-inflammatory Il-1alpha and Il-18 and an increase in anti-inflammatory Hmox-1 and Tgf-beta during PMN to MN transition.
Summary/Conclusion: Our data demonstrate that TEV capture in the PMN precedes tumor cell homing and induces changes in gene expression favorable to metastatic progression. They provide a better understanding of the role of TEV in the PMN and its transition to the MN in pathophysiological relevant models of metastasis.
Funding: UA National Institute of Health/National Cancer Institute R01 CA207983 to YDC

OT10.04 | Circulating extracellular vesicles as biomarkers of response for immune-checkpoint inhibitors in advanced NSCLC

Orazio Fortunato[1]; Diego Signorelli[2]; Patrizia Ghidotti[2]; Claudia Proto[2]; Marta Brambilla[3]; Elena jachetti[3]; Valentina Capizzuto[3]; Francesca Pontis[1]; Ilaria Petraroia[1]; Mattia Boeri[1]; Gabriella Sozzi[1]; Marina Garassino[4]
[1]Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy, Milan, Italy; [2]Fondazione IRCCS Istituto Tumori Milano, Milan, Italy; [3]Fondazione IRCCS Istituto Tumori Milano, milan, Italy; [4]University of Chicago, Chicago, USA

Introduction: Programmed death ligand-1 (PD-L1) expression is the only predictive biomarker in clinical practice for immune-checkpoints inhibitors (ICI) in NSCLC. Extracellular vesicles (EVs) were used as biomarkers for cancer progression and can express PD-L1 on their surface. The aim of this study was to evaluate EVs in advanced NSCLC patients (pts) with low PD-L1 expression in order to find biomarkers for ICI.
Methods: EVs were isolated using ultracentrifuge from plasma of 64 advanced NSCLC pts treated with ICI or from conditioned medium of lung cancer cells. Pts were classified in responders (R) if they achieved a complete or partial response by RECIST 1.1, non-responders (NR) otherwise. EVs characterization was performed following MISEV guidelines (Nanoparticles Tracking analysis, Flow cytometry, TEM). The presence of co-inhibitory or co-stimulatory molecules on EVs was assessed by flow cytometry (FC). T cells of healthy donors were isolated and stimulated with CD3/CD28 beads in presence of EVs (15ug) and analyzed by FC.

Results: -EVs show significant higher levels of CD9, CD81, CD63, CTLA-4, 41BB, OX40L and CD86 and low levels of TIGIT compared to NR-EVs. Stratifying baseline surface EVs molecules according to median value, higher values of PD-L1 and VISTA were related to worse progression free survival (HR 1.78 and 2.15 respectively). In 20 patients (10 R, 10 NR), evaluated during ICI therapy, EVs-PD-L1 levels increased in R compared to NR (PD-L1 fold change 1.89). Interestingly, changes in CD9, CD81 and CD63 levels on both EVs were observed after ICI.
Strikingly, EVs isolated from NR (n=12) or R (n=8) pts increased CD4 and CD8 T cell proliferation and activation, evaluated as CFSE dilution and IFNg and Granzyme B production, respectively. We did not find any significant difference in checkpoint receptors on T cell surface (PD1, Tim3, Vista, Lag3). Notably, in the same experimental setting, EVs from a NSCLC cell line efficiently inhibited T cell proliferation
Summary/Conclusion: Plasma EVs from NSCLC pts treated with ICI showed different features in terms of surface markers. PD-L1 and VISTA on EVs could represent promising biomarkers for ICI response in NSCLC.
Funding: The study was supported by grants from Italian Ministry of Health (GR-2019-12369047)

OT10.05 | Early response prediction with EV-miRNA signatures in patients with Diffuse large B cell Lymphoma

Steven Wang[1]; esther Drees[1]; cristina gomez martin[1]; Monique van Eijndhoven[1]; Nils Groenewegen[1]; Karin Oudshoorn[2]; josee Zijlstra[1]; daphne de Jong[1]; Martine chamuleau[1]; Dirk M. Pegtel[3]
[1]amsterdam university medical centers, amsterdam, Netherlands; [2]twenty university, enschede, Netherlands; [3]Amsterdam University Medical Centers, Amsterdam, Netherlands

Introduction: Introduction: Previously, we have showed that classical Hodgkin lymphoma (cHL) patients with active disease sustain increased levels of a defined set of cell-free extracellular vesicle (EV)-bound circulating miRNAs by qRT-PCR. We postulated that, if elevated levels of EV-bound miRNAs in individual lymphoma patients can predict metabolically active disease, consistent alterations in the EV-miRNA landscape may predict predict response to therapy. Diffuse large B-cell lymphoma (DLBCL) is the most common hematological malignancy with heterogeneous outcomes. Current monitoring methods cannot reliably predict response early in treatment. RNAseq is a powerful method to explore the miRNA landscape in biological samples ad generate diagnostic signatures.
Methods: We generated a small RNAseq protocol (isoSeek) that is optimized for small RNA profiling from ultra-low input amounts. All blood samples (PAX gene tubes) were processed upon shipment within 48hours. Plasma EVs were isolated with Size-exclusion chromatography and after an EV-miRNA enrichment step, libraries were generated and Illumina sequencing was performed with our isoSeek method. We analyzed over 250 libraries from 100 patients collected at three to five different time points before and during treatment from three clinical trials that were collected at over 80 hospitals in the Netherlands, Belgium and Luxembourg. Technical evaluations including PCA/HC analysis, batch effect, and spike-inns and control libraries were highly reproducible demonstrating robustness of our protocol. The data was analyzed with sRNA-bench bioinformatics pipeline and EV-miRNA signatures were developed by using LASSO regression a form of machine learning.
Results: We developed several microRNA 'signatures' from samples collected pre-treatment, after one cycle, and post-treatment. EV-miRNA signatures could best validated after one cycle of treatment in predicting response after internal and external validations with training and test sets yielding a highly promising AUC of 0.88 (0.79-0.98).
Summary/Conclusion: NGS with isoSEEK protocol in plasma EVs yielded a robust EV-miRNA signature that predicts response to standard therapy (R-CHOP) early during treatment. At later stages during treatment the method seems to lose predictive power. This novel approach could potentially guide risk-adapted treatment strategies in lymphoma patients and may help assess efficacy of novel front-line therapies.
Funding: This work was funded by the Ducth Cancer Foundation, The CCA-foundation and the Health-Holland TKI project Accurate.

OT10.06 | Surface marker analysis of extracellular vesicles from tumor extracellular matrix

Sarah Tassinari[1]; Edoardo d'Angelo[2]; Federico Caicci[3]; Marco Agostini[2]; Federica Collino[4]; Benedetta Bussolati[5]
[1]*Department of Medical Sciences, University of Turin, 10126 Turin, Italy, Turin, Italy;* [2]*First Surgical Clinic, Department of Surgery, Oncology and Gastroenterology, University of Padova, Padua, Italy., Padua, Italy;* [3]*Department of Biomedical Sciences, University of Padova, Padua, Italy., Padua, Italy;* [4]*Laboratory of Translational Research in Paediatric Nephro-urology, Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico, Milan, Italy., Milan, Italy;* [5]*Department of Medical Sciences, University of Turin, 10126 Turin, Italy, Torino, Italy*

Introduction: Extracellular vesicles (EVs) are an important player in tumor progression and diffusion, and their characterization within the extracellular matrix (ECM) will represent a tumor molecular fingerprint. This project aims to identify and characterize EVs from decellularized tumor biopsies at different stages.
Methods: ECM has been obtained by decellularization of human colorectal cancer (CRC) and corresponding normal colon biopsy (HT), as control. Twelve primary 2/3 stage colorectal cancer (2/3-CRC), 7 metastatic (M-CRC) and the corresponding HT samples, were processed to isolate EVs (as approved by local ethical committee). We developed a protocol of mild enzymatic digestion, followed by ultra-centrifugation. Nanosight and transmission electron microscopy (TEM) were performed to characterize EVs concentration and structure. EVs surface markers were analyzed by super-resolution microscopy and cytofluorimetric analyses.
Results: EVs were recovered from decellularized CRC with an EVs average yield of 1.70*108 particles/mg of decellularized tissue. TEM confirmed their morphology, integrity and size, supporting that the decellularization maintains a bioactive microenvironment. Tetraspanins (CD9, CD63, CD81) were detected by cytofluorimetric analysis and super-resolution microscopy, highlighting a homogenous distribution; CD81 had the lowest expression among the samples. Moreover, M-CRC and 2/3-CRC expressed high CD3, CD29 and CD133, as a signature of cancer tissue. The expression of CD25, CD56 and HLA-DR was different in the metastatic population and associated with tumor-infiltrating lymphocyte's subsets. We confirmed EVs cell-origin and the abundance of PD-1 and PD-L1 markers on ECM-EVs by super-resolution microscopy.
Summary/Conclusion: ECM-EVs can be a tool to identify biomarkers for cancer diagnosis and staging. The surface markers characterization may provide useful information on the role of EVs in the tumor microenvironment communication.

Oral with Poster

OWP1: Oral with Poster: Biomarkers & Technologies and Methods

Chair: Raymond M. Schiffelers – University Medical Center Utrecht, Utrecht, The Netherlands

Chair: Pieter Vader, University Medical Center Utrecht, Utrecht, The Netherlands, alongside Qing-Ling Fu

OWP1.01=PT10.04 | Engineered surface expression of combined CD11a and CD18 (LFA-1) on extracellular vesicles to target ICAM-1 expressing cells

Markus Bergqvist[1]; Kyong-su Park[2]; Nasibeh Karimi[3]; Cecilia Lässer[3]; Jan Lötvall[3]
[1]*Krefting Research Centre, Institute of Medicine at Sahlgrenska Acadamy, University of Gothenburg, Gothenburg, Sweden, Göteborg, Sweden;* [2]*Krefting Research Centre, Institute of Medicine at Sahlgrenska Acadamy, University of Gothenburg, Gothenburg, Sweden, Gothenburg, Sweden;* [3]*Krefting Research Centre, Institute of Medicine at Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, Gothenburg, Sweden*

Introduction: Lymphocyte function-associated antigen 1 (LFA-1) is an integrin present on lymphocytes and other leukocytes and has ICAM-1 and other adhesion molecules as its ligands. LFA-1 is a heterodimeric glycosylated receptor with two non-covalently linked subunits, CD11A and CD18. We hypothesized that genetically overexpressing LFA-1 in HEK293f cells would result in release of extracellular vesicles (EVs) that a) express high quantities of LFA-1, and b) that these EVs have a propensity to bind to and be taken up by cells overexpressing ICAM-1.
Methods: HEK293f cells were sequentially transfected with plasmids for CD11a and CD18 using either the FectoProTM system or the LipofectamineTM 2000 system and multiple clones with stable expression of LFA-1 were identified by flow cytometry, and the expression of LFA-1 on the EVs was confirmed by nanoFCM analysis and Western blot of respected protein. The isolation of EVs from this clone was performed by ultracentrifugation and density cushion. Both direct and a reversed binding assays

were utilized to determine whether the cells and EVs expressing LFA-1 would associate efficiently to ICAM-1. Further, uptake experiments in ICAM-1 overexpressing HMEC-1 cells compared wild type and LFA-1 expressing EVs.
Results: Flow cytometry verified that the transfections with CD18 and CD11a on HEK293f was successful and that a stable LFA-1 clone were developed successfully. Binding assays with ICAM-1 and anti-CD18 antibody coated plates showed a higher binding for the LFA-1 clone than the 293f-wildtype and CD18 clone for both wells coated with ICAM-1 and anti-CD18 antibody. Western blot and nanoFCM analysis on the EVs isolated from these three types of cells showed that CD18 and CD11a were expressed on the EVs isolated from the LFA-1 clone but were absent on the EVs from the 293f-WT and the CD18 clone. In the reverse binding assays more cells expressing ICAM-1 were able to bind to the wells with the LFA-1 EVs than the wells with other EVs and the neutralized LFA-1 EVs. Incubating ICAM-1 expressing cells with different types of EVs showed that the cells had a higher uptake for the EVs with LFA-1 on their surface.
Summary/Conclusion: In conclusion, LFA-1 overexpressing EVs bind to ICAM-1 and can be more readily taken up by ICAM-1 expressing cells. These EVs can therefore potentially be utilized to target inflamed tissues, and to delivery therapeutics to cells expressing ICAM-1.

OWP1.02=PT10.02 | Development of a FRET-based assay for biomarker pairs in small extracellular vesicles

Déanna F. Shea[1]; Paul H. Teesdale-Spittle[2]; Nathaniel J.L.K. Davis[2]
[1]University of Otago, Dunedin, New Zealand; [2]Victoria University of Wellington, Wellington, New Zealand

Introduction: The early stages of lung cancer are difficult to detect, leading to high levels of mortality. This highlights the need for new diagnostic methods. Exhaled breath condensate (EBC) is a promising source of biomarkers for monitoring lung and respiratory system function. Small extracellular vesicles (sEVs) from EBC are an ideal source of biomarkers, as they can be purified away from contaminants. In this study we developed a FRET assay that can distinguish between sEVs isolated from a cellular model of lung cancer (A549 cells) and those of normal lung (WI-38 cells) based on pairs of biomarkers.
Methods: Cell and breath sEVs were isolated via ultracentrifugation and characterised by NTA, TEM, and western blot. Breath sEVs were non-invasively obtained from 55 healthy participants. To identify biomarkers that distinguish a cancer cell model from a cell model of normal lung, proteomic analysis was used. A photoluminescence (PL)-quenching-based FRET detection assay was developed using antibodies against known EV markers (CD63 and CD81) and the proposed cancer biomarkers conjugated with either a donor (AF488) or acceptor (AF546) fluorophore.
Results: Proteomic analysis revealed 158 proteins that are unique to A549 sEVs compared to WI-38 sEVs. Four of these (CD151, GLUT, MYOF, RhoC) were chosen for investigation. After confirmation that the FRET assay can detect known EV markers, several pairwise combinations of proposed biomarkers were first tested on A549 sEVs showing the highest FRET efficiency with pairs containing CD151. Successful pairs were tested on negative controls (WI-38 cell and breath sEVs) reporting no change between the isolated donor dyes' PL and that of the conjugated complexes.
Summary/Conclusion: The study shows that a simple FRET assay can distinguish between sEVs isolated from different sources. That opens the possibility for future development of FRET assays that can be used for assessment of patients with impaired lung function, including lung cancer.

OWP1.03=PT10.13 | A microfluidic device for extracellular vesicle purification

Alessio Meggiolaro; Valentina Moccia; Paola Brun; Valentina Zappulli; Davide Ferraro
University of Padua, Padova, Italy

Introduction: Extracellular vesicles (EVs) were found to play crucial role in intercellular exchange of molecular constituents and tumor growth. The study of their properties requires an efficient isolation and this task is not yet satisfied by the available techniques. Microfluidics, that allows handling small amount of liquids in microchannels, combined with solid phase extraction, represent a promising strategy to solve this fundamental task. However, these systems showed very low throughput due to the poor mixing between EVs and the solid support. This can be solved by droplet microfluidics, in which tiny and independent aqueous droplets are generated in an oil phase, and presents strong mixing of the contained liquids. We present a droplet microfluidic device devoted to apply this capabilities to EVs isolation to improve recovery rate and throughput, and to reduce protein background.
Methods: A customized microfluidic device was used to co-encapsulate in droplets both cell culture media (MDA-MB-231, pre-purified by 1 UC step) and magnetic beads coated with anti-CD63 or anti-CD81 antibodies. After incubation, droplets were driven

towards a magnetic region within the microfluidic device which acts as a trap for the magnetic beads. EVs resulting attached to them were characterized by Flow Cytometry (FC), Western Blot (WB, markers: Alix, Calnexin, TSG101, CD63, CD81) and Nanoparticle Tracking Analysis (NTA). Results were compared with a more conventional method.

Results: NTA showed that microfluidic approach allows good extraction rate, since the amount of EVs was comparable with the sample after pre-purification. Both WB and FC proved the presence of EVs on the isolated beads using both the microfluidic device and conventional approach.

Summary/Conclusion: EVs purification assays were performed within a droplet microfluidic device and validated by three characterization methods (NTA, FACS and WB), resulting in agreement with conventional method. Currently, purification from more complex samples are under investigation.

Funding: STARS 2019 - UNIPD

OWP1.04=PT10.01 | Biofluid specificity of non-coding RNA profile associated with systemic lupus erythematosus activity, relevance of exosomal fraction

Olga Martinez-Arroyo[1]; Ana Ortega[1]; Ana Flores-Chova[1]; Carlos Bea[2]; Maria J Forner[2]; Raquel Cortes[3]

[1]Cardiometabolic and Renal Risk Research Group, Biomedical Research Group Clinic Hospital - INCLIVA, Valencia, Spain; [2]Internal Medicine Clinic Hospital, Valencia, Spain; [3]Cardiometabolic and Renal Risk Research Group, Biomedical Research Institute Clinic Hospital - INCLIVA, Valencia, Spain

Introduction: Systemic lupus erythematosus (SLE) is a chronic autoimmune disease where the exosomes have a pathogenic role throughout their cargos, especially non-coding RNA (ncRNA). The aim of this study is to identify a global ncRNA profile specific of biofluid, plasma or plasma exosomes, associated to SLE activity.

Methods: Plasma samples were obtained from 96 SLE patients and 25 healthy controls to isolate EXO-P by differential ultracentrifugation. RNA was extracted from both EXO-P and plasma and ncRNAs were identified using high-throughput SmallRNA sequencing analysis. Then results were validated in a higher cohort by qPCR.

Results: MicroRNAs (miRNAs) were the group of small ncRNAs with the highest mapped reads in all groups for both biofluids, followed by piRNAs, lncRNA and Y-RNA. Then, making comparisons between groups, it was observed that the plasma presented the greatest diversity of differentially expressed ncRNA biotypes, being the miRNA and lncRNA the most representative. Analysing only miRNAs in SLE, it was observed that they biofluid-specific, being up-regulated in exosomes and down-regulated in plasma, and only 1.2% were common in both fractions. Finally, miR-144-3p and miR-144-5p were the highest up-regulated in EXO-P versus P in all patient groups (3.92 and 3.03, $p < 0.001$, respectively). The ROC curve analysis showed a significant area under the curve indicating the discriminatory power only of the levels of hsa-miR-144-3p in EXO-P for the presence of SLE ($AUC = 0.71$, $p < 0.01$).

Summary/Conclusion: The results obtained show a biofluid specificity for the molecular profile of ncRNA identified, being up-regulated in exosomes and down-regulated in plasma. MiRNAs are the most representative biotype, and exosomal miR-144-3p could be a potential biomarker of SLE activity.

Funding: This research was funded by "Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III" [PI18/01405 and PI21/00249 (FIS Projects for M.J.F. and R.C.) and CD18/00166 (Sara Borrell A.O.) and the European Regional Development Fund (ERDF).

OWP1.05=PT10.09 | MicroRNA signatures from tumor-derived extracellular vesicles for detection of early-stage of breast cancer

Sunyoung Park[1]; Min Woo Kim[2]; Hyojung Lee[2]; Hogyeong Gwak[1]; Kyung-A Hyun[1]; Seoyeon Choi[1]; Jee Ye Kim[2]; Hyo-Il Jung[1]; Seung Il Kim[2]

[1]School of Mechanical Engineering, Yonsei University, Republic of Korea, Seoul, Republic of Korea; [2]College of Medicine, Yonsei University, Republic of Korea, Seoul, Republic of Korea

Introduction: Tumor-derived EVs (TDEs), which carry oncogenic microRNAs (miRNAs), have been emerging biomarkers in liquid biopsy. However, TDEs are surrounded by normal cell-derived EVs and abundant free-miRNAs in the plasma. An essential step to practically analyze TDE-derived miRNAs is needed to purify and enrich the cancer-associated EVs from the plasma samples.

Methods: Cancerous EVs expressing breast cancer-associated surface markers (Integrin alpha 6 and EpCAM) were rapidly and selectively isolated by using our novel microfluidic device within two minutes. The miRNAs in TDE isolated by microfluidic

chip were compared with miRNAs in total EV isolated by the conventional precipitation method. After that, we selected seven-candidate miRNAs from the TDEs and profiled them in 82 plasma samples of breast cancer patients and healthy controls.
Results: The miRNAs from TDEs were successfully enriched with our device. Among seven-candidate miRNAs, four miRNAs (miR-9, miR-16, miR-21, and miR-429) from the EVs were highly elevated in early-stage breast cancer patients compared with healthy donors. The combination of significant miRNAs from specific EVs has a high sensitivity of 0.90, 0.86, 0.88, and 0.84 of AUC (Area under the curve) in each subtype (Luminal A, luminal B, HER-2, and triple-negative) of early-stage breast cancer.
Summary/Conclusion: Our results suggest that the combination of four miRNA signatures of TDEs can open a door to enable the early diagnosis of breast cancer using liquid biopsy.
Funding: This study was supported by a grant from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (2020R1I1A1A01067448, 2021R1A2C3011254l1, 2020M3A9I4039045).

OWP1.07=PT10.14 | A modular microfluidic chip for continuous enrichment and isolation of extracellular vesicles

Hyo-Il Jung[1]; Hogyeong Gwak[2]; Sunyoung Park[1]; Haeun Yu[2]; Kyung-A Hyun[2]
[1]School of Mechanical Engineering, Yonsei University, Republic of Korea, Seoul, Republic of Korea; [2]Yonsei University, Seoul, Republic of Korea

Introduction: Nanometer-sized extracellular vesicles (EVs) are secreted by all cells and circulate in blood body fluids and carry various biomarkers, including RNA, DNA, lipids, and play crucial roles in cell-to-cell communications in the microenvironment. EVs have been explored as diagnostic biomarkers and therapeutic substances. In order to utilize EVs in clinical applications, it is required to isolate and enrich EVs from abundant other biomarkers such as lipoproteins. Conventional methods of EV isolation have several drawbacks such as poor yields, low purity, and time-consuming operations.
Methods: Herein, we present a modular microfluidic platform for enriching EVs on micron-sized beads based on affinity capture and purely eluting EVs from the beads. The modular microfluidic platform consists of two microfluidic chips: a horseshoe-shaped orifice micromixer (HOMM) and a fish-trap-shaped microfilter unit (fish trap). The HOMM effectively enhances collisions between the EVs and antibody-coated carrier beads. Subsequently, these EV-carrying beads are trapped by the square structures in the fish trap. Finally, Injection of the elution buffer releases only pure EVs from the beads.
Results: The binding efficiency of EVs and microbeads on the HOMM was 97% at the flow rate of 150 μL/min. The capture efficiency of microbeads in the fish trap was approximately 99% at flow rates of 50 to 150 μL/min. PD-L1 functions as an evasion to immune cells in the tumor microenvironment and is emerging to play pivotal roles in immunotherapy response. PD-L1 positive EVs collected from the modular chip were co-cultured with T cells for 12 hours. After then, we investigated the expression of PD-L1 EV-internalized T cells by using flow cytometry. The expression levels of PD-L1 positive EVs were 50% higher internalized into T cells compared to control.
Summary/Conclusion: Based on these results, the modular chip could be employed as a simple therapeutic and diagnostic tool for clinical applications.
Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2021R1A2C3011254l1 and No. 2020M3A9I4039045) and 2021 Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2021R1C1C2007646)

OWP1.08=PT10.06 | Extracellular Vesicle MicroRNA That Are Involved in β-Thalassemia Complications

Carina Levin[1]; Ariel Koren[2]; Annie Rebibo-Sabbah[3]; Maya Levin[4]; Na'ama Koifman[5]; Benjamin Brenner[3]; Anat Aharon[4]
[1]Pediatric Hematology Unit, Emek Medical Center, Afula, Israel; [2]Pediatric Hematology Unit "Emek" Medical Center - Afula, Afula, Israel; [3]Department of Hematology and Bone Marrow Transplantation, Rambam Health Care Campus, Haifa, Haifa, Israel; [4]Tel Aviv Sourasky Medical Center, Tel Aviv, Israel; [5]Department of Chemical Engineering and the Russell Berrie Nanotechnology Institute, Technion-Israel Institute of Technology, Haifa, Haifa, Israel

Introduction: Beta thalassemia major (βT) is a hereditary anemia characterized by transfusion-dependency, lifelong requirement of chelation, and organ dysfunction. MicroRNA (miRNA) can be packed into extracellular vesicles (EVs) that carry them to target cells. We explored EV-miRNA in βT and their pathophysiologic role.

Methods: Circulating EVs were isolated from 35 βT-patients and 15 controls. EV miRNA was evaluated by nano-string technology and real-time quantitative polymerase chain reaction (RT-qPCR). We explored effects of EVs on cell culture proliferation, apoptosis, and signal transduction.
Results: Higher amounts of small EV (exosomes) were found in patients than in controls. The expression of 21 miRNA was > two-fold higher, and of 17 miRNA < three-fold lower in βT-EVs than control-EVs. RT-qPCR confirmed differential expression of six miRNAs in βT, particularly miR-144-3p, a regulator of erythropoiesis. Exposure of endothelial, liver Huh7, and pancreatic 1.1B4 cells to βT-EVs significantly reduced cell viability and increased cell apoptosis. βT-EV-induced endothelial cell apoptosis involved the MAPK/JNK signal-transduction pathway. In contrast, splenectomized βT-EVs induced proliferation of bone marrow mesenchymal stem cells (BM-MSC).
Summary/Conclusion: In summary, the miR-144-3p was strongly increased; βT-EVs induced apoptosis and decreased endothelial, pancreatic, and liver cell survival while supporting BM-MSC proliferation. These mechanisms may contribute to βT organ dysfunction and complications.
Funding: ISF , The Israel Science Foundation

OWP1.09=PT10.15 | Detection of arginase containing small extracellular vesicles in biological fluids of endometriosis patients as a potential immunosuppressive factor

Karolina M. Soroczyńska[1]; Tobias Tertel[2]; Oumaima Stambouli[2]; Bernd Giebel[2]; Malgorzata Czystowska-Kuzmicz[1]
[1]Medical University of Warsaw, Warsaw, Poland; [2]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany

Introduction: Endometriosis is a chronic gynaecological disorder characterized by the growth of the endometrium outside its cavity. Despite its high prevalence, at present there are no effective treatments or biomarkers for early diagnosis. Recent reports suggest that endometriosis may be related to the impaired immune response. Our preliminary results indicate an increased level of two isoforms of arginase (ARG) enzyme, arginase-1 (ARG1) and arginase-2 (ARG2) as well as increased ARG activity in the peripheral blood of patients. Arginases are well-known regulators of amino acid metabolism, with a strong immunosuppressive effect, especially in tumors. We assume that an immunosuppressive mechanism mediated by (ARG)-carrying extracellular vesicles (ARG+EVs), may be responsible for the observed dysfunction of the immune system in endometriosis, resulting in the progression of this disease. The aim of the project is to identify ARG+EVs in the serum and peritoneal fluid (PF) of endometriosis patients and to decipher their impact on the dysfunction of the immune system in endometriosis.
Methods: Small EVs were isolated from serum and PF of endometriosis and control patients using SEC and were verified by Western blotting, NTA and imaging flow cytometry. The presence of arginases in EVs was determined by Western blotting, ELISA and flow cytometry. The functionality of ARG+ EVs was investigated using a multi-donor mixed lymphocyte reaction (mdMLR) assay.
Results: We detected small EVs in serum and PF samples from endometriosis patients and controls. According to NTA measurements, there was a trend towards higher total number of particles in endometriosis patients in comparison to controls both in serum and PF. We confirmed the presence of ARG1 and ARG2 in single EV samples from serum and PF of endometriosis patients along with the detection of some classical markers of small EVs. Based on single EV analyses by imaging flow cytometry, we showed a decrease in ARG1 expression in serum-derived EV samples after the laparoscopic surgery. According to ELISA results, the concentration of vesicular ARG2 was several times higher than the ARG1 concentration, that was in the range of several ng per ml of serum or PF. In addition, EVs showed a minor immunomodulatory effect in the mdMLR, with a decline in activated CD4 and CD8 cells (CD25+CD54+), which was reversed by the addition of an ARG inhibitor.
Summary/Conclusion: Our findings provide the first evidence for the presence of the immunosuppressive enzyme - ARG in the cargo of small EVs isolated from serum and PF of endometriosis patients. We believe that ARG+EVs may impact endometriosis progression, in terms of immune dysfunction, as well as provide a potential diagnostic/prognostic biomarker or therapeutic target.
Funding: NAWA Polish National Agency for Academic Exchange grant PPI/APM/2019/1/00051/U001
National Science Centre (NCN) grant OPUS21 2021/41/B/NZ6/02291

OWP1.10=PT10.05 | EV derived Proteomics profile to stratify Angiogenic and Vessel co-opting Lesion in Colorectal Cancer Liver metastasis

Migmar Metrakos[1]; Peter Metrakos[2]; Anthoula Lazaris[2]; Lucyna Krzywon[1]; Stephanie Petrillo[2]
[1]McGill University, Montreal, Canada; [2]RIMUHC, Montreal, Canada

Introduction: Colorectal cancer (CRC) is the 3rd most common type of cancer worldwide with liver being the dominant metastatic site. Liver metastases present as two major histopathological growth patterns (HGPs): Angiogenic and Non-angiogenic (vessel co-opting). We have published that CRCLM patients treated with chemotherapy and bevacizumab have a worse five-year overall survival when their lesions employ vessel co-option instead of angiogenesis. Thus, the stratification of these HGP is of clinical relevance. There are currently no biomarkers available to stratify these patients. Our study aims at generating extracellular vesicle (EV) based signature using blood-based biomarkers (Liquid Biopsy) to stratify patients into angiogenic or vessel coopting lesions for personalized treatment strategy.
Methods: We isolated EVs from plasma of 13 Chemonaive patients (Angiogenic n= 7 and Vessel co-option n=6). Based on MISEV guideline, EVs were characterized using nanoparticle tracking analysis, electron microscopy and Western blot. Mass Spectrometry was performed to study the EV proteomics profiles followed by analysis using MAXQuant software. We validated the presence of the proteins in the tissue of origin using immunostaining methods.
Results: Using FunRich to perform cellular enrichment analysis, we observed that the majority of proteins were of exosome origin. Principal component analysis (PCA) was performed using Perseus analysis tool and we generated a list of significantly differentially expressed proteins. ROC-AUC analysis was performed to obtain diagnostic power of individual candidate proteins and refine the list of proteins in order of the diagnostic power. Interestingly, S100A9 protein was the top protein observed and the expression of the S100A9 in the EV correlated well with the tumor tissue thus showing significant differences in expression at the tumor interphase in co-opting lesions only. Further we observed that S100A9 proteins on the tissue were expressed by the macrophage and neutrophils which has been suggested to be involved in the inhibition of T-Cell response.
Summary/Conclusion: Our findings indicate that blood derived EV biomarkers can be used to stratify patients into angiogenic and non-angiogenic tumor types and may predict response to current treatment regimes.
Funding: MEDTEQ Innovation for Health

OWP1.11=PT10.10 | miR-210 expression is strongly hypoxia-induced in anaplastic thyroid cancer compared with papillary thyroid cancer cell lines and is associated with extracellular vesicles and extracellular ribonucleoproteins

Bonita H. Powell[1]; Andrey Turchinovich[2]; Yongchun Wang[3]; Martha A. Zeiger[4]; Christopher B. Umbricht[3]; Kenneth W. Witwer[5]
[1]Johns Hopkins University School of Medicine, Baltimore, USA; [2]Sciberg e.Kfm., Mannheim, Germany; [3]Johns Hopkins University School of Medicine, Department of Surgery, Baltimore, USA; [4]Surgical Oncology Program, National Cancer Institute, National Institutes of Health, Bethesda, USA; [5]Johns Hopkins University School of Medicine, Department of Molecular and Comparative Pathobiology, Baltimore, USA

Introduction: Anaplastic thyroid carcinoma (ATC) is a rare but deadly thyroid cancer. Hypoxia is frequent in aggressive solid tumors like ATC and can promote resistance to therapy via the hypoxia-inducible factor (HIF) pathway. Identifying and targeting hypoxic cells is an attractive approach to treating ATC.
Methods: Ligation-based and -independent small RNA-seq (sRNA-seq) methods were used to compare miRNA expression in ATC lines (SW1736 and C643) vs. papillary thyroid cancer (PTC) lines (TPC-1 and BCPAP) at 2% (hypoxia) or atmospheric (normoxia) O2 levels. Differentially expressed miRNA was validated by Stem-Loop RT-qPCR. Extracellular vesicles (EVs) and ribonucleoproteins (RNPs) were isolated from cell- and debris-pre-cleared conditioned media by size-exclusion chromatography (SEC). EVs were characterized by electron microscopy (EM), resistive pulse sensing (RPS), and Western blot (WB).
Results: sRNA-seq and qPCR showed 2x lower normoxic expression of miR-210 in ATC vs. PTC lines. In a 0, 2, 4, 8, 24, & 48hr time-course, miR-210 was >10x induced in hypoxic vs normoxic ATC cells but only 2-4-x in PTC. Both sRNA-seq and qPCR showed that miR-210 was the only miRNA significantly up-regulated (>2x) in hypoxic SW1736 (ATC) cells. In SW1736, miR-210 levels declined after 72hr of hypoxia, consistent with lower HIF-1alpha protein levels, but remained elevated (>2x) relative to normoxia. However, miR-210 increased (>2x) in media collected at 72hr, suggesting extracellular release. miR-210 in hypoxia relative to normoxia was enriched ~3x in EV and RNP fractions separated by SEC. Lastly, similar particle counts per cell were observed for EVs released during hypoxia vs. normoxia (RPS), but there was an enrichment of CD63 and CD9 after 72hr hypoxia (WB).
Summary/Conclusion: mir-210, a HIF target, indicates hypoxia in ATC lines and associates with EVs and extracellular RNPs. Therefore, miR-210 is a potential cellular and extracellular marker for hypoxia within ATC tumors. Further analysis of miR-210 levels in ATC tissues and plasma is warranted for the potential use of drugs that target hypoxia effectors such as HIF-1 and HIF-2.

OWP1.12=PT10.12 | Vesicle subpopulations from the nanoscale to the microscale: development and optimization of a separation protocol with asymmetrical flow field-flow fractionation (AF4)

Daniele D'Arrigo[1]; Arianna Gennari[2]; Chiara Arrigoni[1]; Marco Vanoni[3]; Marco Delcogliano[4]; Christian Candrian[5]; Nicola Tirelli[6]; Matteo Moretti[1]

[1]Regenerative Medicine Technologies Lab, Service of Orthopaedics and Traumatology, Department of Surgery, EOC, via Federico Chiesa 5, Bellinzona, Bellinzona, Switzerland; [2]Laboratory of Polymers Biomaterials, Istituto Italiano di Tecnologia, Via Morego 30, 16163, Genoa, Italy, Genoa, Italy; [3]Department of Biotechnology and Bioscience, Università degli studi di Milano-Bicocca, 20126, Milan (IT), Milan, Italy; [4]Service of Orthopaedics and Traumatology, Department of Surgery, EOC, Via Tesserete 46, Lugano, Switzerland, Lugano, Switzerland; [5]Service of Orthopaedics and Traumatology, Department of Surgery, EOC, via Tesserete 46, Lugano, Switzerland, Lugano, Switzerland; [6]Laboratory of Polymers Biomaterials, Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genoa, Italy, Genoa, Italy

Introduction: Extracellular vesicles (EVs) are described as lipid particles with a limited size distribution, nonetheless different EV subpopulations exist and can have important biological roles. For instance, large EVs have a role in inflammation, key process in joint diseases like osteoarthritis (OA). However, most of the studies looking for new OA biomarkers are only focused on small EVs. Thus, our aim is to develop a protocol based on the asymmetrical flow field-flow fractionation (A4F) technique to separate different sized EV subpopulations from the synovial fluid (SF), also applicable to other biofluids.

Methods: We collected shoulder and knee SF from a total of 6 donors after biceps tenotomy or knee replacement. We also got culture medium from synovial fibroblasts isolated from 4 arthritic patients and plasma from a healthy donor. Firstly, we optimized flow values in the A4F to obtain the whole EV profile from the different samples. Then we separated different sized EV subpopulations, evaluating EV average size, relative abundance, Z potential, and concentration of proteins and nucleic acids. We performed transmission and immune electron microscopy (EM) to confirm particle size and identity. Finally, with proteomics we assessed EV markers and protein content.

Results: With our protocol we obtained the EV profile and isolated EVs with a radius ranging from 20 up to 700 nm, divided into 4 subpopulations. As confirmed by EM, the EV size, profile and the relative percentage of each subpopulation were different in each biofluid. In addition, EVs from SF showed a decreasing trend of Z potential and protein concentration, but not of nucleic acid content, with increasing size. We demonstrated the presence of EV-specific markers, also by immune EM and, as expected from a size-based separation approach, we found lipoproteins in the smaller EV subpopulations.

Summary/Conclusion: Our A4F-based protocol allows the isolation of EVs with a radius ranging from 20 up to 700 nm and the separation of different sized subpopulations. It also provides the whole EV profile of samples and we showed that it differs based on the biofluid. The isolation and the characterization of different subpopulations in biofluids would give intriguing new opportunities to find diagnostic OA biomarkers.

Funding: Swiss National Foundation, SNF grant number P1SKP3_199444

OWP1.13=PT10.07 | Imaging flow cytometry challenges the usefulness of classically used EV labelling dyes and qualifies that of a novel dye, named Exoria™ for the labelling of MSC-EV

Tobias Tertel[1]; Melanie Schoppet[2]; Oumaima Stambouli[1]; Ali Al-Jipouri[1]; Patrick F. James[2]; Bernd Giebel[1]

[1]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany; [2]Exopharm Ltd, Level 17, 31 Queen St, Melbourne, Victoria, Australia, Melbourne, Australia

Introduction: Extracellular vesicles (EVs) are involved in mediating intercellular communication processes. An important goal within the EV field is the study of the biodistribution of EVs and the identification of their target cells. Considering that EV uptake is important for EVs in mediating intercellular communication processes, labelling with fluorescent dyes has emerged as a broadly distributed strategy for the identification of the EVs target cells and tissues. However, the accuracy and specificity of commonly utilized labelling dyes has not been sufficiently analyzed.

Methods: By combining recent advancements in imaging flow cytometry for the phenotypic analysis of single EVs and aiming to identify target cells for EVs within therapeutically relevant MSC-EV preparations, we explored the EV labelling efficacy of various fluorescent dyes, specifically of CFDA-SE, Calcein AM, PKH67, BODIPY-TR-Ceramide and a novel lipid dye named Exoria.

Results: Our analyses qualified Exoria as the only dye which specifically labels EVs within our MSC-EV preparations. Furthermore, we demonstrate Exoria labelling does not interfere with the immunomodulatory properties of the MSC-EV preparations as tested in a multi-donor mixed lymphocyte reaction assay. Within this assay, labelled EVs were differentially taken-up by different immune cell types.

Summary/Conclusion: Overall, our results qualify Exoria as an appropriate dye for the labelling of EVs derived from our MSC-EV preparations, this study also demonstrates the need for the development of next generation EV characterization tools which are able to localize and confirm specificity of EV labelling.

OWP1.14=PT10.11 | New targets and new approaches for multiple myeloma: extracellular vesicles as functional liquid biomarkers

Antonia Reale[1]; Andrew Spencer[1]; Tiffany Khong[1]; David W. Greening[2]; Haoyun (Alexx) Fang[2]; Maoshan Chen[3]; Rong Xu[1]; Irena Carmichael[1]; Nicholas Bingham[1]; Malarmathy Ramachandran[1]
[1]Monash University, Melbourne, Australia; [2]Baker Heart and Diabetes Institute, Melbourne, Australia; [3]NA, Melbourne, Australia

Introduction: HS5 stromal cells pre-treated with small extracellular vesicles (sEV) enriched from blood plasma of myeloma (MM) patients promoted adhesion of human MM cells (HMCL), with preliminary proteomic profiling of MM-sEV (vs healthy donors-HD) revealing enrichment of factors implicated in cell migration and adhesion.
Aims: To demonstrate that plasma-derived sEV induce a microenvironment favouring MM progression and identify the protein content of plasma-sEV that promotes this.
Methods: sEV were enriched from plasma (1mL) using a commercial kit. Proteomic profiling (nLC and high-resolution mass spectrometry, Orbitrap HF-X) of plasma-sEV derived from HD (x10) and patients with MM (x8) or pre-malignant conditions (monoclonal gammopathy of undetermined significance-MGUS x10; smouldering/asymptomatic MM-SMM x4), and functional studies (co-culture system HS5:HMCL) were performed.
Results: Stromal cells pre-treated with MM-sEV induced both HMCL proliferation ($p< 0.05$) and drug resistance ($p< 0.0001$) to anti-MM drugs (proteasome inhibitors) when compared to untreated cells.
A total of 412 proteins were detected and quantified by proteomic profiling of PBPL-sEV with 13 reported as highly enriched in EV-marker databases (ExoCarta-top100) and 8/13 corresponding to universal cancer EV-markers proposed by Hoshino-Cell2020. Gene ontology analysis of identified proteins (G:Profiler; $p< 0.05$) revealed enrichment for cellular component terms, e.g. extracellular vesicles, exosomes, and for several biological processes, e.g. cell communication, endocytosis, cell migration, cellular response to stimulus. Comparative analysis between our dataset and several publicly available datasets revealed sEV-markers with potential discriminatory specificity for MM, MGUS or SMM. Comparative analysis revealed 40, 40 and 41 proteins differentially regulated between HD-sEV and MM-sEV or MGUS-sEV or SMM-sEV ($P < 0.05$; log2 fold change ≥ 2). A specific protein signature identified in MM-sEV was found in ≥30% of MM-sEV vs < 30% HD-sEV. Specific protein signatures were also identified in MGUS-sEV (≥30% of MGUS-sEV but < 30% HD- or MM- or SMM-sEV) and SMM-sEV (≥30% of SMM-sEV but < 30% HD- or MM- or MGUS-sEV). These proteins were not found in human whole plasma (Lehallier-Nat Med2019) or solid tumors-derived sEV described by Hoshino-Cell2020 and Vinik-SciAdv2020).
Summary/Conclusion: MM-sEV may play an important role in disease progression by re-programming the tumour microenvironment. The characterization and proteomic profiling of disease-specific circulating sEV as a biomarker discovery strategy may provide translational applications in MM.
Funding: Australian Government Research Training Program Scholarship and Departmental Scholarship (Monash University, to Antonia Reale)

OWP2: Oral with Poster: Fundamental Biology and Pathophysiology

Chair: María Yáñez-Mó – CBM-SO, IIS-IP, Universidad Autónoma de Madrid

OWP2 | Oral with Poster: Fundamental Biology and Pathophysiology

María Yáñez-Mó
CBM-SO, IIS-IP, Universidad Autónoma de Madrid, Madrid, Spain

OWP2.01=PT11.13 | Release of immunosuppressive prostate cancer-derived extracellular vesicles impairs with cytotoxic CD8+ T-cell activity in response to radiotherapy

Yohan Kim; Roxane Lavoie; Haidong Dong; Sean Park; Fabrice Lucien
Mayo Clinic, Rochester, USA

Introduction: Stereotactic ablative radiotherapy (SABR) is a promising treatment for oligometastatic prostate cancer by optimizing local control and inducing a systemic antitumor immune response at non-irradiated distant metastatic sites. However, most patients still experience distant progression. We recently showed that patients with high levels of tumor derived EVs (tdEVs) following SABR tend to have lower levels of circulating effector CD8+ T cells, leading to higher risk of developing metastasis. Based on the clinical observations, we hypothesized that radiotherapy can induce the release of immunosuppressive tdEVs resulting in impaired antitumor immune response.
Methods: Prostate cancer cells (PC3 and DU145) were irradiated with single dose or fractionated radiotherapy. Levels of tdEVs were measured by nanoscale flow cytometer (Apogee A60MP). Human CD8+ T-cells were co-cultured with tdEVs to analyze T-cell phenotype and cytotoxic function. Molecular composition of cancer cell surface and tdEVs in response to radiotherapy was analyzed by mass spectrometry, western blotting, and flow cytometry.
Results: Radiotherapy significantly increased release of tdEVs (>2-fold, $p< 0.05$). EVs from irradiated CRPCa cells inhibited proliferation and killing function of CD8+ T cells. Proteomics revealed upregulation of B7-H3 at surface of cancer cells and enrichment within EVs in response to radiotherapy. Co-culture of CD8+ T-cells and radiotherapy-induced tdEVs significantly decreased T-cell cytotoxic activity (1.3-fold, $p< 0.05$). Overexpression of B7-H3 protein in tdEVs suppressed cytotoxic activity whereas tdEVs with deletion of B7-H3 expression was associated with partial recovery of T-cell cytotoxic function.
Summary/Conclusion: This study unveils a novel cellular mechanism that can impair with the efficacy of SABR and warrants tdEVs (i.e B7-H3+) as a promising target to elicit radiotherapy-induced antitumor immune response.
Funding: This research was supported by departmental grant (FL) and generous benefactors (FL). FL was recipient of a post-doctoral fellowship from the Fonds de Recherche du Quebec-Sante (FRQS).

OWP2.02=PT11.07 | MALAT1+ EVs from inflammatory airway neutrophils promotes hyperexocytosis across generations of airway recruited neutrophils via HDAC11

Brian S. Dobosh; Diego Moncada Giraldo; Rabindra Tirouvanziam
Emory University, Atlanta, USA

Introduction: Cystic fibrosis (CF) is characterized by recruitment and hyperexocytosis of granules from polymorphonuclear neutrophils (PMNs) yet paradoxical failure to kill pathogens in the airways - a phenotype termed granule releasing, immunomodulatory and metabolically active (GRIM). Newly arrived, naïve PMNs are exposed to EVs from other cells including earlier arrived PMNs. We utilized a model in which blood PMNs can be conditioned into GRIM cells by transmigration toward CF sputum. We sought to determine what component of the sputum caused PMNs to become GRIM.
Methods: EVs were purified by centrifugation at 800, 3000, 20000xg then by 300kDa MWCO column considered an "intermediate recovery, intermediate specificity" EV enrichment method by MISEV2018. EV integrity, purity and concentration were determined by EM and NTA and western blot of TSG101, CD63 and calnexin. Cell-specific EVs were validated by immunoprecipitation and nanoflow cytometry with antibodies against CD66b (PMN), CD326 (epithelial) or CD115 (macrophage).
Results: EVs from CF sputum were necessary and sufficient to generate GRIM PMNs. Removal of CD66b+, but not CD326+ or CD115+EVs prevented the GRIM phenotype. EVs from GRIM PMNs were generated and applied to naïve neutrophils, which became GRIM.
RNA-seq of GRIM and non-GRIM PMN EVs identified lncRNA MALAT1 as differentially expressed. Naïve PMNs became GRIM when transfected with MALAT1, which localized to EVs, and caused other naïve PMNs to become GRIM. HDAC11 was differentially expressed in GRIM vs non-GRIM PMNs. Expression of HDAC11 in GRIM PMNs caused upregulation and packaging of MALAT1 into EVs which could convert naïve PMNs to GRIM PMNs.
Summary/Conclusion: PMNs become GRIM due to HDAC11 which caused expression and packaging of MALAT1 into EVs. MALAT1+ CD66b+ EVs caused naïve PMNs to express HDAC11 and become GRIM. This suggests that MALAT1 and HDAC11 are targets to lessen inflammation in CF and other airway diseases with PMNs such as COPD, asthma and COVID19.
Funding: CF Foundation (TIROUV15A0), Emory Pediatrics Flow Core.

OWP2.03=PT11.03 | Chronic subtoxic oxidative stress in retinal pigmented epithelium identifies polarized desmosome shedding via exosomes as an early biomarker of cellular dysfunction

Belinda Hernandez[1]; Nikolai Skiba[2]; Martha Cady[2]; Mikael Klingeborn[2]; Catherine Bowes Rickman[2]
[1]Duke Univeristy, Durham, USA; [2]Duke University, Durham, USA

Introduction: Oxidative stress is implicated in the pathobiology of age-related macular degeneration (AMD) and initially targets the retinal pigmented epithelium (RPE). We hypothesize that oxidative stress leads to RPE dysfunction, affecting RPE exosome release and their role in extracellular matrix (ECM) changes leading to early signs of AMD.
Methods: Fully differentiated and polarized primary porcine RPE cells grown on cell culture inserts were treated daily with 0.2mM H2O2 for 4 weeks to induce chronic oxidative stress. Exosome isolation from basal-side conditioned media was done by differential centrifugation followed by Iodixanol density gradient flotation. Exosome yield was assessed by Nanoparticle Tracking Analysis. Purity and protein content of exosome preparations were analyzed by immunoblotting for exosomal markers, and markers for known contaminants. In-depth proteomic mass spectrometry analyses were done on both exosomes and ECM preparations. EV track for methods: EV170046 and EV200042.
Results: Unbiased proteomic analyses of the content of highly purified basolateral exosomes isolated from RPE cultures under oxidative stress revealed changes to a number of ECM and desmosomal proteins. Keratin 10 and Junction Plakoglobin were increased greater than 3-fold in basolateral exosomes. Increased release of desmosomal proteins were correlated with a 4-fold increase in basolateral exosome secretion. Treatment of RPE cultures with an inhibitor of exosome release reduced basolateral EV secretion and reduced deposition of a subset of proteins into the ECM.
Summary/Conclusion: We show for the first time that oxidative stress in primary RPE cultures induces basolateral shedding of desmosome components via exosomes, ECM changes, and increased basolateral EV secretion which could be prevented by inhibition of exosome release. These findings open up a completely novel avenue for therapeutic intervention and access to early biomarkers of cellular dysfunction in AMD.
Funding: NIH/NEI R21 EY033057 , R01 EY031748, and P30 EY005722 (Duke Eye Center).

OWP2.04=PT11.09 | miRNAs enriched in extracellular vesicles as actors and markers of papillary thyroid cancer

Ophélie Delcorte
de DUVE Institute - UCL, Brussels, Belgium

Introduction: Papillary thyroid carcinoma (PTC) is the most frequent subtype of thyroid cancers. Despite good prognosis in most cases, postsurgery recurrences and metastases can occur. Moreover, differential diagnosis between benign and malignant nodules is still challenging. Gaining knowledge about extracellular vesicles (EVs) in PTC could have a double benefit: a better understanding of PTC clinical behaviour, and the discovery of accessible (and accurate) diagnostic tools.
The goals of this project are to (i) identify miRNAs actors and markers, released via EVs by the tumor, (ii) decipher the mechanisms by which they impact thyroid cancer and its microenvironment, and (iii) evaluate the diagnostic value of circulating miRNAs.
Methods: Using a mouse model mimicking human PTC, we isolated EVs from dissociated control- and early and late PTC-tissue by differential ultracentrifugations. Vesicles in the high-speed pellet were characterized in-depth and sequencing was performed to identify tumor-derived EV-miRNAs. We investigated the distribution of EV-miRNAs according to their cellular source using the Nanoview technique. In parallel, the miRNAs candidates were quantified in tissues, in plasma and in plasma-EVs from patients treated for thyroid diseases.
Results: We focused on 4 miRNAs differentially more abundant in EVs from PTC tissues. In silico analysis revealed their enrichment in immune-related pathways, consistently with the massive recruitment of macrophages observed in the model. We showed that the number of EVs bearing epithelial and immune markers was increased in PTC tissues. The 4 miRNAs were mostly expressed, and deregulated, in epithelial cells. We thus propose that their increased abundance in epithelial-EVs could affect the immune microenvironment. Finally, the analysis on human samples demontrated that two miRNAs, miR-146b-5p and miR-21a-5p were more abundant in tissue and plasma-EVs isolated from patients with PTC, as compared to benign diseases.
Summary/Conclusion: We provided a gradual tissue- and EV-miRNAs profiling during PTC development which allowed the identification of EV-miRNAs that could (i) support the establishment of a permissive microenvironment for tumor development and (ii) contribute to thyroid cancer diagnosis.
Funding: Fondation Roi Baudouin (Fund Yvonne Smits), Université catholique de Louvain (Actions de Recherche concertées, ARC 15/20-065), National Lottery, FNRS, Télévie and de Duve Institute.

OWP2.05=PT11.04 | Controlled release of epigenetically-activated extracellular vesicles from a gelMA/nanoclay composite hydrogel to promote bone repair

Kenny Man[1]; Inês A. Barroso[1]; Mathieu Y. Brunet[1]; Ben Peacock[2]; Angelica S. Federici[3]; David A. Hoey[3]; Sophie C. Cox[1]
[1]University of Birmingham, Birmingham, United Kingdom; [2]NanoFCM Co., Ltd., Nottingham, United Kingdom; [3]Trinity College Dublin, Dublin, Ireland

Introduction: Extracellular vesicles (EVs) have garnered growing attention as promising acellular tools for bone repair. Although EVs potential has been shown, issues associated with their therapeutic potency and short half-life in vivo hinders their clinical utility. Epigenetic reprogramming with the histone deacetylase inhibitor Trichostatin A (TSA) has been reported to promote the osteoinductive potency of osteoblast-derived EVs. Gelatin methacryloyl (GelMA) hydrogels functionalised with the synthetic nanoclay laponite (LAP) have been shown to enhance the retention of bioactive factors. This study investigated the potential of utilising a GelMA-LAP hydrogel to improve local retention and control delivery of epigenetically-activated EVs as a novel bone repair strategy.
Methods: GelMA/nanoclay composites were fabricated by combining 5wt% GelMA with different concentrations of LAP (0.5, 1 and 2 wt%) prior to visible light crosslinking. The hydrogels compressive modulus, shear-thinning behaviour, 3D printing fidelity and osteogenic potency was evaluated. EVs were derived from 5 nM TSA-treated or untreated osteoblasts over a 2-week period. EVs size, morphology and concentration were assessed via nanoflow cytometry and transmission electron microscopy. Isolated EVs were incorporated within the composites and their release kinetics were determined using the CD63 ELISA. Human bone marrow stromal cells (hBMSCs) osteogenic differentiation within the EV-functionalised hydrogel was evaluated by biochemical and histological analysis.
Results: LAP incorporation improved GelMA compressive modulus, shear-thinning properties and 3D printed shape fidelity in a dose-dependent manner when compared to LAP-free gels. Moreover, hydrogels containing LAP increased hBMSCs mineralisation capacity (1.41-fold) over 14 days. EV release kinetics from these nanocomposites were strongly influenced by LAP concentration with significantly more vesicles released from LAP-free constructs. EVs derived from TSA-treated osteoblasts (TSA-EVs) enhanced proliferation (1.09-fold), migration (1.83-fold), and mineralisation (1.87-fold) of hBMSCs when released from the GelMA-LAP hydrogel compared to the untreated EV gels. Importantly, the TSA-EV functionalised GelMA-LAP hydrogel significantly promoted encapsulated hBMSCs extracellular matrix collagen production (≥1.3-fold) and mineralisation (≥1.78-fold) in a dose-dependent manner compared to untreated EV constructs.
Summary/Conclusion: Taken together, these findings demonstrate the potential of combining epigenetically-activated EVs with a nanocomposite photocurable hydrogel to enhance the therapeutic efficacy of acellular vesicle approaches for bone repair.

OWP2.06=PT11.15 | The role of Notch2 in osteoclastogenic and angiogenic potential of extracellular vesicles in multiple myeloma

Domenica Giannandrea[1]; Natalia Platonova[1]; Michela Colombo[1]; Mara Mazzola[2]; Valentina Citro[1]; Raffaella Adami[1]; Filippo maltoni[1]; Silvia Ancona[1]; Vincenza Dolo[3]; Ilaria Giusti[3]; Andrea Basile[4]; Anna Pistocchi[2]; Laura Cantone[5]; Valentina Bollati[6]; Lavinia Casati[1]; Elisabetta Calzavara[7]; Mauro Turrini[7]; Elena Lesma[1]; Raffaella Chiaramonte[1]
[1]Department of Health Sciences, Università degli Studi di Milano, Milano, Italy; [2]Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Milano, Italy; [3]Department of Life, Health and Environmental Sciences- University of L'Aquila, L'Aquila- Italy, L'Aquila, Italy; [4]Department of Oncology and Hemato-oncology, Università degli Studi di Milano, Milano, Italy; [5]Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milano, Italy; [6]Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy, Milano, Italy; [7]Division of Hematology, Valduce Hospital, Como, Italy

Introduction: Multiple myeloma (MM) is still uncurable due to the crosstalk between MM cells and bone barrow (BM) cells. Extracellular vesicles (EVs) play a key role in the pathological communication between MM and BM. Notch2 receptor and Jagged1 and 2 are overexpressed in MM, triggering Notch pathway also on BM population and inducing their pro-tumorigenic activity. Here, we investigate the effect of MM-derived EVs in eliciting the pro-tumoral effect of BM in MM progression and the role of vesicular Notch2.
Methods: MM cells constitutively inhibited for Notch2 (MMN2KD-EVs) and the shed EVs (MM-EVs) were characterized for Notch receptors by Western blot, for size and number by nanoparticle tracking analysis and electronic transmission microscopy, and for their uptake in recipient cells by confocal and flow cytometry. The transfer of Notch2 via EVs was evaluated by an in vitro system of sending cells expressing HA-tagged Notch2, their EVs and receiving cells. Notch pathway activation was assessed by Notch reporter assays in vitro (HeLa cells) and in vivo (transgenic zebrafish embryos). The pro-tumorigenic effect of MM-EVs and MMN2KD-EVs were evaluated by measuring their osteoclastogenic and angiogenic potential in vitro. To confirm the role

of vesicular Notch, the effect of MM-EVs or EVs from the BM of MM patients was assessed in the presence of a g-secretase inhibitor (GSI), which affects Notch activation (Informed consent and IRB of Insubria approval was obtained n. 1/2018.).
Results: MM-EVs carry and transfer Notch2 increasing Notch signaling in recipient endothelial cells and osteoclasts. MM-EVs induce the osteoclast formation and angiogenesis in a Notch2 dependent way. GSI effectively decreased the osteoclastogenic and angiogenic potential of MM-EVs in vitro and the angiogenic effect of EV from MM patients' BM.
Summary/Conclusion: These results suggest that targeting Notch pathway may hamper the pro-tumorigenic activity of MM-EV in the BM.

OWP2.07=PT11.02 | CD63 Regulates Cholesterol storage within endosomes and its distribution via exosomes

Roberta Palmulli[1]; Mickael Couty[2]; Frederik Verweij[3]; Stéphanie Charrin[4]; Maharajah Ponnaiah[5]; Melissa C. Piontek[6]; Florent Dingli[7]; Wouter H. Roos[6]; Eric Rubinstein[8]; Marie Lhomme[9]; Anatol Kontush[9]; Damarys Loew[7]; Graça Raposo[10]; Guillaume van Niel[11]
[1]Department of Pathology, University of Cambridge, Cambridge. CB2 1QP. United Kingdom, Cambridge, United Kingdom; [2]Université de Paris, Institute of Psychiatry and Neuroscience of Paris (IPNP), INSERM U1266, F-75014 Paris, France, paris, France; [3]Université de Paris, Institute of Psychiatry and Neuroscience of Paris (IPNP), INSERM U1266, F-75014 Paris, France, Paris, France; [4]Centre d'Immunologie et des Maladies Infectieuses, Inserm, CNRS, Sorbonne Université, CIMI-Paris, 75013 Paris, France, Paris, France; [5]National Institute for Health and Medical Research (INSERM), UMR-ICAN 1166, Paris, France, paris, France; [6]Faculty of Science and Engineering Molecular Biophysics — Zernike Institute for Advanced Materials Nijenborgh 4 9747 AG Groningen The Netherlands, Gronigen, Netherlands; [7]Institut Curie, PSL Research University, LSMP - Curie CoreTech Mass Spectrometry Proteomics, Paris, France; [8]Centre d'Immunologie et des Maladies Infectieuses, Inserm, CNRS, Sorbonne Université, CIMI-Paris, 75013 Paris, France, paris, France; [9]National Institute for Health and Medical Research (INSERM), UMR-ICAN 1166, Paris, France, Paris, France; [10]Institut Curie CNRS, PARIS 05, France; [11]Institute of Psychiatry and Neurosciences of Paris, INSERM U1266, France, Paris, France

Introduction: Exosomes are extracellular vesicles (EV) of endosomal origin that function as a clearance cellular pathway and in intercellular communication. CD63 is a tetraspanin enriched on intraluminal vesicles of multivesicular endosomes (MVEs) and is hence commonly used as exosomal marker and for functionalizing exosomes. Yet, the role of CD63 in Intra-Luminal Vesicles (ILVs) and exosomes biogenesis remains unclear.
Methods: To determine the role of CD63 in intracellular trafficking and ILVs biogenesis we generated in vitro cell lines KO for CD63 by CRISPR editing in HeLa and melanoma MNT1 cell lines. We isolated extracellular vesicles by size exclusion chromatography (SEC) for phenotypic analysis and differential ultracentrifugation (UC) for functional assays. We combined lipidomic and proteomic analysis to live cell imaging, photon and electron-microscopy coupled to immunolabeling to decipher pathways where CD63 is involved in generated KO cell lines and derived extracellular vesicles.
Results: We report here that depletion of CD63 neither impairs size and distribution of EV isolated by SEC nor enrichment of canonical exosome marker. OMICs analysis showed reveals changes in lipid composition, in particular cholesterol between Wild-Type (WT) and CD63 KO cells. This led us to study the role of CD63 in cholesterol sorting into ILVs and exosomes. Live cell imaging of cholesterol trafficking and staining of cholesterol enriched microdomains by EM show that CD63 controls sorting of cholesterol to ILVs and exosomes. In absence of CD63, cholesterol is redirected to the Trans-Golgi Network using actin-related tubulation from MVEs. In feeding assay we show that ILVs can serve as endosomal storage entities for cholesterol and exosomes can be used as alternative source of cholesterol by recipient cells.
Summary/Conclusion: Overall, these results demonstrate a role for CD63 in sorting cholesterol to ILVs and exosomes, a process consistent with the roles previously attributed to CD63. Our work also establishes ILVs and exosomes as an alternative source of cholesterol for cells that would complement that provided by lipoparticles.
Funding: RP was a beneficiary of a fellowship from the Institute Curie International PhD programme and the ARC foundation. MC is grateful to the ARC foundation (PGA1 RF20190208474). GvN is grateful to the Institut National du Cancer (PLBIO19-059.

OWP2.08=PT11.06 | Fluorescent Nanoprobes Based on Goat Milk Small Extracellular Vesicles for In Vivo Detection of Inflammation Processes

María Isabel González[1]; Ana Santos-Coquillat[2]; Agustín Clemente-Moragón[3]; Mario González-Arjona[2]; Gorka Sobrino[3]; Virginia Albaladejo-García[2]; Héctor Peinado Selgas[4]; Javier Muñoz[5]; Pilar Ximénez Embún[4]; Borja Ibañez[3]; Eduardo Oliver[3]; Manuel Desco[2]; Beatriz Salinas[6]

[1]Instituto de Investigación Sanitaria Gregorio Marañón (Madrid, Spain), Madrid, Spain; [2]Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain; [3]Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain; [4]Spanish National Cancer Research Center (CNIO), Madrid, Spain; [5]Centro Nacional de Investigaciones Oncológicas Carlos III (CNIO), Madrid, Spain; [6]Instituto de investigación Sanitaria Gregorio Marañón, Madrid, Spain

Introduction: In the last decade, small extracellular vesicles (sEVs) are emerging as substitutes of synthetic nanoparticles in the field of molecular imaging due to their morphological and structural similarities, along with its proven biological role in the regulation of inflammatory processes. We present the potential use of goat milk sEVs labeled with commercial fluorophores as natural probes for the detection of inflammation by optical imaging.
Methods: sEVs isolated from goat milk by combined ultracentrifugation and size exclusion chromatography were fully characterized (TEM, DLS, NTA, proteomics and western blot) and fluorescently labeled with BDP and SCy5 fluorophores for 90 min at pH 8.5 and 4°C. After physicochemical characterization, in vitro internalization and cytotoxicity assays of sEVs-BDP were performed in RAW 264.7 macrophages (M0, M1 and M2). sEVs-SCy (20μg/PBS) were in vivo evaluated by optical imaging (6 and 21h) after the intravenous injection in a peritonitis mouse model. sEVs-SCy uptake by myeloid population from peritoneal exudates was analyzed by flow cytometry.
Results: sEVs-based nanoprobes were synthesized with high reaction yields (99% by flow cytometry), purity (>95%) and stability (up to 72h by HPLC), maintaining the original properties of non-labeled vesicles. The probes did not exhibited cytotoxicity or adverse effects in macrophages. In vitro assessment showed internalization in polarized and non-polarized macrophages, with highest uptake by M1. In vivo validation of the nanoprobes demonstrated their ability to detect the inflammatory process in the peritoneal region. Subsequent confocal and flow cytometry studies confirmed in vivo incorporation into inflammatory populations, with 28% of total macrophage population and 19% of neutrophils from peritoneal exudates.
Summary/Conclusion: We present a novel fluorescent probe based on goat milk sEVs able to in vivo detect inflammation underlying diseases, through its uptake by myeloid cells.
Funding: This study has been funded by Instituto de Salud Carlos III, through the project "PI20/01632", co-funded by European Regional Development Fund (ERDF), "A way to make Europe" and by Comunidad de Madrid, project "Y2018/NMT-4949 (NanoLiver-CM)" and "S2017/BMD-3867 (RENIM-CM)", co-funded by European Structural and Investment Fund. This work has been also supported by "Diagnosis and treatment follow-up of severe Staphylococcal Infections with Anti-Staphylococcal antibodies and Immune-PET - Grant Fundación BBVA a Equipos de Investigación Científca 2018". A. Santos-Coquillat is grateful for financial support to Consejería de Educación e Investigación, co-financed by European Social Fund (ESF) grant PEJD-2018-POST/BMD-9592. A. Santos Coquillat is also funded by Instituto de Salud Carlos III, co-funded by European Social Fund "Investing in your future" (grant CD19/00136).

OWP2.09=PT11.10 | Miscommunication in Amyotrophic Lateral Sclerosis and Frontotemporal Dementia: how to see it?

Paolo Vincenzo Fioretti[1]; Alice Migazzi[1]; Giorgina Scarduelli[1]; Luisa Donini[1]; Silvia Del Longo[1]; Manuela Basso[2]
[1]Department of Cellular, Computational and Integrative Biology – CIBIO, University of Trento, Trento, Italy; [2]Department of Cellular, Computational and Integrative Biology – CIBIO, University of Trento; Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano (Italy)., Trento, Italy

Introduction: Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal dementia (FTD) are progressive neurodegenerative diseases characterized by protein inclusions enriched in a protein called TDP-43. Among several factors, the progression of these diseases could be mediated by altered intercellular communication between neurons and glial cells. Published data and our preliminary results suggest that extracellular vesicles (EVs) derived from astrocytes of a transgenic mouse model of ALS, overexpressing TDP-43Q331K, transmit toxicity to wild type neurons. Whether EVs modulate toxicity by directly entering the receiving neurons or by other mechanisms is still unknown.
Methods: Primary cultures were prepared as previously described and comply with the Declaration of Helsinki (authorization from the Italian Ministry of Health # 576/2021-PR). To isolate EVs, we used a charged-based methodology, called nickel-based isolation (NBI), on conditioned media concentrated through Amicon filters. EVs visualization was performed with confocal and spinning disk microscopy, on immortalized cells and glial and neuronal primary cultures derived from ALS and FTD mice. Preliminary single-particle tracking experiments were carried out at the Nanoimager, ONI.
Results: We isolated EVs from astrocytes overexpressing EGFP-CD63 and incubated them with receiving neurons, resulting in the detection of internalized signal. We developed new fluorescent tools with proteins belonging to the tetraspanin (CD9 and CD81) and annexin (ANXA2 and ANXA11) families to compare uptake kinetics and toxicity of different EV subpopulations. To

further investigate the uptake mechanisms, experiments of EV co-localization with lysosomes and endoplasmic reticulum, are ongoing in the lab, together with treatments with inhibitors of distinct uptake pathways.

Summary/Conclusion: Deciphering the neuronal uptake mechanisms of glial EVs in a TDP-43Q331K model uncover novel pathogenic mechanisms of ALS and FTD.

Funding: Supported by the MSCA-IF ExItALS (752470), the Italian Ministry of Health (GR-2016-02361552) and 1° Bando Eccellenze 2021 - Fondazione VRT.

OWP2.10=PT11.01 | Cancer extracellular vesicles promotes lymph node metastasis via neutrophil extracellular traps

Xin SU[1]; Ariane Brassard[1]; Ramin Rohanizadeh[2]; Iqraa Dhoparee-Doomah[1]; Betty Giannias[3]; France Bourdeau[3]; Roni Rayes[3]; Julia Burnier[2]; Jonathan Spicer[2]; Veena Sangwan[2]; Lorenzo Ferri[2]; Jonathan Cools-Lartigue[2]

[1]RI-MUHC/McGill University, MONTREAL, Canada; [2]RI-MUHC/McGill University, Montreal, Canada; [3]McGill University, Montreal, Canada

Introduction: Lymph nodes (LNs) are the first sites of metastasis for most of solid cancers. The initiation of LN metastasis is mediated by immune cells including polymorphonuclear neutrophils (PMNs), and tumor derived factors, such as tumour extracellular vesicles (tEV). Our earlier work showed that PMNs and neutrophil extracellular traps (NETs) can capture circulating tumour cells and promote metastasis in distant sites. Thus, one potential mechanism of increased LN metastasis is that tEV recruit PMNs and induce NETs formation.

Methods: Human tissue micro-arrays (TMAs) of gastroesophageal (GEA) cancer patients were stained with PMN and NETs markers and quantified by HALO software. C57BL/6 or pad4-/- mice were injected with B16F10 or H59 cells alone or treated with neutrophil elastase inhibitor (NEi) or neutrophil depletion antibody. LN sections were stained with NETs markers and quantified by ImageJ (NIH). EV were isolated from cell culture media by ultracentrifugation.

Results: In the study of 175 GEA cancer patients, lymphatic NET deposition was observed. This was associated with reduced survival even in the absence of overt metastasis($p=0.03$). Next, we demonstrated the lymphatic accumulation of tEVs both in vitro and in vivo. In both settings this was associated with PMN recruitment and NETs deposition. In vivo, NETs deposition was associated with increased development of lymphatic metastasis, and LN metastasis was abrogated through different kinds of NETs inhibition (neutrophil depletion, pad4 knockout and NEi treatment, $n=10$, $p< 0.001$), demonstrating the consequences of LN NETs deposition and its potential as a treatment target. Finally, we showed that tEV induced PMN recruitment and NETs formation was mediated by increased production of IL-8 by Lymphatic Endothelial Cells (LEC).

Summary/Conclusion: Together, we demonstrated that tEVs can contribute to LN metastasis as PMN recruiter and NETs inducer, as well as a potential therapeutic targets. By further investigating the detailed mechanism, this project will lead to major advances in the management of cancer patients.

Funding: The Thoracic Surgery Foundation and MGH start-up fund

OWP2.12=PT11.14 | Surface proteins dictate the diffusion of extracellular vesicles derived from myofibroblasts through various components of the extracellular matrix during skin wound healing

Syrine Arif[1]; Sébastien Larochelle[2]; Véronique J. J. Moulin[3]

[1]Centre de recherche en organogénèse expérimentale de l'Université Laval/LOEX - Centre de recherche du CHU de Québec-Université Laval, Québec, QC, Canada, Québec, Canada; [2]Centre de recherche en organogénèse expérimentale de l'Université Laval/LOEX - Centre de recherche du CHU de Québec-Université Laval, Québec, QC, Canada, Quebec, Canada; [3]Centre de recherche en organogénèse expérimentale de l'Université Laval/LOEX - Centre de recherche du CHU de Québec-Université Laval, Québec, QC, Canada; Département de chirurgie, Faculté de médecine, Université Laval, Québec, QC, Canada, Quebec, Canada

Introduction: The intercellular communication through the extracellular matrix (ECM) of a tissue is essential for skin wound healing. Myofibroblasts, specialized cells of this process, produce extracellular vesicles (EVs) that stimulate angiogenesis and tissue remodeling in vitro, two key steps in wound healing. EVs can target local or distal cells based on their free diffusion or binding to their environment. However, the diffusion mechanisms of EVs through solid tissues remain largely unexplored. Binding of EVs to the ECM could modulate EVs diffusion and therefore modify their action during healing.

Methods: EVs were extracted from myofibroblast culture medium by differential centrifugation. To evaluate EVs binding to ECM molecules, we mixed fluorescent EVs, pretreated or not with proteinase K, with solutions of type I collagen or type III collagen. Following hydrogel polymerization, a buffer was added allowing a passive exchange between the two compartments.

EVs presence in the buffer was quantified for 4 days by evaluating fluorescence appearance. The presence of some integrins were detected by flow cytometry and specific peptides were used to evaluate EVs-ECM binding.
Results: EVs did not diffuse outside of collagen I gel compared to the rapid diffusion observed when collagen III was used. The lack of diffusion observed when EVs were added into collagen I was cancelled when EVs were pretreated with proteinase K or RGD-peptide. Several integrins including $\alpha5\beta1$ and $\alpha2\beta1$ have been detected on EVs.
Summary/Conclusion: Our data provide evidence that the bioavailability of EVs as well as their action during healing might be dictated by the local organization of the ECM: collagen III is predominant at the onset of healing whereas collagen I is mainly predominant at the end of healing process.
Funding: This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) (RGPIN2014–04404).

OWP2.13=PT11.12 | Platelet concentrate and red blood cell product derived extracellular vesicles: composition and specific targeting into different immune cells

Petra Ilvonen; Ulla Impola; Saara Laitinen
Finnish Red Cross Blood Service, Helsinki, Finland

Introduction: Blood-derived extracellular vesicles (EVs) are shown to have great therapeutic potential. Until recently, characterizing the composition of clinically used platelet concentrate (PC) and red blood cell (RBC) product-derived EVs in modern technology has been possible. We have previously shown that PCs used for transfusions contain an increasing amount of EVs after a longer storage period and the number of EVs and platelet activation is also affected by platelet additive solutions (PASs). Few recent publications show that platelet EVs from plasma can target specifically certain mononuclear cell populations but little is known about PC-derived EVs and their immunological function as varying manufacturing and purification methods also yield different EV populations.
Methods: Different isolation methods were tested and ultracentrifugation (UC) was selected to collect a pure population of PC-derived EVs. EVs were further analyzed for surface proteins and contaminating lipoprotein particles using Imaging Flow Cytometer and western blot analysis. Targeting into different mononuclear cells as well as their immunological relevance was investigated in vitro using imaging flow cytometry and IIF microscopy.
Results: With UC we get a population of EVs with low contamination of plasma protein impurities. The main population of the PC-derived EVs are CD41 positive, whereas the population of EVs derived from RBC product contain several CD235 negative EVs. We show that the origin of EVs affects their targeting and uptake kinetics into mononuclear cells affecting their immunophenotype.
Summary/Conclusion: In this study, we identified differences in the immunological function of PC and RBC-product EVs. Different EV populations isolated from blood products might be utilized either as therapeutic components or as drug delivery vehicles. This would give new applications for donated blood components.
Funding: EV Ecosystem for Theranostic Platforms, funded by Business Finland

OWP2.14=PT11.08 | Mapping the biodistribution of CD63 endogenously produced pancreatic cancer EVs

Bárbara Adem[1]; Nuno Bastos[1]; Carolina F Ruivo[1]; Barbara Seidler[2]; José C Machado[3]; Dieter Saur[2]; Dawen Cai[4]; Sonia A Melo[3]
[1]i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal; IPATIMUP - Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Portugal; Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Portugal, Porto, Portugal; [2]Medical Clinic and Polyclinic II, Klinikum rechts der Isar, Technical University Munich, Germany; German Cancer Research Center (DKFZ) and German Cancer Consortium (DKTK), Heidelberg, Germany, Munich, Germany; [3]i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal; IPATIMUP - Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Portugal; Departamento de Patologia, Faculdade de Medicina, Universidade do Porto, Portugal., Porto, Portugal; [4]University of Michigan Medical School, Ann Arbor, Michigan, USA., Ann Arbor, USA

Introduction: Cancer cells use extracellular vesicles (EVs) as a mean to exchange information with its close-contact microenvironment and at distance, coordinating a myriad of biological functions. Still, how endogenously produced cancer EVs spontaneously flow in a multicellular organism, and with which organs/cells do they communicate remains highly unexplored.

Methods: We developed a CD63 multireporter transgenic mouse model in which tissue-specific derived EVs are fluorescently labelled, the ExoBow. Together with transgenic mice that faithfully recapitulate the pathogenesis of pancreatic ductal adenocarcinoma (PDAC) we have mapped the network of communication mediated by pancreatic cancer EVs within its microenvironment, and systemically, with other organs.
Results: Notably, the observed network of communication mediated by pancreas cells is plastic and varies according to the biological context. Prior to disease onset, pancreas EVs are scarcely exchanged within the normal pancreas microenvironment, and we found significant inter-organ communication with the thymus and stomach. Upon cancer establishment, the level of communication increases both locally and systemically and, the biodistribution of cancer EVs changes along disease progression. At early PDAC stages, communication occurs with intestines and mesenteric lymph nodes, while at late stages occurs with thymus and kidneys. Within the tumor, communication from cancer cells occurs significantly with cancer associated fibroblasts, endothelial and distinct subsets of immune cells.
Summary/Conclusion: Our work shows for the first time that the communication routes originated from cancer cells are not preferentially related with organs of metastasis. Rather, it suggests that cancer EVs are more actively involved in other processes of disease progression. Also, the dynamic nature of this communication network potentially contributes to the plastic capacity of the tumor.

OWP2.15=PT11.11 | Morphine-induced the same proteome modulation on Human Brain Microvascular Endothelial Cells and associated Extracellular Vesicles

Tatjana Vujic
University of Geneva, Genève, Switzerland

Introduction: Morphine, a member of opioid family, is commonly used as a gold standard for pain medication. This antinociceptive drug is transported to the Central Nervous System (CNS) by crossing the blood-brain barrier. The latter ensures the CNS homeostasis with brain endothelial cells, which are at the interface between the blood and the CNS. Moreover, one relatively unexplored form of cell-cell communication associated with brain in response to morphine exposure is extracellular vesicles (EVs). EVs are a nanosized particle released from the cells into the circulation and are a promising cargo reflecting the state of the cell of origin. Some studies suggested that morphine is involved in oxidative stress as well as inflammation and affects BBB permeability, but the biological processes are still not well understood. The purpose of this research was to characterize EVs derived from morphine-exposed brain endothelial cells. EVs were then analyzed with a combination of mass spectrometry-based proteomics and pathway enrichment strategies.
Methods: Human Brain Microvascular Endothelial Cells (HBMECs) were exposed to morphine (0, 1, 10, 25, 50 and 100 μM) for 24h. EVs were isolated using EVtrap beads (Tymora Analytical Operations) and characterized by their size distribution (NTA), their concentration (NTA) and the presence of well-known EV markers (western blots). High-throughput mass spectrometry-based quantitative proteomics by Data Independent Acquisition was then applied on whole HBMECs and HBMECs-derived EVs. Signature pathways of morphine-exposed HBMECs and EVs were analyzed by gene ontology terms and pathway enrichment (MetaCoreTM).
Results: EVs isolation was successful as the size of these particles was within the range (50-150nm) and that they possess common EV markers. The main results highlighted that HBMECs and HBMECs-derived EVs exposed to morphine have common modulated pathways, namely cell adhesion, oxidative stress and immune system.
Summary/Conclusion: In conclusion, our data confirmed that morphine has a detrimental effect on HBMECs-derived EVs by modulating proteins involved in the same three main biological processes (cell adhesion, oxidative stress and immunity). These biological pathways might be associated. Indeed, a high level of oxidative stress may lead to a lack of cell adhesion triggering the immune system defense. This effect is also observable on the whole cell. As EVs mirror their cell of origin, their common biological pathways may potentially be molecular signatures of the morphine-induced damaged in the circulation. However, more studies have to be done to validate these results.
Funding: Swiss National Funding

Oral Presentations

OF11: EV Detection

Chair: Marca H.M Wauben – Division of Cell Biology, Metabolism & Cancer, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Chair: André Görgens – Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

OF11.01 | In-chip characterization of EV population heterogeneity with single EV sensitivity

Alexia Stollmann[1]; Jaime Ortega Arroyo[1]; Jose Garcia Guirado[1]; Hyungsoon Im[2]; Romain Quidant[1]
[1]*Nanophotonic Systems Laboratory, Department of Mechanical and Process Engineering, ETH Zürich, Zurich, Switzerland;* [2]*Massachusetts General Hospital, Boston, USA*

Introduction: Early diagnostics is an area where humanity can improve its fight against many diseases, especially cancer. One of the most promising tools for improving early diagnostics are liquid biopsies, since they are minimally invasive, portable and low cost compared to other options. Extracellular vesicles (EVs) have been shown to have great potential as liquid biopsies; unfortunately, however, not enough is understood about these biological nanoparticles yet. This is because EVs are extremely heterogeneous in size, molecular composition and biogenesis, thus making them very difficult to isolate and characterize with single EV sensitivity and high throughput. Here, we developed an integrated microfluidics and interferometric scattering (iSCAT) microscopy platform to perform label-free multiplexed single-EV experiments that characterize the underlying heterogeneity of EV populations.
Methods: We designed a microfluidic chip that allows for multiplexed screening of different EV subpopulations within a sample. We tested different surface passivation strategies to reduce non-specific EV binding. EVs were then immobilized using tethered capture antibodies. We used CD63, CD9, CD81 and EpCAM, CD24, EGFR and EGFRvIII as EV and cancer specific biomarkers, respectively. We then performed EV binding experiments, using cell cultured derived EVs from Caov-3, ES-2, Gli36-WT and Gli36-EGFRvIII cell lines. The captured EVs were imaged using a custom iSCAT microscope, which is able to resolve single EVs in a label-free manner. The collected images were analyzed to obtain quantitative statistical information on all captured EV subpopulations.
Results: We found that passivating our microfluidic chip with a supported lipid bilayer reduced non-specific EV binding the most, all whilst being compatible with in-chip functionalization. The protocol to achieve this passivation was then optimized to control the number of binding sites. Multiplexed EV screening experiments were performed, allowing us to characterize the subpopulations present in EV samples. Our chips allow us to perform fast (total experiment time 3-4 hours), high-throughput (imaging area of 300x3000um2) and multiplexed (6 channels with a positive and negative control) immunoassays with minimal sample volumes (10 ul).
Summary/Conclusion: We developed an integrated platform with a protocol which enables in-chip surface passivation and experiment run-through, to study EV population heterogeneity. Our iSCAT detection method enables label-free single particle imaging and subsequent image analysis results in quantitative information about targeted EV subpopulations. These experiments prove that our platform is ideally suited for the characterization of EV subpopulations and show its potential to be used for multiplexed, high-throughput, specific and sensitive immunoaffinity assays.

OF11.02 | Machine learning-enabled quantitative detection of extracellular vesicle mixtures using nanoplasmonic sensors

Colin L. Hisey[1]; Mohammadrahim Kazemzadeh[2]; Miguel Martinez-Calderon[3]; MoiMoi Lowe[2]; Cherie Blenkiron[2]; Weiliang Xu[2]; Neil Broderick[2]; Lawrence W. Chamley[2]
[1]*The Ohio State University, Columbus, USA;* [2]*University of Auckland, Auckland, New Zealand;* [3]*CERN, Meyrin, Switzerland*

Introduction: Many extracellular vesicle (EV) studies rely on cell cultures for EV production, but the exogenous EVs from fetal bovine serum (FBS) or other sera supplements are difficult to remove and may cause confounding effects on downstream experiments. However, there are currently no adequate methods for routinely determining the relative amounts of FBS EVs within a given EV preparation. One potential solution involves the use of surface-enhanced Raman spectroscopy (SERS) to biochemically fingerprinting EVs in combination with machine learning algorithms to classify the otherwise convoluted spectra.

In this study, we combined EV SERS and a custom Autoencoder algorithm to quantitatively determine the relative amounts of FBS EVs to EVs from cultured cells within a mixture.
Methods: We first tested the feasibility of this approach using known ratios of SERS chemical standards Rhodamine B and Rhodamine 6G, which have similar but distinguishable spectra, much like EVs. We then isolated both commercial FBS EVs (Sigma) and bioreactor-produced MDAMB231 breast cancer EVs using differential ultracentrifugation and size exclusion chromatography. Following MISEV validation, including western blotting, transmission electron microscopy, and nanoparticle tracking analysis, we performed EV SERS on known mixtures of the two EV populations and trained the autoencoder to find a linear function that describes their relative amounts within any mixture.
Results: Our results show that EV SERS can be used to effectively fingerprint fetal bovine serum EVs and EVs from cell cultures, and that by applying a customized Autoencoder algorithm, their relative amounts within an EV mixture can be quantitatively determined. This approach is now being applied to EVs from mesenchymal stromal cell cultures, which often require serum supplementation, and which will require this type of quality control measure for future clinical translation.
Summary/Conclusion: We demonstrate the non-destructive, label-free, ultrasensitive, and quantitative detection of the relative amounts of FBS EVs in mixtures with EVs from cell cultures. This approach should provide several useful applications in diverse EV studies, such as monitoring the integrity of semipermeable membranes within EV bioreactors, ensuring the quality or potency of therapeutic EV preparations, or even determining relative amounts of EVs produced in cocultures.
Funding: Breast Cancer Foundation New Zealand Innovation and Technology Grant

OF11.03 | Plastic antibodies tailored as photonic materials for sensing extracellular vesicles

Manuela F. Frasco; Mariana S. Amorim; Joana F. Pires; M. Goreti G. F. Sales
BioMark@UC, Faculty of Sciences and Technology, University of Coimbra, Coimbra, Portugal, Coimbra, Portugal

Introduction: Plastic antibodies (PAs) are biomimetic polymer materials with high affinity and selectivity to a target biomolecule. PAs act as synthetic receptors because they retain an imprinted molecular memory obtained during polymer formation in the presence of the target. After template removal, these binding sites are selective and mimic specific biological recognition mechanisms with higher stability and robustness. Thus, PAs have been extensively applied in biosensors. Novel technologies for separation of extracellular vesicles (EVs) are urged to surpass the existing limitations, owing to the intrinsic complexity of EVs. Simultaneously, tailoring PAs as photonic materials enables a label-free optical detection upon recognition and binding of the EVs.
Methods: Various strategies of preparing PAs as structured hydrogels or as core-shell imprinted materials were pursued. Surface proteins on EVs, namely CD9 and CD81, were chosen as target molecules, and the polymer networks were combined with self-assembled colloids having photonic properties.
Results: The photonic PAs-based biosensors demonstrated a selective and sensitive recognition of the target proteins, as observed by changes in the reflectance spectra of the sensing materials, while the non-imprinted controls had random variations. Also, the developed sensing materials responded to the presence of EVs.
Summary/Conclusion: PAs can be tailored with different formats and conjugated with a label-free detection approach are very promising biomimetic materials to improve the sensing of EVs. Moreover, the selectivity presented by these materials, allied to their low cost of production and stability, are relevant features considering the capture of EVs by specific surface markers, and thus can be useful to analyse subpopulations of EVs.
Funding: The authors gratefully acknowledge funding from the European Commission trough the project MindGAP (FET-Open/H2020/GA829040).

OF11.04 | Detecting the collective nanomechanical fingerprint of EV populations by COlloidal NANoplasmonics (CONAN)

Lucrezia Caselli[1]; Jacopo Cardellini[2]; Lucia Paolini[3]; Andrea Zendrini[3]; Annalisa Radeghieri[3]; Giuseppe Pomarico[3]; Andrea Ridolfi[4]; Marco Brucale[4]; Francesco Valle[4]; Costanza Montis[5]; Paolo Bergese[3]; Debora Berti[6]
[1]Università degli Studi di Firenze, Firenze, Italy; [2]Università degli Studi di Firenze, Florence, Italy; [3]Università degli studi di Brescia, Brescia, Italy; [4]Italian National Research Council (CNR), Institute for the Study of Nanostructured Materials (ISMN), Bologna, Italy, Bologna, Italy; [5]University of Florence, Italy, Firenze, Italy; [6]University of Florence, Italy, Florence, Italy

Introduction: We focus on some unique characteristics of the interaction of citrated gold nanoparticles (AuNPs) with lipid membranes (Montis et al. 2014 doi:10.1039/C4NR00838C, Montis et al. 2020 doi:10.1016/j.jcis.2020.03.123) and combine them with nanoplasmonics to propose an accessible, versatile, fast and cost-effective method for assaying the collective nanomechanical properties of EV populations.

Methods: Separation and characterization of EV samples from different sources compliant with MISEV2018. EV hybrids obtained by post-separation functionalization with selected proteins via click chemistry. All the other materials and methods as reported in Caselli et al. 2021 doi:10.1039/D1NH00012H.
Results: By combining UV-Vis Spectroscopy, Small-Angle X-ray Scattering (SAXS) and Atomic Force Microscopy (AFM)-based Force Spectroscopy, we demonstrate that the stiffness of vesicles modulates the surface plasmon resonance (SPR) of the AuNPs adsorbed on their surface, which can be readily monitored by UV-Vis spectroscopy. We then leverage this discovery to show that SPR tracking provides quantitative access to the mean stiffness of samples of liposomes as well as of native and hybrid EVs.
Summary/Conclusion: A "plasmon-based nanoruler" has been set up for collectively fingerprinting EV populations by their stiffness and successfully tested on native and hybrid EVs. These findings open new perspectives for fundamental analytical investigation of EVs, while promoting nanomechanical properties as an appealing complement/alternative for large-scale EV characterization, grading and monitoring.
Funding: European projects "evFOUNDRY" (H2020 FETOPEN, n. 801367) and "BOW" (H2020 FETPROACT, n. 952183).

OF11.05 | Benchmarking purification and proteomic characterization of human plasma extracellular vesicles

Alin Rai; David W. Greening
Baker Heart and Diabetes Institute, Melbourne, Australia

Introduction: Although mass spectrometry (MS) remains a powerful tool to rapidly identify extracellular vesicle (EV) proteins, yet our understanding of circulating EVs in human remains limited mainly due to technical limitations in detecting low-abundant EV proteins against a background of abundant non-EV plasma components that co-purify using current EV isolation strategies.
Methods: We introduce direct density-gradient separation protocol to isolate circulating EVs from human plasma and performed their biophysical/biochemical (using MS) characterization.
Results: From clinically relevant volume of human plasma (500 μl, ~100 mg), we isolate circulating EVs (~2 μg, buoyant density 1.08-1.10 g/ml) of high purity (EV-METRIC: 100%), achieving a ~50 × 106 fold enrichment. Cryo electron microscopy revealed that purified EVs were 100-800 nm in size, spherical in shape and morphologically intact, with minimal contamination of lipoprotein particles/protein aggregates. EVs display enrichment of EV markers (CD9/44/81, Syntenin 1), and depletion of abundant plasma proteins (albumin, transferrin), lipoprotein particles (APOA1/B100) and non-EV proteins (AGO2, complement proteins). MS quantified 1787 proteins including receptors (integrins, ephrins), kinases, small EVs and large EVs proteins (markers, hallmark ontologies and surfaceome), cell-specific protein signatures and those implicated in Disease Ontologies. Comparative Ontology analysis of our Plasma EV proteome and well-characterized EV proteome from different cell lines enabled careful assessment of reported Plasma EV proteomes, highlighting limitations in current strategies (including commercial kits).
Summary/Conclusion: Our study provides an effective protocol to isolate circulating EVs from human plasma, particularly for discovery and functional studies, and will serve as a benchmark for future studies of plasma EV research. We propose that our study provides a way forward for standardization of plasma EV protein landscape.

OF11.06 | Metabolically labeled exosomes for biogenesis and functional studies

Maria L. fiani; Valeria Barreca; Deborah Polignano; Lorenzo Galli; Valentina Tirelli; Massimo Sanchez; Mario Falchi; Lucia Bertuccini; Massimo Tatti; Massimo Sargiacomo
Istituto Superiore di Sanità, Rome, Italy

Introduction: Exosomes are small Extracellular Vesicles (sEV) formed by an endosomal route by inward budding of the late endosome/multivesicular body (MVB) membrane. Despite in recent years much progress has been made to better define sEV composition and biogenesis pathways, their small size and heterogeneity pose challenges to find new reliable labelling strategies to identify specific exosome populations. We developed an innovative methodology to metabolically label fluorescent sEV through the use of a fluorescent lipid (BODIPY C16) that is readily internalized by cells and is transformed into phospholipids which will form part of the lipid bilayer of the secreted vesicles.
Methods: Fluorescent sEV secreted in the conditioned media of melanoma cells pulsed with BODIPY FL C16 were purified by differential ultracentrifugation, quantified by Flow Cytometry (FC) and Nanoparticle Tracking Analysis (NTA), sorted by Fluorescence Activated Cell Sorting (FACS) and further characterized by density gradient separation and Western Blot analysis for typical sEVs markers. Colocalization studies were performed by confocal microscopy and electron microscopy.
Results: Confocal images showed colocalization of BODIPY lipids with lipid transformation sites such as ER and mitochondria and with specific markers of late endosomes/MVB or other organelles (tetraspanins, Golgi markers, lysosomes) but not with the

plasma membrane. Secretion of fluorescent sEV (Bodipy sEV) was followed over time showing an early release of Bodipy sEV into the extracellular medium with a constant ratio of Bodipy sEV/total EVs, as determined by NTA, up to 6 hours. Bodipy sEV secreted in the conditioned media purified by differential ultracentrifugation were separated by density gradient fractionation. Fractions analysed by FC displayed a single low density peak at 1,08-1,09 g/ml that is detergent sensitive demonstrating that fluorescent particles are indeed lipid vesicles and contain tetraspanins (CD63, CD81 and CD9), syntenin and ESCRT components when analysed by Western Blot. Electron microscopy analysis of ultracentrifuged and sorted Bodipy sEV showed that Bodipy sEV have the typical shape and size (about 80 nm) of a subpopulation of sEV often referred to as small exosomes (Exo-S). Finally, colocalization studies of single Bodipy sEV with tetraspanins fluorescent antibodies showed colocalization of Bodipy sEV with CD63, CD81 and CD9.

Summary/Conclusion: Taken together these results show a very specific and effective labelling of a discrete sEV subpopulation that can be further exploited for biogenesis, internalization and functional studies.

Funding: This work was supported by the Italian Ministry of Health (grant RF-2019-12369719)

OF12: EVs in Cancer Immunology and Cancer Immunotherapy

Chair: Theresa L. Whiteside – UPMC Hillman Cancer Center, University of Pittsburgh, Cancer Institute Pittsburgh, Pennsylvania, USA

OF12.01 | Antigen-loaded extracellular vesicles induce responsiveness to anti-PD-1 and anti-PD-L1 treatment in a checkpoint refractory melanoma model

Rosanne E. Veerman[1]; Gözde Güclüler Akpinar[2]; Annemarijn Offens[1]; Loïc Steiner[1]; Pia Larssen[1]; Andreas Lundqvist[3]; Mikael Karlsson[4]; Susanne Gabrielsson[1]

[1]Division of Immunology and Allergy, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden, Stockholm, Sweden; [2]Division of Immunology and Allergy, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden, Solna, Sweden; [3]Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden, Stockholm, Sweden; [4]Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden, Stockholm, Sweden

Introduction: Extracellular vesicles (EVs) are important mediators of intercellular communication and are candidates for cancer immunotherapy. Immune checkpoint blockade, specifically of the PD-1/PD-L1 axis, mitigates T cell exhaustion but is only effective in a subset of cancer patients. Therefore, combination strategies are extensively explored. Reasons for therapy resistance can be low primary T cell activation to cancer antigens, poor antigen presentation and reduced T cell infiltration into the tumor. We here investigated if EV therapy could induce susceptibility to anti-PD-1 or -PD-L1 therapy in a checkpoint refractory B16 melanoma model.

Methods: EVs carrying the model antigen ovalbumin (OVA) and adjuvant α-galactosylceramide were isolated from murine bone marrow-derived dendritic cells by ultracentrifugation and characterized by nanoparticle tracking analysis, transmission electron microscopy, western blot and bead-based flow cytometry. Mice were inoculated with B16 melanoma cells and treated with anti-PD-1 or -PD-L1. EVs were administered therapeutically or prophylactically. Tumor growth was monitored and immune cells from the tumor and spleen were analyzed by flow cytometry and ELISPOT.

Results: Injection of EVs but not checkpoint blockade induced a potent antigen-specific T cell response and reduced tumor growth. The combination of EVs and anti-PD-1 or -PD-L1 in a therapeutic model caused a trend towards higher numbers of OVA-specific CD8+ T cells. Moreover, the combination therapy prolonged survival in a prophylactic model.

Summary/Conclusion: EVs induced potent anti-tumor immune responses and furthermore induced anti-PD-1 or anti-PD-L1 sensitivity in a checkpoint-refractory mouse tumor model. This shows that EVs have potential as a treatment for patients not responding to checkpoint blockade.

OF12.03 | ICAM-1-mediated Adhesion is a Prerequisite for PD-L1 Exosome-induced T Cell Suppression

Wei Guo[1]; Wei Zhang[2]; Wenqun Zhong[2]; Beike Wang[2]; Jiegang Yang[2]; Jingbo Yang[2]; Ziyan Yu[2]; Xiaowei Xu[3]

[1]University of Pennsylvania, Philadelphia, USA; [2]University of Pennsylvania Department of Biology, Philadelphia, USA; [3]University of Pennsylvania Department of Pathology, Philadelphia, USA

Introduction: Tumor-derived extracellular vesicles (TEVs) suppress the proliferation and cytotoxicity of CD8+ T cells, thereby contributing to tumor immune evasion. The molecular mechanisms by which TEVs interact with CD8+ T cells and suppress their function remains elusive.
Methods: Nanoparticle tracking analysis, electron microscopy, differential centrifugation, Iodixanol density gradient centrifugation, fluorescence microscopy, western blotting, ExoView single vesicle assay, flow cytometry, Extracellular vesicle-Target cell Interaction Detection through SorTagging (ETIDS), Immunohistochemistry, tumor xenograft in mice.
Results: The adhesion molecule ICAM-1 co-localizes with PD-L1 on the exosomes, and are both upregulated by interferon-g. Exosomal ICAM-1 interacts with LFA-1, which is upregulated on activated T cells. The reciprocal upregulation of ICAM-1 and LFA-1 promotes exosome-T cell interaction. Blocking ICAM-1 on TEVs reduces the interaction of TEVs with CD8+ T cells, and attenuates PD-L1-mediated suppressive effects of TEVs. During this study, we have established an Extracellular vesicle-Target cell Interaction Detection through SorTagging (ETIDS) system to assess the interaction between a TEV ligand and its target cell receptor. This system, for the first time, allowed the detection of the interaction between PD-L1 on TEVs and PD-1 on T cells, and this interaction is significantly reduced in the absence of ICAM-1.
Summary/Conclusion: ICAM-1-LFA-1 mediated adhesion between TEVs and T cells is a prerequisite for exosomal PD-L1 mediated immune suppression.
Funding: NIH R35 GM141832 and NCI CA174523.

OF12.04 | Tumor-derived extracellular vesicles, nanoparticles and co-isolated viral particles from endogenous retrovirus, contribute differently to the effect of bulk tumor-EVs/ENPs on immune modulation of antigen presenting cells

Federico Cocozza[1]; Lorena Martin-Jaular[2]; Aurélie Di Cicco[3]; Daniel Levy[3]; George Kassiotis[4]; Mercedes Tkach[1]; Clotilde Théry[2]
[1]INSERM U932, Institut Curie Centre de Recherche, PSL Research University, Paris, France, Paris, France; [2]Institut Curie, PSL Research University, INSERM U932, Paris, France; [3]CNRS UMR3215, Institut Curie Centre de Recherche, PSL Research University, Paris, France, Paris, France; [4]Retroviral Immunology, The Francis Crick Institute, London, NW 1AT, UK, London, United Kingdom

Introduction: Tumor cells secrete extracellular vesicles (EVs) and nanoparticles (ENPs) that have been associated with a plethora of immune regulatory functions. In particular, EVs have been proposed to act as a cell-free source of tumor antigens (Ag) and to carry together pathogen- or damage-associated molecular patterns that may induce immune priming of antigen presenting cells (APC) possibly resulting in immune responses against tumors. However, the EV composition and their cargo can be remarkably diverse, and different EVs and ENPs may have varying abilities to interact and modify recipient cells. Therefore, understanding the mechanisms by which the different types of EVs and ENPs modulate the responses of APCs in the context of tumor progression is crucial, and may identify suitable EV-based candidates to use as immunotherapeutic agents against tumors.
Methods: Here we characterize the heterogeneity of the secreted EVs and ENPs of E0771 cells, a murine mammary adenocarcinoma. Unexpectedly, we found viral-like particles (VLPs) of infectious endogenous murine leukemia virus (MLV) in the bulk isolates of EVs/ENPs. We set up a protocol that isolate these subpopulations of nanoparticles, to analyze separately 10k (large EVs + VLPs), small EVs, VLPs and ENPs.
Results: These subpopulations were differentially uptaken and induced different phenotypic changes in a dendritic cell line and primary DCs, as assessed by flow cytometry to measure upregulation of maturation markers and cell viability.
Summary/Conclusion: EVs isolated by regular protocls from mouse tumor cells contain VLPs coming from endogenous MLV with infectious potential.
Combination of filter concentration, centrifugation and density gradient allows to separate 10k, sEV, VLP and ENP.
These subtypes of particles contribute differently to modification of target immune myeloid cells in vitro.
These results call for re-evaluation of previous studies of mouse tumor-derived EVs to decipher the respective proportions and functions of non-viral EVs and virus-like particles.
Funding: "TRAIN-EV" a MSCA ITN, grant agreement No 722148. French funding sources: ARC, FRM, INCa, ANR.

OF12.05 | Colorectal cancer-derived Te-EVs function as tumor promoter by targeting monocytes via EVs-tRNA

Yuya Monoe[1]; Kentaro Jingushi[2]; Yoshiaki Takano[3]; Kohei Taniguchi[4]; Kazumasa Komura[5]; Kazutake Tsujikawa[6]
[1]Laboratory of Molecular and Cellular of Physiology, Graduate school of pharmacuetical scirnces, Osaka university, suita, Japan; [2]Laboratory of Molecular and Cellular Physiology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [3]Department of General and Gastroenterological Surgery, Osaka Medical and Pharmaceutical University, Takatsuki, Japan;

[4]Department of Translational Research, Osaka Medical and Pharmaceutical University, Tkatsuki, Japan; [5]Department of Translational Research, Osaka Medical and Pharmaceutical University, Takatsuki, Japan; [6]Laboratory of Molecular and Cellular Physiology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan

Introduction: Extracellular vesicles (EVs) have emerged as a novel mediator of tumorigenesis. Many reports suggest that EVs are inseparable part of cancer microenvironment from the experiments using cell lines and body fluids. However, these observations have not yet been experimentally proven in vivo. To understand the physiological function of cancer cell-derived EVs, we have analyzed the tumor tissue-derived EVs, which reflects physiological characteristics and behavior in human body.
Methods: Clinical specimens: The colon cancer specimens were obtained from patients undergoing primary resection at the Osaka Medical and Pharmaceutical University, Japan. Tumor-associated normal colon tissue was also obtained from a subset of these patients when possible. Written informed consent was obtained from each patient, and the study was approved by the ethics review board of the Osaka Medical and Pharmaceutical University and was conducted according to the principles of the Declaration of Helsinki.
Purification of tumor tissue-derived EVs: Following excision, the tissue samples were immediately immersed in 4 ml DMEM medium without FCS and stored at 37oC for 2 h. Tissue-immersed medium was then centrifuged at 2,000 g, for 30 min, and the collected supernatants were subjected to the ultracentrifuge method (100,000 g x 2) for recovery of tissue-exudative EVs (Te-EVs). The size and concentration of EVs were determined using qNano.
RNAseq for EVs-RNA: RNA was isolated from EVs samples using the miRNeasy Kit and subjected for RNAseq analysis.
Results: Compared to normal tissue, tumor released more EVs (tumor Te-EVs) which had larger particle diameter and higher small RNA content than those released from adjacent normal tissue (normal Te-EVs). RNAseq showed that 5'-tRNA fragment Glycine-GCC (5'-tRF-Gly-GCC) was much higher in tumor Te-EVs compared to normal Te-EVs. Phenotypical analysis showed that the tumor Te-EVs but not normal Te-EVs, upregulated the secretion of inflammatory cytokines in monocytes via Toll-like receptor 8 (TLR8). In vitro-transcribed 5'-tRF-Gly-GCC also upregulated the secretion of inflammatory cytokines in monocytes. Furthermore, supernatant obtained from tumor Te-EVs-educated monocytes, significantly upregulated the cell growth of colon cancer cells, which was abolished by TLR8 knockdown in monocytes.
Summary/Conclusion: Colorectal cancer EVs-RNA function as tumor promoter by targeting monocytes via TLR8.

OF12.06 | Reprogrammed T cell exosomes by surface engineering of cytokine induce potent anti-tumor effects

Kyungmoo Yea[1]; Moon-Chang Baek[2]; Dokyung Jung[3]
[1]Department of New Biology, DGIST, Daegu, Republic of Korea; [2]Kyungpook National University School of Medicine, Daegu, Republic of Korea; [3]School of Medicine, Kyungpook National University, Daegu, Republic of Korea

Introduction: Exosomes play a key role in the interaction between the immune system and cancer. Immune cell-derived, particularly T cell exosomes are known for their anti-cancer effect. However, to enhance the clinical applicability of T cell exosomes, it is essential to reinforce their anti-cancer potential.
Methods: First, we engineered T cell exosomes by attaching cytokine to the cell surface using a flexible linker. Then, physical characteristics of engineered exosomes were verified by using NTA and TEM analysis. Protein and mRNA levels of T cell activation and proliferation markers were determined by western blot and quantitative real-time PCR. Cancer cell viability was measured by in vitro bioluminescent or MTS assay. To identify the altered payload of EXO-cyts causative for their anti-cancer activity, we conducted miRNA-sequencing analysis of EXO-cyts to profile miRNAs, respectively. To investigate in vivo efficacy of EXO-cyts, tumor growth and metastasis were analyzed after exosome injection in melanoma mouse models. In addition, to test the combination effect, EXO-cyts and existing anti-cancer drugs were administered together.
Results: EXO-cyt increased the anti-cancer ability of CD8+ T cells, but interestingly, it did not affect the activity of regulatory T cells. Expression levels of cellular programmed cell death ligand-1 on melanoma cells and their exosomal PD-L1 were downregulated by EXO-cyt treatment, resulting in increase of CD8+ T cell-mediated cytotoxicity. The respective effects of EXO-cyt on CD8+ T cells and melanoma cells were reproduced by several miRNAs upregulated by the cytokine autocrine effect present inside EXO-cyt. In mouse models, EXO-cyt treatment inhibited tumor progression in melanoma-bearing immunocompetent mice but not in nude mice. The combination therapy of EXO-cyt and existing anti-cancer drugs significantly improved anti-tumor efficacy with decreasing PD-L1 in tumor tissues and plasma.
Summary/Conclusion: In summary, the expression of cytokine through a flexible linker on the surface of T cells induces dramatic changes in anti-cancer miRNA contents of exosomes in addition to the direct immune stimulation by cytokine. Remarkably, these EXO-cyts preferentially increased the proliferation and activity of CD8+ T cells without affecting Treg cells. Furthermore, EXO-cyts inhibited cPD-L1 and ePD-L1 levels both in vitro and in vivo melanoma models. Here, we propose that reprogrammed EXO-cyt is a potent cancer immunotherapy that works by modulating both immune cells and cancer cells. We anticipate that the

use of EXO-cyts alone or in combination with conventional immunotherapy could lead to significant advances in cancer therapy. In addition, our method to engineer exosome shows considerable potential in terms of robust reprogramming for exosome-based therapy beyond artificial payloads.

OF13: Neuronal and Neurodegenerative Diseases

Chair: Andrew F. Hill – Victoria University

Chair: Graça Raposo – Institut Curie UMR144, PSL Research University

OF13.01 | Efficacy of extracellular vesicles from hiPSC-derived neural stem cells in alleviating cognitive and mood dysfunction and neuropathologies in a model of Alzheimer's disease

Leelavathi N Madhu; Raghavendra Upadhya; Sahithi Attaluri; Maheedhar Kodali; Bing Shuai; Maha Kirmani; Jenny Jaimes Gonzalez; Ashok K. Shetty

Institute for Regenerative Medicine, Dept of Molecular and Cellular Medicine, Texas A&M Univ College of Medicine, College Station, USA

Introduction: Current therapies do not improve brain function in Alzheimer's disease (AD). Hence, there is a need for new approaches capable of restraining disease progression and improving brain function. In this regard, extracellular vesicles (EVs) from human induced pluripotent stem cell-derived neural stem cells (hiPSC-NSCs) exhibiting the therapeutic effects of NSCs are of great interest.
Methods: We investigated whether hiPSC-NSC-EV treatment in the early stage of AD would maintain better brain function. EVs from hiPSC-NSC cultures were purified through chromatography and then administered intranasally to 3-months old 5XFAD mice (~200 billion/week for two weeks). A month later, the efficacy of EV treatment on cognitive and mood function was probed via several behavioral tests, following which brain tissues were examined for neuropathologies and hippocampal neurogenesis.
Results: Both male and female AD mice receiving the vehicle exhibited impaired object location memory and pattern separation function, and anhedonia. Intranasally administered hiPSC-NSC-EVs incorporated into neurons and microglia in virtually all regions of the AD brain. Furthermore, both genders of AD mice receiving EVs displayed improved proficiency for object location memory and pattern separation with no anhedonia. Such functional benefits in EV-treated AD mice were associated with reductions in astrocyte hypertrophy, the density of microglia, oxidative stress markers malondialdehyde and protein carbonyls, and proinflammatory cytokines TNF-a, IL-1b, and MIP-1a in the hippocampus. EV-treatment also reduced amyloid-beta load and maintained a higher level of neurogenesis in AD mice.
Summary/Conclusion: The results imply that intranasal administration of hiPSC-NSC-EVs in the early stage of AD can maintain better cognitive and mood function by restraining the progression of neuroinflammatory changes and amyloid-beta accumulation and facilitating higher levels of hippocampal neurogenesis.
Funding: Supported by a grant from the National Institute of Neurological Disorders and Stroke (1R01NS106907 to A.K.S.)

OF13.02 | Relationship between plasma extracellular vesicle miRNAs and measures of neuropsychiatric function in methamphetamine use disorder

Ursula S. Sandau[1]; Erika Duggan[2]; Sierra J. Smith[3]; Marilyn Huckans[3]; John P. Nolan[2]; Jennifer M. Loftis[3]; Julie A. Saugstad[1]

[1]Oregon Health & Science University, PORTLAND, USA; [2]Scintillion Institute, San Diego, USA; [3]Oregon Health & Science University, Portland, USA

Introduction: The long-term health effects of methamphetamine including cognitive impairments, anxiety, and depression, which can persist during recovery and are associated with poor treatment outcomes. Thus, biosignatures based on objectively quantifiable blood biomarkers that relate to neuropsychiatric data could be used clinically to monitor recovery. We previously identified seven plasma extracellular vesicle (EV) miRNAs that are differentially expressed in humans with active methamphetamine disorder (MA-ACT) vs. controls (CTL). Here we related the expression of plasma EVs and their miRNAs to measures of neuropsychiatric function.
Methods: We measured the concentration of tetraspanin+ (CD9, CD63, CD81) and platelet EVs (CD41) in MA-ACT and CTL participants by vesicle flow cytometry. MiRNA expression in plasma EVs isolated by size exclusion chromatography were assayed by TaqMan arrays. Plasma EV concentrations and miRNA expression levels were related to measures of anxiety (GAD), depression (PHQ-9), and memory (PRMQ).

Results: CD41+ EVs correlated with depression scores in MA-ACT plasma, while TS+ EVs did not correlate with any of the neuropsychiatric measures. For the seven EV miRNAs with differential expression in MA-ACT, four correlated with clinical features of methamphetamine use disorder: miR-374b-5p and -628-3p correlated with anxiety and memory scores, respectively, while miR-301a-3p, -382-5p, and -628-3p correlated with methamphetamine use characteristics (e.g., frequency of use and lifetime exposure). The predicted gene targets of these four miRNAs identified pathways related to synaptic plasticity, neurodegeneration, neuroinflammation, and dopamine neuron function, which are all factors of methamphetamine dependency.
Summary/Conclusion: These studies demonstrate the potential utility of plasma EVs to serve as metrics for recovery by relating EVs and miRNA cargo to clinical features of methamphetamine use.

OF13.04 | Retinal pigment epithelium extracellular vesicles are potent inducers of age-related macular degeneration disease phenotype in the outer retina

Marzena Kurzawa-Akanbi[1]; Phillip Whitfield[2]; Florence Burté[1]; Birthe Hilgen[1]; Mark Platt[3]; Mary Doherty[4]; Rachel Queen[1]; Jonathan Coxhead[1]; Andrew Porter[1]; Maria Öberg[5]; Daniela Fabrikova[5]; Chia Shyan Beh[1]; Maria Georgiou[1]; Veronika Boczonadi[1]; Joseph Collin[1]; Anetta Härtlova[5]; Michael Taggart[1]; Jumana Al-Aama[6]; Viktor Korolchuk[1]; David Steel[1]; Lyle Armstrong[1]; Majlinda Lako[1]
[1]Newcastle University Biosciences Institute, Newcastle upon Tyne, United Kingdom; [2]Glasgow Polyomics, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom; [3]Loughborough University, Loughborough, United Kingdom; [4]Lipidomics Research Facility, University of the Highlands and Islands, Inverness, United Kingdom; [5]Wallenberg Center for Molecular and Translational Medicine, University of Gothenburg, Gothenburg, Sweden; [6]Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia

Introduction: Age related macular degeneration (AMD) is a leading cause of legal blindness. Vision loss is caused by the progressive loss of retinal pigment epithelium (RPE) and photoreceptors, and/or retinal and choroidal angiogenesis. It is unclear how disease features spread in the outer retina and whether extracellular vesicles (EVs) play a role in this process. We used AMD patient specific RPE with the high-risk Y402H polymorphism in the complement factor H (CFH) gene to perform a comprehensive analysis of EVs, their cargo and role in AMD pathology.
Methods: EVs were purified from cell conditioned media using size exclusion chromatography and characterised using transmission electron microscopy and western blot for EV markers (CD63, CD81 and Alix). RPE EV secretion was assessed using Tunable Resistive Pulse Sensing. Transcriptomic, proteomic and lipidomic analyses were used to identify the disease specific contents of EVs. Functional assays were employed to investigate the AMD RPE EV signaling in the outer retina.
Results: Analyses showed enhanced and polarised EV secretion in AMD RPE cells. Transcriptomic, proteomic and lipidomic analyses demonstrated that AMD RPE EVs carry a repertoire of RNA, proteins and lipids that reflect disease changes in the RPE cells of origin and mediate key AMD pathological processes including oxidative stress, cytoskeletal dysfunction, angiogenesis and drusen accumulation. We demonstrated that exposure of control RPE to AMD RPE apical EVs leads to the formation of cytoplasmic stress vacuoles, cytoskeletal destabilization, abnormalities in the morphology of the nucleus in the recipient cells, and protein and oxidative stress, these being key pathological features in AMD. Treatment of laminated retinal organoids with apical AMD RPE EVs led to disrupted neuroepithelium and appearance of enlarged cytoprotective alpha B crystallin immunopositive cells, some of which co-expressed retinal progenitor cell markers PAX6 or VSX2, consistent with the activation of regenerative pathways upon injury.
Summary/Conclusion: Our findings indicate that AMD RPE EVs act as signaling messengers in the outer retina with an important role in disease progression.
Funding: This work was funded by the Macular Society UK and MRC Confidence in Concept.

OF13.05 | Small extracellular vesicle remodeling in response to mutant huntingtin

Natayme R. Tartaglia[1]; Francesca Farina[2]; Morgane Fontaine[2]; Lorena Martin-Jaular[3]; Damarys Loew[4]; Florent Dingli[4]; Emmanuel Brouillet[2]; Clotilde Théry[3]; Christian Neri[2]
[1]Sorbonne University, Institute of Biology Paris-Seine (IBPS), Paris, France; [2]Institute of Biology Paris-Seine (IBPS), Sorbonne University, CNRS UMR 8256, ERL INSERM U1164, Paris, France; [3]Institut Curie, PSL Research University, INSERM U932, Paris, France; [4]Institut Curie, PSL Research University, LSMP - Curie CoreTech Mass Spectrometry Proteomics, Paris, France

Introduction: Extracellular vesicles (EVs) have emerged as important mediators of cell-to-cell communication in brain development and they have been associated with neurodegenerative disease (ND) processes. However, the effects of ND insults on specific EV sub-populations remain poorly characterized. Here, we investigated the features of small EVs (SEVs) in cellular

models of Huntington's Disease (HD), a ND caused by CAG expansion in huntingtin, primarily leading to late-onset striatal neurodegeneration, and altering homeostatic mechanisms commonly associated with several NDs.
Methods: We purified SEVs from conditioned medium of striatal cells derived from HD knock-in mice and neural stem cells derived from human HD induced pluripotent stem cells. SEVs were purified using size exclusion chromatography (SEC), followed by nanoparticle tracking analysis (NTA), total protein dosage, western blot analysis using a panel of EV markers, electronic microscopy, and quantitative mass spectrometry analysis of human SEVs.
Results: We found that SEVs undergo several modifications in HD conditions, including impaired secretion, reduced tetraspanin-marker levels, protein-content remodeling with protein increases and decreases detected in HD SEVs compared to control SEVs, raising the possibility that mutant huntingtin-expressing cells may reprogram SEV biogenesis and secretion, during neuronal differentiation.
Summary/Conclusion: Our data point to profound SEV-remodeling in response to ND insults such as mutant huntingtin. We will discuss how our study may lead to better understanding EV dynamics in response to ND insults, highlighting fundamental implications and biomedical potential associated with the study of SEVs.
Funding: Agence Nationale de la Recherche (ANR)

OF13.06 | Molecular characterization of brain-derived EVs from human brain and animal models of Alzheimer's disease by proteomics and novel microglia-specific EV reporter system

Seiko Ikezu[1]; Satoshi Muraoka[2]; Kevin A. Clayton[3]; Jean-Christophe Delpech[4]; Mark P. Jedrychowski[5]; Shawn Herron[6]; Mohammad Abdullah[1]; kristen D. Onos[7]; Kelly J. Keezer[8]; Gareth R. Howell[8]; Tsuneya Ikezu[1]
[1]Department of Neuroscience, Mayo Clinic Florida, Jacksonville, USA; [2]National Institutes of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan; [3]Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Hanover, USA; [4]INRAE University Bordeaux, Bordeaux, France; [5]Department of Cell Biology, Harvard Medical School, Boston, USA; [6]Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, USA; [7]The Jaxon laboratory, Bar Harbor, USA; [8]The Jaxon Laboratory, Bar Harbor, USA

Introduction: Extracellular vesicles (EVs) play an emerging role in progression of neurodegenerative disorders, notably Alzheimer's disease (AD). We have recently reported that brain-derived extracellular vesicles (BDEVs) isolated from AD patients' brains efficiently spread misfolded tau protein in mouse brains. To uncover the specific cell type of BDEVs facilitating this phenomenon, we performed quantitative proteomic profiling of BDEVs isolated from brain tissues of AD patients, mild cognitive impairment (MCI) and control human subjects as well as APP/PS1 rodent AD model and control mice. We further examined EV secretory function of plaque associated microglia in APP knock-in mice.
Methods: BDEVs were separated from human brain samples of 11 AD, 8 MCI and 11 control age-matched subjects as well as 6 brain samples of 8-month-old CAST.APP/PS1 and control female mice, using discontinuous sucrose gradient ultracentrifugation and subjected to tandem mass spectrometry for differentially expressed protein and pathway analysis. To monitor EV secretion specifically from microglia in vivo, we developed and injected a novel lentivirus expressing mEmerald-conjugated CD9 specifically in microglia with APPNL-G-F knock-in mice. Mice were euthanized 10 days after the lentivirus injection in the medial entorhinal cortex of mouse brains and brain tissues were subjected to immunohistochemistry to detect mEmerald+ EVs using GFP antibody and disease associated microglia using Mac2 antibody.
Results: Cell type specific molecular analysis of BDEVs showed gradual shift from neuronal to glial origins between control, MCI and AD patients. Disease-associated microglia proteomic signature was significantly upregulated in BDEVs in AD patients (APOE, ITGAX, and CD63) compared to the control and/ or MCI. ITGAX was also enriched in BDEVs isolated from CAST.APP/PS1 mice, further supporting the involvement of microglial EVs in disease progression. Interestingly, Mac2+ plaque associated microglia secrete significantly more mEmerald-CD9+ EVs compared to Mac2- microglia in AD mouse models.
Summary/Conclusion: We discovered an enrichment of glia-derived EVs in AD brains compared to the control or MCI cases, which was reproduced in APP/PS1 mouse model. Further, microglia appear as potent contributor of disease progression via hyper-secretion of EVs.
Funding: Abbvie Inc. Cure Alzheimer's Fund, NIH RF1 AG054199, NIH R01 AG054672, NIH R01 AG066429, NIH R01 AG072719, NIH R01 AG067763, and NIH R21 NS104609.

OF14: Novel Approaches in Studying EV Nucleic Acids

Chair: Metka Lenassi – Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

Chair: Esther N.M Nolte-'t Hoen – Division of Cell Biology, Metabolism & Cancer, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University

OF14.01 | In situ hybridization to detect DNA amplification in EVs

LUCIA CASADEI[1]; Patricia Sarchet[2]; Fernanda Costas C. de Faria[2]; Sayumi Tahara[2]; Luciano Cascione[3]; Federica Calore[2]; Giovanni Nigita[4]; Valerie Grignol[2]; Raphael E. Pollock[2]
[1]The Ohio State University, COLUMBUS, USA; [2]The Ohio State University, Columbus, USA; [3]Institute of Oncology Research (IOR), Lausanne, Switzerland; [4]The Ohio State University, columbus, USA

Introduction: Extracellular vesicles (EVs) are secreted lipid bilayer-enclosing vesicles. The small size of EVs and the limited quantities that can usually be obtained from patient- and cell-derived samples poses limitations to their study. Currently, no methodologies have been shown that allow EV study within tissues of origin and subsequent probing for specific sequences of DNA within tissue-contained EVs. Retroperitoneal liposarcoma (RL) includes well differentiated/dedifferentiated liposarcoma (WD/DDLPS); these are among the most common sarcoma subtypes. At the molecular level, practically all RL have amplification of 12q13-q22, the locus of MDM2, the most commonly overexpressed RL gene.
Methods: A new methodology allowing embedding and in situ hybridization (ISH) of EVs from tissues/cell lines and sera has been developed. EVs were isolated from cells cultured in serum-free medium for 48 h and from serum patient samples. Serial centrifugations (300 x g for 10', 2,000 x g for 20', 10,000 x g for 30') and ultracentrifugation (100,000 × g for 70') were performed. EV pellet was washed with PBS and another ultracentrifugation at the same speed was performed. As for tumor EVs isolation, once tumor was dissociated, the cell suspensions were filtered (100 μM filter) and serial centrifugation and ultracentrifugation has been performed, as described above. The quality of isolated particles was assessed through TEM, Nanotrack analysis and Western blot.
Results: Here we show, for the first time, the possibility of examining EVs while still within tissues, cell lines and patient sera as substrates to be used for detecting DNA amplification through ISH. Specifically, we report here: (i) a procedure for the successful selective, specific, and contaminant-free ISH examination of EVs contained within liposarcoma tissues; (ii) the detection of MDM2 amplification in EVs within liposarcoma tissues prior to EV release into the circulation; (iii) the detection of MDM2 amplification in EVs isolated from sera and cell line conditioned media.
Summary/Conclusion: Our findings show that EVs isolated from tissues, cell lines and sera can be embedded and used as substrate to detect specific sequences of DNA through ISH. This methodology may allow the visualization of DNA targets, help establish DNA sorting-into-EV processes, and facilitate the study of EV DNA cargo transfer from donor to recipient cells while allowing diagnostic verification in many diseases. Modification of probes may enable researchers to detect targets and specific DNA alterations directly in tumor EVs, thereby facilitating detection, diagnosis, and improved understanding of tumor biology relevant to many cancer types.
Funding: This work was supported by The Ohio State University Comprehensive Cancer Center and the National Institutes of Health under grant number P30 C016058 and DOD CDMRP Grant CA210874.

OF14.02 | Identification and validation of EV reference genes for the normalization of RT-qPCR data

Cláudio A. Pinheiro[1]; Niké Guilbert[2]; Quentin Roux[2]; Suzanne Fischer[2]; Lien Lippens[2]; An Hendrix[3]
[1]Laboratory of Experimental Cancer Research, Ghent, Belgium; [2]Laboratory of Experimental Cancer Research, Gent, Belgium; [3]Department of Urology, Ghent University Hospital, Ghent, Belgium; Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Ghent University, Ghent, Belgium; Cancer Research Institute, Ghent, Belgium, Ghent, Belgium

Introduction: Reference genes (RG) for extracellular vesicles (EV) are central to the high-throughput and accurate profiling of selected EV-associated genes. We identify and validate four EV-associated mRNAs as RG for the normalization of EV-associated gene expression by RT-qPCR across multiple cell lines and biofluids.
Methods: From our previous work (PMID:25317274) we selected the top 4 differentially enriched protein coding genes in EV preparations obtained by density gradient ultracentrifugation (DGUC): SNRPG, OST4, TOMM7 and NOP10. We evaluated and compared the expression of the candidate RG and commonly used RG (including GAPDH) across 15 cell lines and analyzed their presence in EV separated by DGUC from 4 biofluids (conditioned medium (n=10), blood plasma, urine and tumor interstitial

fluid) in three biological replicates. EV preparations were characterized by EM, western blot, NTA and RT-qPCR. Gene stability was assessed by 4 algorithms for RG identification.
Results: Similar to commonly used RG, candidate RG were stably expressed across 15 cell lines, under varying serum conditions (0-10%). Protease K and RNase A treatment of DGUC fractions confirmed the selectivity of the candidate RG for EV-enriched fractions (density 1.09–1.10g/mL), in contrast to GAPDH. Disruption of culture conditions by 10Gy irradiation did not affect RG levels in EV. Algorithm-based stability analysis of randomized samples confirmed constant levels of candidate RG in EV across all biofluids. In addition, their Cq values directly correlated with particle numbers measured by NTA. Importantly, the 4 candidate RG demonstrated to be more stable compared to the commonly used RG.
Summary/Conclusion: We have successfully identified and validated four EV-associated RG, that can be used for the normalization of EV RT-qPCR data and ultimately assist in advancing our understanding of the EV-associated mRNA cargo.
Funding: This work was supported by UGent, FWO, KOTK (the Flemish cancer society) and European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No [722148].

OF14.04 | Extracellular vesicles carrying Firmicutes predict response to pembrolizumab in urothelial carcinoma

Kentaro Jingushi[1]; Atsunari Kawashima[2]; Takuro Saito[3]; Akinaru Yamamoto[2]; Toshihiro Uemura[2]; Norio Nonomura[2]; Kazutake Tsujikawa[4]
[1]Laboratory of Molecular and Cellular Physiology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan; [2]Department of Urology, Graduate School of Medicine, Osaka University, Suita, Japan; [3]Department of Surgery, Graduate School of Medicine, Osaka University, Suita, Japan; [4]Laboratory of Molecular and Cellular Physiology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan

Introduction: Bacterial flora in the body has clinical significance for the host. The metabolic environment created by this flora influences immunotherapy in urothelial carcinoma. However, there have been no report on the clinical significance of bacterial flora in the host bloodstream. Here, we aimed to clarify the clinical significance of blood flora in urothelial carcinoma patients.
Methods: Clinical specimens: Serum were obtained from 50 patients with localized urothelial cancer (UC) who had undergone TURBT and from 31 metastatic UC patients who had undergone treatment with pembrolizumab, and 20 healthy donors. Written informed consent was obtained from each patient, and the study was approved by the ethics review board of the Osaka University Medical Hospital and was conducted according to the principles of the Declaration of Helsinki.
Serum EVs isolation: Serum samples (200 ul) were centrifuged at 2,000 g for 30 minutes and filtered with 0.2 um syringe filter before applying into the following extracellular vesicles (EVs) isolation. Serum EVs were isolated using ultracentrifugation method or qEV columns, Exosome Isolation Kit and MagCapture Exosome Isolation Kit. DNA was isolated from EVs samples using the QIAamp Circulating Nucleic Acid Kit.
16S metagenomic sequencing: 16S rRNA metagenomic sequencing was performed on a MiSeq platform. QIIME was used to process all raw sequencing data. Linear discriminant analysis effect size (LEfSe) was used to elucidate bacterial genus classification taxa that were associated with malignancy of UC and immunological classification of T cells.
Results: The expression of peripheral EVs carrying DNA of Firmicutes phylum was significantly correlated with the number and activation surface marker expression of infiltrating T cells within tumor tissues. In addition, Firmicutes abundance could predict the response and prognosis of patients with metastatic urothelial carcinoma treated with immunotherapy.
Summary/Conclusion: Information of Firmicutes abundance derived from EVs in the blood can reflect the local immune status of the tumor microenvironment and could be used to predict the efficacy and prognosis of cancer immunotherapy.

OF14.05 | DNA bound to the surface of urine extracellular vesicles as potential kidney allograft injury biomarker

Ivana Sedej[1]; Maja Štalekar[2]; Magda Tušek Žnidarič[2]; Katja Goričar[3]; Nika Kojc[4]; Polona Kogovšek[2]; Vita Dolžan[3]; Miha Arnol[1]; Metka Lenassi[5]
[1]Department of Nephrology, Division of Internal Medicine, University Medical Centre Ljubljana, Ljubljana, Slovenia, Ljubljana, Slovenia; [2]Department of Biotechnology and Systems Biology, National Institute of Biology, Ljubljana, Slovenia, Ljubljana, Slovenia; [3]Institute of Biochemistry and Molecular Genetics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, Ljubljana, Slovenia; [4]Institute of Pathology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, Ljubljana, Slovenia; [5]Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, Ljubljana, Slovenia

Introduction: Numerous studies support elevated donor-derived cell-free DNA (dd-cfDNA) in blood and urine as a biomarker of kidney allograft injury (KAI) in kidney transplant recipients (KTR). We aimed to explore whether DNA is bound to extracellular vesicles in urine (uEVs), and if so, is EV-DNA associated with KAI.
Methods: Using size exclusion chromatography (EV210292), we isolated EVs, EV-DNA and cfDNA from second morning spot urine of 41 study consenting KTR. According to histopathology of surveillance/for-cause biopsies, we divided the patients into normal histology (NH) and KAI (rejection or non-rejection injury) groups. NTA was used to determine EV size and concentration, while fluorometry, donor-recipient genotyping and digital-droplet PCR were used to determine DNA parameters (yield, copy number, integrity index). DNase assay and immunogold TEM were applied to study EV-DNA location. Association between EV-DNA and histological phenotype of KAI was analysed. The National Ethics Committee approved the study.
Results: We isolated pure uEVs of KTR. The median uEV concentration was 8.47 x 1010/mmol U-creatinine, while uEV mode size was 125.8 nm. EVs mean size was significantly larger in KAI groups compared to NH (177.5 nm and 174.1 nm vs. 160.7 nm, $P = 0.045$, respectively). DNA co-isolated with uEVs and correlated with cfDNA in several parameters. EV-DNA and cfDNA yield, DNA copy numbers or ddDNA copy numbers (for cfDNA) were significantly increased in KAI groups compared to NH. Importantly, EV-DNA copy numbers were greater in allograft rejection and differed significantly in antibody- vs. cell- mediated rejection. Compared to cfDNA, EV-DNA was less fragmented and bound to the surface of EVs as per TEM after DNA labelling. uEV-DNA characteristics correlated with the degree of inflammation in KTR.
Summary/Conclusion: DNA is bound to the surface of uEVs and may be a potential non-invasive KAI biomarker.
Funding: Slovenian Research Agency Grants P3-0323, P1-0170, P4-0165, P4-0407.

OF15: Metabolism Obesity Nutrition

Chair: Soazig Le Lay – Inserm U1087 Institut du Thorax Nantes & SFR ICAT Univ Angers

Chair: Olivier Blanc-Brude – Paris Center for Cardiovascular Research, Institut National de la Santé et de la Recherche Médicale U970, Université de Paris, France

OF15.01 | MicroRNAs from Adipocyte-derived Small Extracellular Vesicles in Patients with Alzheimer's Disease are Associated with Cognitive Impairment and Insulin Resistance

Rachael Batabyal[1]; Ankush Bansal[2]; Evan Nadler[3]; Kazue Hashimoto-Torii[3]; Robert J. Freishtat[2]
[1]Children's National Hospital, Columbia, USA; [2]Children's National Hospital, Washington, USA; [3]Children's National Hospital and The George Washington University, Washington, USA

Introduction: We are experiencing parallel epidemics of obesity and Alzheimer's disease (AD). Obesity increases the risk of AD. This suggests adipose-derived factors may provide a mechanistic link in AD pathogenesis. Adipocyte-derived small extracellular vesicles (ad-sEVs) induce pathology critical to developing obesity-related disease (e.g insulin resistance). Insulin resistance may play a key role in AD pathogenesis. We hypothesized that circulating ad-sEVs from AD patients carry miRNAs predicted to cause insulin resistance and are associated with cognitive impairment.
Methods: We studied serum and cerebrospinal fluid (CSF) from 19 participants with AD and 14 non-AD controls. Ad-sEVs were isolated from samples by precipitation and immunoselection. Ad-sEV miRNA expression was profiled and the biofluids of the two groups compared. Weighted gene co-expression network analysis (WGCNA) was applied to investigate the relationship between miRNA expression changes and phenotypic traits, including Mini-Mental State Exam (MMSE). Enrichment analysis was performed on the top 30-40 miRNAs in each co-expression module for MMSE.
Results: Serum and CSF miRNA expression correlated strongly ($r2=0.98$). In serum, 189 miRNAs were differentially expressed by a fold change $\geq|1.1|$ in the AD and control groups ($p\leq0.1$) and 251 were differentially expressed in CSF. Using WGCNA, 5 miRNA modules were identified in each biofluid. MiRNAs associated with AD and insulin signaling were enriched in both serum and CSF in the two modules that most strongly correlated (directly or indirectly) with MMSE scores. Within these two modules, 26/30 and 14/30 miRNAs were associated with AD in the serum and 22/30 and 26/30 miRNAs targeted insulin signaling pathways. In CSF, 22/30 and 27/40 miRNAs were associated with AD and 22/30 and 29/40 miRNAs targeted insulin signaling pathways. Using Ingenuity Pathway Analysis, we found that downregulation of insulin receptor and IGF-1 signaling was predicted by miRNAs from AD patients compared to controls.
Summary/Conclusion: These findings support our hypothesis that, in AD, altered expression of ad-sEV miRNAs target insulin signaling pathways and are associated with cognitive impairment. These findings support using serum ad-sEVs as a surrogate for CSF ad-sEVs. Functional validation of these findings is ongoing.
Funding: Supported by UW ADRC NIH:P50AG005136

OF15.02 | Effects of n-3 PUFAs on the coagulatory behaviour of EVs generated in vitro from washed platelets

Esra Bozbas; Ruihan Zhou; Keith Allen-Redpath; Parveen Yaqoob
University of Reading, Reading, United Kingdom

Introduction: Platelet-derived EVs (PDEVs) comprise the most abundant EV population in the circulation, at least in healthy individuals, and may play a role in the development of cardiovascular diseases (CVDs) through their procoagulant activities. PDEVs can potentially be modified by dietary fatty acids since they carry bioactive lipid mediators and their cell membrane fatty acid composition can be modified by dietary fatty acids, including n-3 polyunsaturated fatty acids (PUFA), which are abundant in oily fish and fish oil and reported to reduce risk of cardiovascular diseases. This study investigated whether consumption of n-3 PUFA alters the coagulatory behaviour of PDEVs.
Methods: Forty subjects with moderate risk of CVDs were supplemented with either 1.8 g/d n-3 PUFA or control (high-oleic safflower oil) for a 12-week period. This randomised, double-blind, placebo-controlled crossover study was approved by the University of Reading Research Ethics Committee.
To isolate PDEVs in vitro, washed platelets, obtained before and after each intervention period, were incubated in the presence or absence of the stimulant, 30 μM Thrombin Receptor Activator Peptide-6 (TRAP-6), for 2h at 37°C. Platelets were then removed by centrifugation and EVs were pelleted and washed. The thrombogenicity of these in vitro-generated PDEVs was assessed using a tissue factor-induced thrombin generation assay (Technothrombin TGA kit), which evaluates a change in fluorescence as a result of cleavage of a fluorogenic substrate by thrombin over time upon activation of the clotting cascade by tissue factor. Pooled vesicle free plasma (VFP) from healthy subjects (n=3) was used for benchmarking purposes in the assessment of thrombin generation induced by in-vitro-generated PDEVs.
Results: VFP alone was significantly less able to support thrombin generation than VFP reconstituted with in vitro-generated PDEV. Thrombin generation supported by in vitro-generated PDEVs derived from subjects supplemented with fish oil was significantly lower than that supported by PDEVs from subjects supplemented with control oil, as reflected by increased lag time and time to peak thrombin, and decreased peak thrombin, velocity index and area under the curve.
Summary/Conclusion: PDEVs produced in vitro from both unstimulated and stimulated platelets are able to support thrombin generation, but their thrombogenic capacity is reduced when platelet donors are supplemented with n-3 PUFA, indicating potential anti-coagulatory properties of n-3 PUFAs mediated through PDEVs.
Funding: This study was funded by the BBSRC Diet and Health Research Industry Club (BBSRC DRINC) and the Republic of Türkiye (the Graduate Education Scholarship).

OF15.03 | Sorting mechanisms for MicroRNAs into extracellular vesicles in placental cells in gestational diabetes mellitus

Soumyalekshmi Nair[1]; Nanthini Jayabalan[1]; Andrew Lai[2]; Dominic Guanzon[1]; Flavio Carrion[3]; Katherin Scholz-Romero[1]; Martha Lappas[4]; Carlos Salomon Gallo[1]
[1]The University of Queensland, Brisbane, Australia; [2]Exosome Biology Laboratory, University of Queensland Centre for Clinical Research, The University of Queensland, Brisbane, Australia; [3]Universidad del Alba, Santiago, Chile; [4]University of Melbourne, Melbourne, Australia

Introduction: Gestational Diabetes Mellitus (GDM) is the glucose intolerance in pregnancy and leads to pregnancy complications and metabolic disorders in mother and offspring. Placenta-derived small extracellular vesicles (sEVs) play key roles in the regulation of maternal metabolism by transfer of miRNAs mediating cell communication. The aim of the present study is to identify the molecular mechanisms associated with selective packaging of miRNAs sEVs in placental cells in normal glucose tolerant (NGT) and GDM pregnancies.
Methods: sEVs were isolated from cell-conditioned media of primary human trophoblast cultures from NGT and GDM patients. The miRNA profile in trophoblast cells and sEVs were analyzed using next generation sequencing and the proteins associated with the sEV enriched miRNAs were identified by biotin pull down and mass spectrometry. Further, siRNA mediated knock down of proteins and analysis of miRNA expression in sEVs using real time PCR were performed.
Results: GDM alters the miRNA content of placental cells and sEVs. A specific set of miRNAs were highly enriched in sEVs compared to their cells of origin in NGT and GDM. We classified these miRNAs into three groups as miRNAs enriched in sEVs (1) only in NGT (2) only in GDM and (3) in both NGT and GDM. Candidate miRNAs were chosen for each group (miR-150-5p and miR-1246 for NGT; miR-1285-5p for GDM; and miR-486-5p for shared) based on their abundance and enrichment in sEVs. Further, we identified a repertoire of unique proteins interacting with miRNAs within each group. We found that RNA binding protein YBX3 is required for the sorting of miR-1246 in sEVs in NGT and proteins CWF19L1 and DISC3 for the sorting of

miR-486 in NGT and GDM condition. Furthermore, proteins FASTKD2 and HDLBP inhibits the sorting and release of miR-1285 in sEVs from GDM trophoblast cells.
Summary/Conclusion: These findings provide insights into the mechanisms by which miRNA-protein interactions lead to the selective packaging of miRNAs into sEVs in healthy pregnancy and GDM.
Funding: This research was funded by National Health and Medical Research Council (NHMRC 1195451)

OF15.04 | Hepatoprotective effect of cranberry vesicles in a mouse model of diet-induced obesity

Amélie Légaré[1]; Arianne Morissette[1]; Christine Dallaire[1]; Joanie Dupont-Morissette[1]; Geneviève Guèvremont[1]; Thibault Varin[2]; Geneviève Pilon[1]; Julien Boucher[3]; Valérie Chénard[3]; André Marette[1]
[1]Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec, Canada, Québec, Canada; [2]Institut sur la Nutrition et les Aliments Fonctionnels, Université Laval, Canada, Québec, Canada; [3]Centre de recherche du CHU de Québec, Canada, Québec, Canada

Introduction: Numerous studies, notably carried out by our team, have shown that cranberries counteract obesity-related metabolic disorders. It has been reported that fruit vesicles can alleviate gut-associated diseases, such as obesity. Yet, the impact of cranberry extracellular vesicles (CranEVs) on metabolic and liver health has never been studied. The objective of this project is therefore to evaluate the effect of CranEVs intake on metabolic health in a mouse model of diet-induced obesity.
Methods: CranEVs were isolated from freshly pressed cranberry juice by differential ultracentrifugation followed by a sucrose density gradient. The vesicles were then characterized by Nanoparticle Tracking Analysis, Dynamic Light Scattering, and transmission electron microscopy. Male C57bl/6J mice were subjected to an obesogenic diet for 10 weeks. The first group received daily gavage containing CranEVs diluted in PBS, while the control group received a daily gavage of PBS (n = 12 per group). The food intake and body weight were monitored daily. Insulin and glucose tolerance tests were performed at week 6 and week 8 respectively. Liver transcriptome profiles will be obtained by Illumina NovaSeq sequencing.
Results: Despite a similar calorie intake, mice given CranEVs gained significantly less weight than the control group, in addition to experiencing a reduction of adipose tissue weight. In the liver, a significant decrease of the triglyceride and cholesterol content was observed in the mice given the extract. Analysis of the liver transcriptomic data will perhaps shine a light on the mechanisms underlying the observed benefits of CranEVs on mice health.
Summary/Conclusion: This project is the first to assess the metabolic impact of CranEVs. Based on our results, CranEVs reduce adiposity and hepatic lipid accumulation. Thus, these cranberry-derived vesicles could represent a promising therapeutic avenue to counteract obesity-related metabolic disorders, such as nonalcoholic fatty liver diseases.
Funding: This work was funded by the Cardiometabolic Health, Diabetes and Obesity Research Network. A. Légaré was financially supported by the Canadian Institutes of Health Research, Quebec Research Funds, and Natural Sciences and Engineering Research Council of Canada.

OF15.06 | Glycolysis in hepatic stellate cells induces fibrogenic sEV release and promotes liver fibrosis

Shalil Khanal; Vijay Shah; Enis Kostallari
Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, USA

Introduction: Hepatic stellate cells (HSCs) are the main drivers of liver fibrosis by secreting high amounts of collagen. Our group has previously demonstrated that activated HSCs increase the release of fibrogenic small extracellular vesicles (sEVs). However, the mechanism leading to their release is not fully understood. Activated HSCs also display a highly glycolytic profile, where hexokinases (HK) catalyze the first committed step in glucose metabolism by phosphorylating glucose. The aim of this study is to investigate the role of glycolysis, and more specifically of HK2, in driving fibrogenic sEV release and liver fibrosis.
Methods: In vitro, primary human HSCs were treated overnight with glucose to induce glycolysis or 2-deoxy-glucose (2DG) to inhibit HK2. sEVs were purified by differential ultracentrifugation and characterized by western blot and mass spectrometry. In vivo, mice lacking one allele of Hk2 gene selectively in HSCs (PDGFRβCreERT2/HK2fl/+) and littermate controls (HK2fl/+) were utilized. Liver fibrosis was induced by carbon tetrachloride (CCl4) administration.
Results: Our publicly available single cell RNA sequencing dataset demonstrated that HK2 mRNA expression was 1.7-fold higher in fibrogenic HSCs isolated from CCl4-treated mice than in non-fibrogenic HSCs (adjusted p = 1.82E-09, GSE175939). In vitro, 1 g/L of glucose enhanced HK2 protein expression by 2-fold compared to no glucose condition (n=4, $p < 0.05$). Glucose treatment also increased sEV release by 7-fold as demonstrated by CD81 protein levels, which was abrogated by treating cells with the HK2 inhibitor, 2DG (1 g/L n=3, $p < 0.05$). Mass spectrometry analysis demonstrated that sEVs derived from HSCs cultured in the

presence of glucose were enriched with fibrogenic proteins such as collagens, which was reversed by 2DG treatment. Based on these data, we examined the role of HK2 in fibrosis in vivo. CCl4 administration to HK2fl/+ littermate control mice enhanced Sirius red staining of the liver by 4.7-fold, collagen 1 alpha 1 mRNA levels by 12-fold and alpha smooth muscle actin mRNA levels by 3-fold, suggesting enhanced fibrogenesis. However, Hk2 deletion selectively in HSCs in PDGFRβCreERT2/HK2fl/+ mice attenuated liver fibrosis (3-6 mice/group, $p < 0.05$).

Summary/Conclusion: Our results suggest that glycolytic HK2 mediates fibrogenic sEV release and liver fibrosis.

Funding: Pinnacle Research Award/American Association for the Study of Liver Diseases (AASLD)
P30DK084567 - Pilot and Feasibility award,Mayo Clinic Center for Cell Signaling in Gastroenterology/HHS | NIH | National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

OF16: Biodistribution of EVs

Chair: Oscar Wicklander – Department of laboratory medicine, Karolinska Institutet

Chair: Dirk M. Pegtel – Amsterdam University Medical Centers

OF16.01 | Albumin-binding extracellular vesicles demonstrate extended circulation time and lymph node accumulation

Xiuming Liang[1]; Zheyu Niu[2]; Valentina Galli[3]; Nathalie Howe[3]; Ying Zhao[4]; Oscar Wiklander[5]; Wenyi Zheng[1]; Rim Jawad Wiklander[5]; Giulia Corso[5]; Christopher Davies[6]; Justin Hean[6]; Eleni Kyriakopoulou[6]; Doste Mamand[7]; Risul Amin[8]; Joel Nordin[7]; Dhanu Gupta[7]; Samir El Andaloussi[1]

[1]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, Huddinge, Sweden; [2]Biomolecular Medicine, Clinical Research Center, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, Stockholm, Sweden; [3]Evox Therapeutics Limited, Oxford, United Kingdom, Oxford, United Kingdom; [4]Experimental Cancer Medicine, Clinical Research Center, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden., Stockholm, Sweden; [5]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, Stockholm, Sweden; [6]Evox Therapeutics Limited, Oxford, UK., Oxford, United Kingdom; [7]Department of laboratory medicine, Karolinska Institutet, Stockholm, Sweden; [8]Biomolecular Medicine, Clinical Research Center, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden., Stockholm, Sweden

Introduction: Extracellular vesicles (EVs) have shown promise as potential therapeutics for the treatment of various diseases. However, their rapid plasma clearance after administration could be a limitation in certain therapeutic settings. Hence, there is an unmet need to develop efficient approaches to extend EV circulation time in vivo.

Methods: An engineering strategy is employed to decorate albumin onto the surface of the EVs through surface display of albumin binding domains (ABDs). ABDs are added to the extracellular loops of select EV-enriched tetraspanins (CD63, CD9 and CD81) or directly fused to the extracellular terminal of single transmembrane EV-sorting domains, such as Lamp2B. Nanoluc luciferase is fused to the intracellular terminal of tetraspanins or Lamp2B to allow facile tracing of engineered EVs. The binding of engineered EVs with human plasma albumin (HSA) in vitro and mouse plasma albumin (MSA) in vivo is confirmed by super-resolution imaging. EVs in the circulation and harvested organs are monitored by Nanoluc activity assay and in vivo imaging achieved using the IVIS® optical imager.

Results: The engineered EVs exert robust binding capacity to HSA in vitro and MSA after injection in vivo. By binding to MSA, the circulating time of EVs (derived from different cells) dramatically increases after different routes of administration. Moreover, these engineered EVs show considerable lymph node (LN) and solid tumor accumulation, which can be utilized when using EVs for immunomodulation, cancer- and/or immunotherapy. The increased circulation of EVs may also be important when combined with tissue-specific targeting ligands.

Summary/Conclusion: By introducing ABDs onto EVs, their circulation time is significantly extended, which could provide significant benefit for their therapeutic use in a variety of disease indications.

OF16.02 | Identification of local and systemic exchange of cancer cell derived EVs during pancreatic cancer progression

Kathleen M. McAndrews; Jiha Kim; Amanda Haltom; Patience Kelly; Janine Hensel; Carolina Ruivo; Raghu Kalluri
MD Anderson Cancer Center, Houston, USA

Introduction: Intercellular signaling between cancer cells and stromal cells within the tumor microenvironment (TME) and distant metastatic sites is known to be critical for regulating the progression of cancer. A number of recent studies have indicated a role for ex vivo isolated cancer cell extracellular vesicles (EVs) in communication between distinct cell types of the TME and in promoting metastasis; however, the precise role of endogenous EV transfer in tumorigenesis and autochthonous cancer progression has been elusive. Here, we developed novel mouse models to fluorescently track the endogenous exchange of CD9+ EVs and evaluate the functional consequences of CD9+ EV transfer in the development and progression of pancreatic cancer.
Methods: In order to generate a conditional mCherry-CD9 allele, a cassette encoding loxP-STOP-loxP followed by mCherry-CD9 (LSL-mCherry-CD9) was knocked into the ROSA26 locus. Mice containing this allele were crossed to Pdx1-Cre; LSL-KrasG12D (KC), and Pdx1-Cre; LSL-KrasG12D; LSL-Trp53R172H (KPC) mice with LSL-YFP to express a cytoplasmic YFP, which was used to identify pancreatic epithelial or cancer cells. Transfer of mCherry CD9+ EVs was evaluated by confocal microscopy analysis of cells with mCherry signal and lacking cytoplasmic YFP signal. Single cell RNA sequencing of YFP-mCherry- and YFP-mCherry+ cells from the pancreata of KPC mice was used to evaluate transcriptional changes in stromal cells containing mCherry CD9+ EVs.
Results: Exchange of epithelial cell derived CD9+ EVs (EC EVs) with local cells in the microenvironment of KPC pancreata occurs prior to formation of precancerous lesions and increases throughout cancer progression. Such EC EVs exchange with surrounding stromal cells in the TME, including fibroblasts and immune cells, was associated with transcriptional reprogramming of stromal cells. Analysis of the metastatic organs lung and liver revealed an accumulation of EC EVs with cancer progression and metastasis in KPC mice. EC EVs were also detected in the lung and liver of KC mice in which metastasis does not occur, but at a lower frequency than KPC mice with metastasis. Transfer of EC EVs was detected in organs lacking metastasis, including the kidney and brain; however, such transfer was less common in non-metastatic organs compared to metastatic organs.
Summary/Conclusion: Together, these data suggest that EC EVs are endogenously transferred prior to tumor formation, suggesting that EV transfer may play a role in intercellular communication in the context of normal physiology. EC EV accumulation in both non-metastatic and metastatic organs indicates that EVs may act to promote metastasis and exert other yet to be uncovered functions.

OF16.03 | Radioactive labeling of Extracellular Vesicles and In Vivo Tracking by Non-invasive SPECT/CT Imaging

María Isabel González[1]; Gorka Sobrino[2]; Manuel Desco[3]; Beatriz Salinas[3]
[1]Instituto de Investigación Sanitaria Gregorio Marañón (Madrid, Spain), Madrid, Spain; [2]Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain; [3]Instituto de investigación Sanitaria Gregorio Marañón, Madrid, Spain

Introduction: The clinical application of EVs as natural nanoparticles still requires a deeper understanding of their in vivo behavior after exogenous administration. Nuclear imaging emerges as useful tool due to its non-invasive feature and high selectivity and sensitivity. We present a simple method for the radiolabeling of small EVs (sEVs) and its validation as non-invasive tool for the study of EVs pharmacokinetic properties by nuclear imaging.
Methods: sEVs were isolated from goat milk by combined ultracentrifugation and size exclusion chromatography and radioactively labeled with commercial pertechnetate ([99mTc] NaTcO4) in presence of SnCl2 at 37°C/30min. Purity and in vitro stability (37°C/PBS) were assessed by HPLC and TLC. After physicochemical characterization, 99mTc-sEVs were administrated to healthy mice by intravenous (310–350μCi) and intraperitoneal (190–340μCi) injection and intranasal instillation (140–170μCi). In vivo circulation time was assessed by blood extraction and pharmacology profile was evaluated by in vivo SPECT/CT, ex vivo biodistribution and autoradiography.
Results: Radioactive 99mTc-sEVs presented high purity (>95%) and stability (>95% at 48h), maintaining similar properties to non-labeled sEVs (cup-shape morphology, 114.0±8.0nm). In vivo tracking revealed major changes in biodistribution; intravenous injection showed main sEVs liver uptake (36.6±7.5%ID/g) and short blood half-life (4min). sEVs intraperitoneally injected showed higher circulation time (16min) but typical free 99mTc signal at thyroid (22.0±7.2%ID/g at 24h), suggesting sEVs degradation. Nuclear signal in brain was observed after intranasal instillation, with main uptake in stomach.
Summary/Conclusion: We present an easy method for EVs radiolabeling and its validation as non-invasive tool to study the EVs biological behavior after exogenous administration, demonstrating drastic changes in their pharmacokinetics among administration routes
Funding: This study was supported by the Ministerio de Ciencia, Innovación y Universidades, Instituto de Salud Carlos III, project "PI16/02037", co-funded by the European Regional Development Fund (ERDF), "A way of making Europe", and by Comunidad de Madrid, project "Y2018/NMT-4949 (NanoLiver-CM)" and "S2017/BMD-3867 (RENIM-CM)", co-funded by European Structural and Investment Fund. The CNIC is supported by the Instituto de Salud Carlos III (ISCIII), the Ministerio de Ciencia e Innovación (MCIN) and the Pro CNIC Foundation and is a Severo Ochoa Center of Excellence (SEV-2015-0505).

OF16.04 | Novel Strategies for the Fluorescent Labeling of Extracellular Vesicles for In Vitro and In Vivo Imaging

María Isabel González[1]; Mario González-Arjona[2]; Ana Santos-Coquillat[2]; Gorka Sobrino[3]; Javier Vaquero[2]; Elena Vázquez-Ogando[2]; Antonio de Molina[3]; Héctor Peinado Selgas[4]; Manuel Desco[2]; Beatriz Salinas[5]

[1]Instituto de Investigación Sanitaria Gregorio Marañón (Madrid, Spain), Madrid, Spain; [2]Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain; [3]Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain; [4]Spanish National Cancer Research Center (CNIO), Madrid, Spain; [5]Instituto de investigación Sanitaria Gregorio Marañón, Madrid, Spain

Introduction: Molecular imaging allows non-invasive assessment of the EVs biological properties. Current methods for the fluorescent labeling of EVs involve tedious bioengineering or unstable lipophilic binding, which may lead to false-positive imaging. We present a straightforward strategy for the covalent fluorescent labeling of EVs, and its in vitro and in vivo evaluation by optical techniques.

Methods: Small EVs (sEVs) isolated from goat milk, U87 or B16F10 cell culture by ultracentrifugation were characterized (TEM, DLS, NTA and flow cytometry) and labeled with BDP and SCy7.5 NHS ester fluorophores for 90min at pH 8.5 and 4°C. Physicochemical characterization and in vitro stability (37°C/PBS) by HPLC were performed in all samples. In vitro evaluation of fluorescent sEVs by confocal imaging was assessed in hepatocytes employing 5 and 0.5 μg/ml of milk SCy-sEVs at 30, 60, 120 and 240min, and 24h. Milk SCy-sEVs were in vivo tested after intravenous injection in healthy mice (25μg/PBS) by IVIS imaging at 1, 4 and 24h. Mice were sacrificed, organs were ex vivo imaged and livers were histologically analyzed by H&E and confocal microscopy.

Results: Fluorophores were strongly attached to sEVs with high reaction yield confirmed by flow cytometry (>99%) and high stability at 72h, maintaining the original properties of starting sEVs (cup-shape morphology, 123-140nm). Milk SCy-sEVs was in vitro located in the cytoplasm of hepatocytes even at short time-points, presenting dose-dependent internalization. In vivo biodistribution evaluation of SCy-sEVs exhibited major accumulation in liver (3.4•108 ± 5.8•106 (p/s/cm2/sr)/(μW/cm2)), without significant alterations in this organ.

Summary/Conclusion: Our strategy for fluorescent labeling of EVs allows the attachment of diverse commercial fluorophores to the vesicle surface with a yield of nearly 100% and proven stability. The novel fluorescent EVs were in vitro and in vivo assessed proving the utility of our chemical tool.

Funding: This study was supported by the Comunidad de Madrid, projects: "Y2018/NMT-4949 (NanoLiver-CM)" and "S2017/BMD-3867 (RENIM-CM)"; it was also co-funded by the European Structural and Investment Fund. The CNIC is supported by the Instituto de Salud Carlos III (ISCIII), the Ministerio de Ciencia e Innovación (MCIN), and the Pro CNIC Foundation, and it is a Severo Ochoa Center of Excellence (SEV-2015-0505). JV was supported by grants from Instituto de Salud Carlos III (PI18/01833), co-funded by European Regional Development Fund (ERDF) and from Comunidad de Madrid, project "S2017/BMD2737 (ExoHep-CM)", co-funded by European Structural and Investment Fund. A. Santos-Coquillat is grateful for the financial support from Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III Sara Borrell Fellowship grant CD19/00136.

OF16.05 | Proteomic characterisation of CD81-tdTomato prostate cancer-derived sEVs and their distribution in two-dimensional dynamic cell systems

Timothea Konstantinou[1]; Rachel Errington[2]; Aled Clayton[2]

[1]Cardiff University, CARDIFF, United Kingdom; [2]Cardiff University, Cardiff, United Kingdom

Introduction: Cancer-derived sEVs promote several critical processes in disease progression; however, the underlying mechanisms and kinetics of sEV uptake and distribution within cell populations are still being explored. In this work, a prostate cancer cell line generating fluorescent sEVs was developed by overexpressing the tdTomato fluorescent protein fused to the tetraspanin CD81. The phenotype and proteome of CD81-tdTomato sEVs was characterised, followed by visualisation and quantification of their uptake and retention in prostate cancer- and mesenchymal stem cell-populations.

Methods: sEVs were isolated by the 30% sucrose/D2O cushion ultracentrifugation method and were phenotypically characterised by Nanoparticle Tracking Analysis, Cryo-Electron Microscopy, Western Blot analysis, ELISA-like assays, Micro Flow Cytometry and Confocal Microscopy. Proteomic analysis was done by SWATH-MS, and Fluorescence Microscopy with a developed Fiji macro were used to visualise and quantify sEV signal. CD81-tdTomato sEVs were compared to age-matched sEVs from non-transfected controls throughout.

Results: Phenotypic analysis showed significant differences in CD81-tdTomato sEV size and morphology, and proteomic analysis further revealed a number of dysregulated proteins. These were implicated in MVB/sEV-, growth-, cytoskeleton, adhesion-, migration- and survival-associated pathways. Analysis of CD81-tdTomato sEV uptake and retention demonstrated differential

dynamics between prostate cancer- and mesenchymal stem cell recipients. However, heterogeneous sEV uptake was observed for both systems and associated with cell area.

Summary/Conclusion: Overexpression of the tetraspanin CD81 fused to a fluorescent protein caused significant alterations to the phenotype and proteome of secreted sEVs, which are under-appreciated in similar studies. Furthermore, the understanding of uptake and distribution of these sEVs in 2D dynamic cell systems provided a quantifiable benchmark and starting point for further investigations in 3D microenvironments.

Funding: Cancer Research UK

Keywords: CD81, tetraspanins, tdTomato, fluorescent proteins, sEVs, MVB, SWATH-MS, proteomics, uptake, retention, fluorescence microscopy

OF16.06 | Biodistribution and Kinetics of Cardiac Exosomes After Myocardial Infarction

Weija Luo[1]; Xiao Du[1]; Zhishi Chen[1]; Yuan Dai[1]; Wei Yu[1]; Jiang Chang[2]

[1]Texas A&M University, Houston, USA; [2]Texas A&M University College of Medicine, Houston, USA

Introduction: Exosomes emerge as new messengers for in vivo communication amongst cells and organs. The beneficial effects of exosomes on the injured heart and potential roles in the prognosis of varied cardiovascular diseases have been suggested with promising results. Understanding the biodistribution and kinetics of endogenous cardiac exosomes during post-myocardial infarction (MI) will significantly impact exosome-mediated basic and translational studies. This study uncovered the tissue distribution and kinetics of the exosomes released from the MI hearts using an inducible exosome reporter mouse model.

Methods: We generated a transgenic mouse expressing the bioluminescent reporter NanoLuc-fusion protein. We fused the NanoLuc reporter with exosome surface marker CD63 for specific labeling of exosomes. The cardiomyocyte-specific αMHC promoter was introduced, followed by a loxP-STOP-loxP cassette for precise spatial labeling of exosomes originating from cardiomyocytes. The cardiomyocyte-specific exosome reporter mice were bred with tamoxifen-inducible Cre mice. The exosome labeling and distribution were assessed by luciferase assay and non-invasive bioluminescent live imaging. We performed a permanent ligation of the left anterior descending artery. All of the assessments were conducted on day three and day fourteen of post-MI, representing the acute and chronic stages of the post-MI injury healing process.

Results: The uptake levels of exosomes among thirteen different organs, including plasma, were detected and quantified. Profound differences of cardiac exosomes in various tissues during the MI healing process were exhibited.

Summary/Conclusion: Exosome-mediated communications between the heart and other organs are differential and dynamic during post-MI recovery. Meanwhile, the exosome tracking mouse model enables elucidating the endogenous exosome trafficking pattern and allows the study of exosome behavior in different biological and pathological conditions.

Funding: American Heart Association Innovative Project Award: 18IPA34180012

OF17: EVs in Breast and Prostate Cancers

Chair: Lucia R. Languino – Thomas Jefferson University

OF17.01 | EVs from therapy-induced senescent breast cancer cells elicit STING-dependent inflammatory signaling

Karen Crasta[1]; Rekha Jakhar[2]; Yen Nguyen[3]; Matius Robert[3]

[1]National University of Singapore, Singapore, Singapore; [2]Research Fellow, Singapore, Singapore; [3]Research Assistant, Singapore, Singapore

Introduction: Breast cancer is a leading cause of cancer death in women. Among the chemotherapeutic drugs used as front-line therapy are microtubule-targeting agents (MTAs) which include paclitaxel. The use of such drugs can lead to therapy-induced senescence (stable cell cycle arrest). The aim of this study was to investigate the effect of extracellular vesicles released from therapy-induced senescence breast cancer cells in breast cancer pathogenesis.

Methods: MDA-MB-231 human breast cancer cell line and mouse mammary 4T1 cells treated with MTA commonly used in lab settings, Noc (100 ng/μl) for 72 h to induced therapy-induced senescence (TIS). For sEV/exosome isolation, senescent conditioned media (CM) was prepared after Noc and DMSO treatment by incubating cells in growth media containing exosome-depleted FBS for 72 h. CM was then collected and centrifuged at 500×g 10min, 2,000×g 30min, and 15,000×g 30min at 4°C to remove cells and large debris. Supernatant was filtered, exosomes (sEVS) pelleted at 120,000×g, 2h, 4°C, washed with PBS, centrifugation at 100,000×g,1h, 4°C. Izon fractionation collector was used to collect sEVs/exosomes. Presence and size evaluation

of exosomes was confirmed by Nanosight, electron microscopy and western blot for Cd63, Alix and TSG101. Exosomes were dissolved in PBS for whole exosome experiments or processed for total RNA, miRNA and protein isolation for microRNA profiling, RNA-seq and mass spectrometry proteomic profiling. Live-cell microscopy using CD63-GFP was used to track exosome transfer in recipient cells. Macrophage polarisation (M1/M2) determination assays, FACS, Western and wide-field microscopy for apoptosis markersNF-kB and STING signalling assays was carried out. Therapy-induced Senescence (TIS) was determined by western detection for p53, p21, lamin B1 degradation, beta-gal senescence along with profiling for senescent associated secretory phenotype (SASP).

Results: We observe both in tissue culture, zebrafish and mouse model metastatic potential reduction and tumor suppression/ tumor regression occurs following EV-dependent transfer into breast cancer recipient cells. Characterization and profiling revealed a cathepsin D-dependent apoptotic driven pathway in conjunction with STING-dependent macrophage killing.

Summary/Conclusion: Therapy-induced senescent cells elicit STING-dependent inflammatory signaling in macrophages and lead to tumour regression.

Funding: This work was funded by Singapore Ministry of Education Tier 2 (MOE2018-T2-2-179) to Karen Crasta.

OF17.02 | Inhibiting EV release from breast cancer cells combats drug resistance

Caitrin Crudden[1]; Maarten Bebelman[2]; Katinka Langedijk[1]; Connie Jimenez[1]; Bart Westerman[1]; Dirk M. Pegtel[2]
[1]Amsterdam UMC, Amsterdam, Netherlands; [2]Amsterdam University Medical Centers, Amsterdam, Netherlands

Introduction: Despite the advent of targeted therapies for breast cancer treatment, a large proportion of patients eventually relapse due to resistance mechanisms. There are currently no drugs available that selectively target the most malignant subpopulations of drug-resistant breast cancer cells and re-sensitizing such cells would hugely benefit patients. Extracellular vesicle (EV) release has been linked to therapy resistance, and crucially, has been a central modality across a wide range of mechanisms of resistance, drugs and cancer types. It is therefore conceivable that selective inhibition of the pathways that drive EV production or release from cancer cells has therapeutic potential. In this project we aimed to identify clinically feasible inhibitors of EV release to combat drug resistance.

Methods: From a panel of human breast cancer cell lines we found that EV secretion is increased in aggressive, resistant cells. We developed an endogenous bioluminescent reporter for EV secretion that could sensitively and robustly report EV secretion. Using CRISPR/Cas9-technology we generated HA-NanoLuc-CD63 knock-in MCF7.

Results: We performed a broad-spectrum kinase inhibitor screen of 400 compounds, and validated potential hits for toxicity, pharmacokinetics and cellular morphology changes. In support of our original hypothesis, we found that Bemcentinib, identified as a putative inhibitor of EV secretion in MCF7, increases the sensitivity of a paclitaxel-resistant MCF7 subline to paclitaxel-toxicity. In a long term clonogenic assay, co-treatment of Bemcentinib and Paclitaxel synergize to prevent paclitaxel-resistant MCF7 from producing progeny, when compared to either agent alone.

Summary/Conclusion: We find evidence that blocking underlying EV secretion pathways is feasible by kinase control, and able to resensitize resistant cells to first-line agents, representing an alternative treatment strategy.

Funding: Dutch Cancer Society (KWF) Unique High Risk Grant (#11308) to D.M Pegtel.

OF17.03 | Tethered exosomes containing MT1-MMP contribute to ECM degradation during breast cancer progression

Roberta Palmulli; Hannah K. Jackson; James R. Edgar
Department of Pathology, University of Cambridge, Cambridge. CB2 1QP. United Kingdom, Cambridge, United Kingdom

Introduction: Membrane-bound metalloproteinase membrane type 1–matrix metalloproteinase (MT1-MMP) plays a central role in extracellular matrix (ECM) degradation during breast cancer progression. MT1-MMP is exposed on the plasma membrane of cancer cells, from where it can degrade the ECM. In addition, MT1-MMP has been found to be enriched on EVs, although the contribution of such EVs to cancer progression has been overlooked.

We previously showed that sEVs/exosomes can be tethered at the surface of producing cells by the protein tetherin. Interestingly, tetherin is highly expressed in metastatic breast cancer where it modulates ECM degradation, tumour cell migration and metastasis formation. The molecular mechanism behind tetherin overexpression and cancer progression remains elusive.

Methods: Tetherin KO and tetherin overexpressing MDA-MB-231 cell lines were generated. Biochemistry, fluorescence microscopy, and electron microscopy were used to characterise MDA-MB-231 cell lines, sEV release, and to study the localisation and function of MT1-MMP and tetherin. In some cases, Bafilomycin-A1 was used to induce release of exosomes. sEVs were isolated by size exclusion chromatography and characterised using biochemistry, electron microscopy and nano-flow cytometry (nanoFCM). Extracellular matrix degradation assays were also performed.

Results: Our data show that MDA-MB-231 cells release sEVs containing MT1-MMP, that are likely derived from multivesicular endosomes (exosomes). MDA-MB-231 cells express tetherin. Tetherin is released through sEVs and can tether sEVs at the surface of producing cells, modulating the release of (MT1-MMP) sEVs in the extracellular space. Tetherin expression modulates ECM degradation by MDA-MB-231 cells.
Summary/Conclusion: Our data show that breast cancer cells release MT1-MMP through exosomes that can be tethered at the cell surface by tetherin. This study will contribute to elucidate the function of exosomal metalloproteases and tetherin during breast cancer progression.
Funding: JRE and RP are supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (216370/Z/19/Z). HKJ is funded by Exosis.

OF17.04 | New strategies using small extracellular vesicles to increase the efficacy of CDK4/6 inhibitors in breast cancer

Paula Carpintero-Fernandez; Marta Barturen-Gomez; alejandro Garcia-Yuste; Joaquin Mosquera; Benigno Acea; Maria D. Mayan
CellCOM Research Group. INIBIC-A Coruña, A Coruña, Spain

Introduction: Connexins are channel-forming proteins involved in cell-to-cell communication. These channel proteins modulate the tumour microenvironment via channel-dependent and channel-independent functions. Connexin43 (Cx43) has been reported to be dysregulated in breast cancer, however there is still controversy of its role in the pathogenesis and development of the disease depending on the subtype. The therapeutic response of tumour cells are also affected by these transmembrane proteins. On the other hand, small extracellular vesicles (sEVs) are already being used in different trials as new advanced therapies as drug vehicles to treat different types of cancer.
Methods: The human ER+/HER2- breast cancer cell line MCF7 was used in this study. Lentiviral vectors and sEVs-enriched in Cx43 isolated from different sources , were used to restore Cx43 in breast cancer cells. sEVs were isolated by ultracentrifugation and characterized by NTA, electron microscopy and WB. Standard methods were used to study the effect of sEVs-enriched in Cx43 in combination with CDK4/6 inhibitors in cellular senescence and apoptosis. The CDK4/6 inhibitors (Palbociclib, Abemaciclib, Ribocilcib) were obtained from Selleckchem.
Results: Here, we have demonstrated that the restoration of Cx43 in ER+/HER2- breast cancer cells using lentiviral vectors enhances senescence and sensitizes breast cancer cells to cell death by apoptosis increasing the efficacy of the CDK4/6 inhibitors (Pabociclib, Abemaciclib, Ribociclib) . We obtained similar results using sEVs-enriched in Cx43, in combination with CDK4/6 inhibitors, which significantly increases CDK4/6 inhibitors efficacy in 2D and 3D models. Further, the combination of Cx43, CDK4/6 inhibitors and senolytic drugs, such as Navitoclax, has been assayed and this combination resulted in the best strategy to enhance senescence, reduce the proliferation of breast cancer cells and increase cell death increasing efficacy and preventing drug resistance.
Summary/Conclusion: The results presented here has been protected in an EU patent application. We propose a new and effective drug combination strategy based on the use of sEVs enriched in Cx43 to reduce CDK4/6 inhibitors resistance by improving their efficacy in breast cancer models. The results obtained could impact in the manage and treatment of tumours that respond to CDK4/6 inhibitors treatment and with a potential clinical benefit in patients.

OF17.05 | A 3D-printed metastatic-niche-on-a-chip for characterizing the cancer-stroma co-adaptation via extracellular vesicle crosstalk

Molly L. Shen[1]; Vahid Karamzadeh[1]; Alia Alameri[1]; Andreas Wallucks[1]; Emilie Solymoss[2]; Andy Ng[1]; Peter M. Siegel[2]; David Juncker[1]
[1]Department of Biomedical Engineering, Faculty of Medicine and Health Sciences, McGill University, Montreal, Canada; [2]Rosalind and Morris Goodman Cancer Research Centre, McGill University, Montreal, Canada

Introduction: Cancers exhibit organ-specific metastasis (organotropism). Studying the underlying mechanism remains challenging due to limitations in co-culture models to recapitulate the spatiotemporally regulated cellular interactions in the tumor microenvironment (TME). Here, we report a microfluidic metastatic-niche-on-a-chip for co-culturing cancer spheroids with up to two relevant TME stromal cell types. Furthermore, we established endogenous fluorescent protein tags to distinguish EVs secreted by cancer and stromal cells. We utilized our device to characterize the migration pattern and EV crosstalk of organotropic breast cancer cells co-cultured in matched (lung-tropic in lung) and un-matched (lung-tropic in bone) metastatic-niche.
Methods: The device was 3D-printed using a novel photocurable bio-ink optimized for biocompatibility, hydrophilicity, and transparency. Cell lines expressing membrane-targeting fluorescent proteins were established to classify EVs secreted by cancer

vs. stroma. Matrigel-embedded spheroids of bone- and lung-tropic subpopulations of MDA-MB-231 triple negative breast cancer cell line were seeded into the central chamber of the device, while in-device capillary stop valves enabled patterning of the lung or bone stromal cells in channels surrounding the spheroid, mimicking the in vivo cancer-stromal morphology. Conditioned media was sampled in a time-course, purified using IZON columns, and single EVs were analyzed using a CytoFLEX flow cytometer and an in-house single EV imaging platform.

Results: EVs collected from the co-culture device were profiled for tetraspanins and cancer-associated integrins. Preliminary data suggested that while both bone- and lung-tropic breast cancer spheroid triggered sprouting of lung fibroblast at as early as day 2, EVs from the lung-tropic spheroid in lung condition had a higher CD63-CD9 co-expression and a 2-fold increase in integrin β1+ EVs. In contrast, EVs secreted by the bone-tropic spheroid had higher abundance of integrin αV, α6, and β4 when co-cultured with matched stromal cells.

Summary/Conclusion: Our platform may potentially help identify changes in EV crosstalk linked to metastatic pre-disposition, plasticity and co-adaptation of cancer and stroma in the metastatic niche.

OF17.06 | Activation of TGF-β signaling modifies the protein content of extracellular cellular vesicles derived from breast cancer cells

Dorival M. Rodrigues-Junior[1]; Konstantina Psatha[2]; Michalis Aivaliotis[2]; Carl-Henrik Heldin[1]; Aristidis Moustakas[1]
[1]*Uppsala University, Uppsala, Sweden;* [2]*University of Thessaloniki, Thessaloniki, Greece*

Introduction: Transforming growth factor-β (TGF-β) signaling forms a complex pathway known to suppress early-stage hyperplasia, but yet induces epithelial-mesenchymal transition, assisting tumors to achieve metastasis. Extracellular vesicles (EVs) secreted by diverse human tumor cells have been associated with the process of metastasis. Although some molecules associated with the TGF-β pathway have been implicated as cargo of EVs, a role of TGF-β signaling on EV biology has been poorly studied. Here, we described the impact of TGF-β signaling on the protein content of breast cancer cell-derived EVs and the biological role of EVs induced by TGF-β (TGF-β+EV).

Methods: To this end, EV-fractions enriched in CD81 or Cholerae toxin B chain (CTB) were isolated from the vesicular secretome fraction (VSF) of human breast cancer MDA-MB-231 cells stimulated or not with TGF-β. The EVs were characterized based on morphology, size distribution and presence of EV-specific antigens.

Results: Of the 971 proteins detected in the CD81+EVs by LC-ESI-MS/MS, 38 were specific to TGF-β+EVs while 137 were present in the Control (Ctrl EV). From the 2,406 proteins identified in the CTB+EVs, 86 were present only in the TGF-β+EVs, while 124 were specific to the Ctrl. PANTHER and STRING database analysis indicated that the content of TGF-β+EVs was related to integrin signaling and inflammation mediated by chemokines. Moreover, such EVs induced TGF-β signaling. Blocking EV uptake did not suppress such effect, whereas EV incubation with a monoclonal anti-TGFβ antibody significantly reduced the activation of TGF-β signaling induced by these EVs. Furthermore, TGF-β pathway activation by EVs led to higher levels of TGF-β responsive genes in the recipient cells, also increasing their motility and resistance to cytotoxic drugs.

Summary/Conclusion: Hence, we uncovered new functions of the TGF-β pathway regulating the cargo of tumor-derived EVs, which drives tumor aggressiveness and may assist the prediction and assessment of cancer patients.

Funding: Ludwig Cancer Research; Cancerfonden [CAN2018/469] to ArM; Vetenskapsrådet [2018-02757-3] to ArM, [2020-01291] to CHH; Barncancerfonden [PR2018-0091] to ArM; European Research Council [787472] to CHH.

OF18: The Bioactive Component in EVs

Chair: Antonia Reale – Monash University

Chair: Neta Regev-Rudzki – Weizmann Institute of Science

OF18.01 | Cancer cell targeting via small extracellular vesicles

ShivRam Krishn[1]; Vaughn Garcia[2]; Nicole Naranjo[2]; Fabio Quaglia[2]; Christopher Shields[2]; Maisha Harris[3]; Qin Liu[4]; Dario Altieri[5]; Lucia R. Languino[6]
[1]*Jefferson University, philadelphia, USA;* [2]*Jefferson University, Philadelphia, USA;* [3]*Jefferson u., Philadelphia, USA;* [4]*Wistar I., Philadelphia, USA;* [5]*The Wistar Institute, Philadelphia, USA;* [6]*Thomas Jefferson University, Philadelphia, USA*

Introduction: Cancer cells crosstalk with the tumor microenvironment (TME) by releasing small extracellular vesicles (sEVs) which are enriched in the epithelial-specific αVβ6 integrin. sEVs mediate protein transfer of αVβ6 to microvascular endothelial

cells and monocytes in the TME; this transfer results in de novo αVβ6 expression and affects these cells' functions. As evaluated by our proteomic and immunoblotting analyses, αVβ6 expression in donor cancer cells and their sEVs inhibits the levels of molecules that have a tumor suppressive role, such as STAT1; in recipient cells, sEV-mediated αVβ6 expression affects STAT1 signaling.

Methods: sEVs were isolated from DU145 prostate cancer cell culture media and characterized by electron microscopy, density gradients, NTA, and immunoblotting analysis, as per MISEV2018 guidelines. sEVs were mixed with non-silencing siRNAs, ITGB6 targeting D1 or D13.1 siRNA, or Cy3 control DsiRNAs, and electroporated; then, they were incubated with RNase A, washed, centrifuged, and tested in functional assays using PC3 cells.

Results: We have now selected αVβ6, which is expressed in many cancer cells but absent in normal cells, as candidate for a sEV-mediated therapeutic approach. We show an efficient strategy to target αVβ6 in cancer cells by using siRNA loaded into sEVs. We first demonstrate that fluorescently labeled siRNAs can be efficiently loaded into sEVs by electroporation; by confocal microscopy, we show internalization of siRNA-loaded sEVs into recipient cells. We then provide evidence that sEV-mediated delivery of β6 targeting siRNAs to cancer cells specifically downregulates αVβ6 expression, without changing the expression of the β5 integrin subunit. As a consequence, sEV-mediated delivery of β6 targeting siRNAs, but not of non-silencing siRNAs, significantly reduces cell adhesion and migration of cancer cells on αVβ6-specific substrates.

Summary/Conclusion: Overall, this study shows that sEVs from cancer cells may contribute to a horizontal propagation of integrin-associated phenotypes from cancer cells to the TME and demonstrates an efficient approach for specific targeting of the αVβ6 integrin in cancer cells using sEVs carrying ITGB6-specific siRNAs.

Funding: NIH-R01CA224769; NIH-P01CA140043

OF18.02 | A map of the extracellular RNA carried by RNA-binding proteins (RBPs) within extracellular vesicles, lipoprotein particles, and ribonucleoprotein (RNP) particles across human biofluids

Emily LaPlante; David Chen; Robert Fullem; Matthew Roth; Eric Van Nostrand; Aleksandar Milosavljevic
Baylor College of Medicine, Houston, USA

Introduction: RNA binding proteins (RBPs) play a critical role in the release and transport of extracellular RNA (exRNA) by sorting and loading exRNA into vesicles/lipoproteins and even directly carrying exRNA. However, the current understanding of RBPs as carriers of exRNA fragments in human biofluids is still lagging behind work on other exRNA carriers.

Methods: We leverage ENCODE eCLIP data, which determines the bindings sites for 150 RBPs and intersect it with 6,930 human small RNAseq samples from the exRNA Atlas to determine the landscape of exRNA fragments bound by these RBPs. We identify exRNA fragments with RBP binding sites from eCLIP experiments that are significantly correlated in the human exRNA Atlas to discover RBPs with detectable exRNA "footprints". We then perform computational deconvolution of RBP exRNA cargo to place RBPs into different carrier types – vesicles, lipoproteins, or RNPs. All files are made available for the community.

Results: At least several hundred thousand short RNA fragments appear to be carried into the extracellular space by RBPs. RBPs with enriched exRNA patterns are identified across different biofluids, including plasma, serum, saliva, CSF, and urine. We find 33 RBPs with detectable footprints in both cell supernatant and healthy human plasma. These RBPs are enriched for 5 different functions – RNA stability and decay, translation regulation, RNA localization, miRNA processing, and splicing. We also place 44 RBPs into vesicular and non-vesicular carrier classes.

Summary/Conclusion: We present the first map of exRNAs carried by specific RBPs within extracellular vesicles, lipoprotein particles, and free RNPs across human biofluids. To empower the community, we extend the exRNA Atlas resource with intersections between all 150 RBPs eCLIP "footprints" and all 6,930 human small RNAseq samples in the exRNA Atlas. The map and the new data resource will improve understanding of the role of RBPs as carriers of extracellular RNA in both normal physiological and disease contexts.

OF18.03 | Malaria-infected red blood cells release two distinct extracellular vesicle sub-populations featuring the ability to target different sub-cellular destinations

Paula Abou Karam[1]; Irit Rosenhek-Goldian[1]; Tamar Ziv[2]; Hila Ben Ami-Pilo[1]; Ido Azuri[1]; Anna Rivkin[1]; Edo Kiper[1]; Ron Rotkopf[1]; Sidney R. Cohen[1]; Raya Sorkin[3]; Ana Claudia Torrecilhas[4]; Ori Avinoam[1]; Alicia Rojas[5]; Mattia Morandi[1]; Neta Regev-Rudzki[1]
[1]Weizmann Institute of Science, Rehovot, Israel; [2]Technion - Israel Institute of Technology, Haifa, Israel; [3]Tel Aviv University, Tel Aviv, Israel; [4]Federal University of São Paulo, Diadema, Brazil; [5]University of Costa Rica, San Jose, Costa Rica

Introduction: Malaria is the most serious mosquito-borne parasitic disease, caused mainly by the intracellular parasite Plasmodium falciparum (Pf). This parasite invades human red blood cells (RBCs) and releases extracellular vesicles (EVs) carrying DNA, RNA, proteins and lipids to alter its host immune responses. Recent findings from various mammalian systems indicate the heterogeneity of the EV pool, based on distinct cargo components. Our aim was therefore to identify the various EV subpopulations produced by malaria infection.
Methods: EV samples were subjected to size-separation analysis, using Asymmetric Flow Field-Flow Fractionation (AF4). The EV subpopulations were then collected and characterized both by atomic force microscopy (AFM) and cryo-transmission electron microscopy (cryo-TEM). Proteins were also isolated and subjected to proteomic analysis. Furthermore, using Förster resonance energy transfer (FRET)-based fusion assay we examined the fusion capabilities of the EV subpopulations. Finally, Laurdan staining and AFM puncture analysis combined with machine learning methods were applied in order to characterize the membrane biophysical properties of the subpopulations.
Results: Interestingly, we identified two distinct EV subpopulations secreted by parasite-infected RBCs that differed in size (26 nm and 69 nm, in average). A mass spectrometry (MS) analysis of each respective EV subpopulation revealed a mixture of human and parasitic proteins. Surprisingly, the enriched cellular pathways differed between the two subpopulations, with the small EVs containing complement-system proteins and the large EVs a pronounced amount of proteasome subunits. We then measured the membrane fusion abilities of each subpopulation with three types of host cellular membranes: plasma, late and early endosome. Remarkably, the small EVs fused to early endosome liposomes at significantly greater levels than did the large EVs. Atomic force microscope puncture tests evaluated with machine-learning methods further emphasize the difference between the two subpopulations through their mechanical properties.
Summary/Conclusion: Our data demonstrate that Pf-iRBCs release two distinct subpopulations of EVs which differ in their protein content, biophysical properties, and fusion abilities. Therefore, this study may shed light on how malaria parasites utilize the powerful EV pathway to secrete particles responsible for discriminated functions in the parasite-host interaction.

OF18.04 | Extracellular vesicles in human body fluids trigger coagulation by exposing functional extrinsic tenase complexes

Yong Hu[1]; Chi Hau[2]; Johannes Thaler[3]; Rienk Nieuwland[4]
[1]Laboratory of Experimental Clinical Chemistry; Vesicle Observation Center; Department of Biomedical Engineering & Physics, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands; [2]Department of Clinical Chemistry; Vesicle Observation Center, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands; [3]Clinical Division of Haematology and Haemostaseology, Department of Medicine I, Medical University of Vienna, Vienna, Austria; [4]Department of Clinical Chemistry; Vesicle Observation Center; Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands

Introduction: Coagulation is the first step of wound healing, and reduces blood loss and the risk of infection. Tissue factor (TF) is the transmembrane receptor of (activated) coagulation factor (F)VII(a), and together they form the "extrinsic tenase" complex, which triggers coagulation by activating FX to FXa. Surprisingly, TF occurs not only in the vessel wall, but is also commonly present on extracellular vesicles (EVs) in body fluids such as amniotic fluid (AF), milk, saliva, and urine. Here we investigated whether such EVs expose extrinsic tenase complexes.
Methods: Milk, saliva, and urine were collected from healthy breastfeeding women (n=6), and AF was collected from healthy women undergoing routine amniocentesis (n=7). EVs were isolated by size exclusion chromatography (SEC) and coagulation (clotting) experiments were performed in the presence and absence of antibodies against TF and FVIIa in normal plasma and in FVII-deficient plasma. The extrinsic tenase activity, i.e. the activation of FX to FXa, was determined by a chromogenic assay.
Results: All body fluids triggered clotting of normal plasma. Milk was the most coagulant body fluid, followed by AF, saliva, and urine. The body fluids also triggered clotting of FVII-deficient plasma, which was inhibited by anti-FVII and anti-TF (milk: p=0.031, p=0.044; AF: p< 0.000, p=0.001; saliva, p< 0.000, p=0.055; urine: p=0.024, p=0.024). SEC showed that only fractions containing EVs triggered clotting and generated FXa, which was inhibited by anti-FVII and by anti-TF.
Summary/Conclusion: Normal human body fluids contain EVs exposing functional extrinsic tenase complexes. These EVs activate FX directly, thereby bypassing a major part of the coagulation cascade and providing direct hemostatic protection.
Funding: Y.H. was supported by a scholarship from the China Scholarship Council (CSC).
J. T. was supported by an unrestricted travel grant from the International Society on Thrombosis and Haemostasis.

OF18.05 | Colostrum exosome-based delivery of siRNAs and inhibition of lung cancer

Ramesh C. Gupta[1]; Margaret Wallen[2]; Raghuram Kandimalla[3]; Jeyaprakash Jeyabalan[2]; Disha Moholkar[3]; Radha Munagala[2]; Wendy Spencer[2]; Farrukh Aqil[4]
[1]3P biotechnologies and Department of Pharmecology & Toxicology and Brown Cancer Center, University of Louisville, Louisville, KY 40202, Louisville, USA; [2]3P biotechnologies Inc., Louisville, KY 40202, Louisville, USA; [3]University of Louisville, Louisville, KY 40202, Louisville, USA; [4]University of Louisville, Louisville, USA

Introduction: We have shown that bovine milk and colostrum provide abundant small extracellular vesicles, also referred to as exosomes, for drug delivery. We now report a novel exosome-polyethyleneimine (PEI) matrix (EPM) for delivery of siRNA targeting KRAS (siKRAS) and nuclear factor erythroid 2-related factor, NRF2 (siNRF2) to inhibit lung cancer by downregulating target protein expression. These two oncogenic targets are known to be highly expressed in lung, as well as other types of cancer.
Methods: Exosomes were isolated from bovine colostrum powder by rehydration and differential centrifugation and characterized for size, pdi and charge (Zetasizer) and hallmark proteins (Western blot). Test siRNAs were entrapped in the EPM by brief incubation of siRNA with the vector, followed by PEG precipitation. The siRNA entrapment efficiency was determined using tracer 5'-32P-labeled siRNA. The transfection efficiency was determined in vitro by measuring gene knockdown via Western blot analysis, while anti-cancer effects were assessed in vivo using lung tumor xenografts in immunocompromised mice.
Results: Colostrum exosomes exhibited similar size and charge as mature milk exosomes and carried similar surface proteins markers. Based on the presence of radioactive siRNA, we found that the EPM entrapped >90% of added siRNA (up to 20 μg). siRNA entrapped in EPM was protected from enzymatic degradation upon exposure to RNases, as detected by gel electrophoresis using radioactive tracer siRNA. EPM-siKRAS decreased mutant KRAS expression in A549 lung cancer cells dose dependently. When composed of folic acid-functionalized exosomes, EPM-siKRAS resulted in significant inhibition of orthotopic lung xenografts (>70%; $p < 0.01$) and downregulation of KRAS expression in the tumor (>50%; $p < 0.05$). Likewise, EPM-siNRF2 delivery resulted in dose-dependent decrease of NRF2 expression in the same cell line and correlated with the inhibition of cell survival. EPM-siNRF2 formulation also inhibited the growth of subcutaneous lung tumors (>57%; $p < 0.01$) in immunocompromised mice.
Summary/Conclusion: The colostrum exosome-based EPM technology provided significant gene knockdown and lung tumor inhibition by targeted delivery of siRNA and represent a simple and effective exosome-based gene therapy approach.
Funding: Supported from funds from 3P Biotechnologies and, in part, from the USPHS grant CA221487-01.

OF18.06 | Matrix vesicles membrane organization and its interaction with PHOSPHO1 investigated by means of biomimetic membrane models

Ana Lara Nanzer[1]; Luiz Henrique Andrilli[1]; Heitor G. Sebinelli[2]; Maytê Bolean[1]; Massimo Bottini[3]; José Luis Millán[4]; Ana Paula Ramos[5]; Pietro Ciancaglini[1]
[1]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) da Univeridade de São Paulo (USP), Ribeirão Preto, Brazil; [2]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) - Universidade de São Paulo (USP), Ribeirão Preto, Brazil; [3]University of Rome Tor Vergata, Roma, Italy; [4]Sanford Burnham Prebys, La Jolla, CA, USA, La Jolla, USA; [5]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) da Univeridade de São Paulo (USP), Av. Bandeirantes, 3900, Brazil

Introduction: Bone biomineralization is a process mediated by osteoblasts through the release of matrix vesicles (MVs). The most accepted theory describes the MVs´ biogenesis by budding from cell membranes, secreted at specific sites in the bone extracellular matrix. The internal MVs reservoir is composed by enzymes such as PHOSPHO1, a phosphatase that hydrolyzes phosphocholine and phosphoethanolamine, and generates Pi through phospholipids degradation, that may be also involved in MVs biogenesis. In this study, we aimed to investigate the interactions of PHOSPHO1 with lipids enriched in the membrane of MVs and to compare the results with monolayers composed by native lipids extracted from MVs.
Methods: For this, we isolated MVs from the femurs of chicken embryos (16-17 days) and purified the lipids using a sequence of dispersion in organic solvents/centrifugation steps. We used DPPC, DPPS and cholesterol (Chol), and Langmuir monolayers as a mimetic membrane model. 1M of lipid chloroformic solutions were dripped at the air-liquid interface on a Tris/HCl (containing 100 mM NaCl and 2 mM MgCl2) buffer, pH 7.4. PHOSPHO1 (0.76 μg/mL) was added to the subphase, then the monolayer was compressed with the aid of a Langmuir trough.
Results: The compressional moduli of the DPPS monolayers was increased in the presence of the enzyme when compared to pure DPPS. Moreover, the area occupied per lipid molecule was increased from 56.9 Å2 to 63 Å2 in the presence of PHOSPHO1, which indicated interaction between DPPS and the enzyme. Although PHOSPHO1 also increased the compressional moduli of DPPC monolayers, its presence reduced the area occupied per lipid, revealing different interaction guided by the composition of the lipids polar head In situ fluorescence microscopy images of the monolayers revealed the formation of larger domains rich

in phospholipids induced by the presence of the enzyme. Monolayers composed by lipids extracted from native MVs were also formed in the presence of PHOSPHO1. The enzyme increased the area occupied per lipid in pH 7.4, but not in more acidic pH. The results were similar to those found in artificial monolayers composed by DPPC:DPPS:Chol, which may be an indication of the enzyme adsorption at lipid-rafts moieties.

Summary/Conclusion: The results reveal that the interaction of PHOSPHO1 with lipids in MVs can be mimicked by adjusting the composition of artificial Langmuir monolayers. The lipid organization is crucial for the interaction of PHOSPHO1 with MVs´membrane model. These results shed light in the mechanisms of biogenesis driven by this enzyme.

Funding: Supported by FAPESP: (2019/08568-2; 2019/25054-2; 2021/02768-0); CNPq (304021/2017-2).

OF19: EV Analysis Flow Micros

Chair: Rienk Nieuwland – Department of Clinical Chemistry; Vesicle Observation Center; Amsterdam University Medical Center, University of Amsterdam

Chair: Tobias Tertel – Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

OF19.01 | Multiplexed protein detection on single extracellular vesicles by DNA Exchange Imaging

Andreas Wallucks[1]; Philippe DeCorwin-Martin[1]; Lucile Alexandre[2]; Johan Renault[1]; Rosalie Martel[1]; Molly L. Shen[1]; Lorenna Oliveira Fernandes De Araujo[1]; Andy Ng[1]; David Juncker[1]

[1]Department of Biomedical Engineering, Faculty of Medicine and Health Sciences, McGill University, Montreal, Canada; [2]Department of Biomedical Engineering, Faculty of Medicine and Health Sciences, McGill University, Montreal, Canada; Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, CNRS, Paris, France, Paris, France

Introduction: There is a need for technologies that profile extracellular vesicles (EVs) on the single vesicle level to uncover functionally different subpopulations which are masked in bulk analyses. Here we report a single EV multiplexed proteomics platform named Digital Omics of Single EVs (DOSE) based on DNA exchange imaging (DEI).

Methods: We use EVs derived from HT29 and A431 cells that are purified using 220 nm filters followed by a size exclusion column step (IZON). The EVs are characterized according to MISEV2018 with TRPS, TEM and their protein content is validated by Western blot.

Results: DOSE uses a flow cell on top of a glass coverslip with antibody functionalized surface which is mounted in an inverted fluorescence microscope (Nikon Ti2). The EV capture on the surface is monitored by label-free live imaging using interferometric scattering imaging (iSCAT). We proceed to block the surface and incubate a panel of antibodies, each of which is conjugated to a target specific DNA oligo. Detection is done using complementary oligos with fluorescent labels which can be hybridized in less than five minutes and be removed after imaging by toehold mediated strand displacement. Liquid handling and imaging are fully automated so that thousands of individual vesicles can be characterized with an initial panel of twenty protein markers. The image analysis is done with a custom Matlab pipeline using the label-free images to detect vesicles, suppressing noise and non-specifically bound labels in the fluorescence channels.

We validate the label free EV detection using silica beads as size standards as well as a CD63-GFP expressing A431 cell line. Furthermore, we show proof-of-principle DEI experiments on HT29 EVs where specific capture of vesicles positive for low abundance targets results in enrichment of subpopulations compared to general capture of EVs.

Summary/Conclusion: The method overcomes several challenges in single EV techniques including spectral limitations in multiplexed fluorescence imaging and a background from non-specific interactions or imperfect EV isolation techniques. It allows for fast cycling times and high throughput at relatively low added cost making it a promising technique to be incorporated into the single EV toolbox.

Funding: Genome Canada Disruptive Innovation in Genomics, A.W. Schmidt Science Fellows, L.A. Action Marie Sklodowska-Curie

OF19.02 | Automation of clinical extracellular vesicle measurement using standardized and calibrated nanoscale flow cytometry

Yohan Kim[1]; Ishwor Thapa[2]; Edwin van der Pol[3]; Ali Arafa[1]; Cameron Britton[1]; Shelly Song[1]; Hesham Ali[4]; Robert Karnes[1]; Fabrice Lucien[1]

[1]Mayo Clinic, Rochester, USA; [2]University of Nebraska-Omaha, Omaha, USA; [3]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Amsterdam, Netherlands; [4]University of Nebraska in Omaha, Omaha, USA

Introduction: Nanoscale flow cytometry (nFC) can enumerate thousands of extracellular vesicles (EVs) within minutes using fluorescence and light-scattering detection, thus allowing us to evaluate the clinical utility of EVs as liquid biomarkers in human diseases. However, gating strategy for large sample cohorts can be time-consuming and subjective with low abundant EV populations and background noise. Here, we developed a computer-assisted gating strategy for rapid and objective EV quantification.
Methods: Blood samples of 4 healthy donors and 92 localized prostate cancer (locPCa) patients were prospectively collected using institutional review board approved protocols. Optimized multiplex labeling of EVs with antibodies was performed to detect different EV subsets (prostate, platelet, leukocyte). The Apogee A60-MicroPlus nanoscale flow cytometer was used to detect EVs. An automated gating model, written in R programming language, transformed flow cytometry data to hyperbolic arcsine and detected dense background noise by 'Kmeans' clustering. Based on the slopes of the density plots around the cluster locations, polygon boundaries were generated to select target EVs for each file. To distinguish EVs of interest from non-specific events, multiple machine learning models were evaluated.
Results: Comparing to manual data processing, computer assisted-data processing generated similar concentrations of prostate ($p>0.08$) and platelet ($p>0.06$) EV subsets in an unbiased and faster manner (less than 10 minutes). Both processes resulted in no significant differences in prostate EV levels between healthy donor and locPCa patients. However, levels of leukocyte EVs and platelet EVs from locPCa patients was significantly higher (1.5-fold, $p< 0.05$ and 10-fold, $p< 0.01$ respectively) than healthy donors.
Summary/Conclusion: This study provides reproducible clinical EV measurement using nFC and automated data analysis, and ultimately will serve as framework for the future development of EV-based liquid biopsies in human diseases.
Funding: This research was supported by departmental grant (FL) and generous benefactors (FL). FL was recipient of a postdoctoral fellowship from the Fonds de Recherche du Quebec-Sante (FRQS).

OF19.03 | Tellurium-based labeling of the extracellular vesicle proteome (TeLEV) enables high-dimensional single-cell analyses of EV recipients by mass cytometry and imaging mass cytometry

Daniel Bachurski[1]; Yong J. Bu[2]; Dominik Priesmann[3]; Tobias Tertel[4]; Paul J. Bröckelmann[5]; Marcel Schmiel[6]; Philipp Gödel[7]; Alexander F. vom Stein[8]; Ron D. Jachimowicz[9]; Katrin S. Reiners[10]; Roland T. Ullrich[7]; Katarzyna Bozek[11]; Nima Abedpour[12]; Marcus Krüger[3]; Christian Preußer[13]; Bernd Giebel[4]; Elke Pogge von Strandmann[13]; Mark Nitz[2]; Michael Hallek[8]
[1]University of Cologne, Faculty of Medicine and University Hospital Cologne, Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf, Mildred Scheel School of Oncology Aachen Bonn Cologne Düsseldorf, Center for Molecular Medicine Cologne, CECAD Center of Excellence on Cellular Stress Responses in Aging-Associated Diseases, Cologne, Germany, Cologne, Germany; [2]Department of Chemistry, University of Toronto, Toronto, Ontario, Canada, Toronto, Canada; [3]University of Cologne, Institute for Genetics, CECAD Center of Excellence on Cellular Stress Responses in Aging-Associated Diseases, Cologne, Germany, Cologne, Germany; [4]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany; [5]University of Cologne, Faculty of Medicine and University Hospital Cologne, Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf, Mildred Scheel School of Oncology Aachen Bonn Cologne Düsseldorf, Max Planck Institute for Biology of Ageing, Cologne, Germany, Cologne, Germany; [6]University of Cologne, Faculty of Medicine and University Hospital Cologne, Department of Translational Genomics, Cologne, Germany University of Cologne, Faculty of Medicine and University Hospital Cologne, Institute of Pathology, Cologne, Germany, Cologne, Germany; [7]University of Cologne, Faculty of Medicine and University Hospital Cologne, Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf, Cologne Essen Lymphoma Working Group, Center for Molecular Medicine, Cologne, Germany, Cologne, Germany; [8]University of Cologne, Faculty of Medicine and University Hospital Cologne, Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf, Center for Molecular Medicine Cologne, CECAD Center of Excellence on Cellular Stress Responses in Aging-Associated Diseases, Cologne, Germany, Cologne, Germany; [9]University of Cologne, Faculty of Medicine and University Hospital Cologne, Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf, Max Planck Institute for Biology of Ageing, Cologne, Center for Molecular Medicine Cologne, CECAD Center of Excellence on Cellular Stress Responses in Aging-Associated Diseases, Cologne, Germany, Cologne, Germany; [10]Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital, University of Bonn, Germany, Bonn, Germany; [11]University of Cologne, Center for Molecular Medicine Cologne (CMMC), Faculty of Medicine and University Hospital Cologne, Data Science of Bioimages Lab, Cologne, Germany, Cologne, Germany; [12]University of Cologne, Faculty of Medicine and University Hospital Cologne, Department of Translational Genomics Center for Molecular Medicine Cologne, Cologne, Germany, Cologne,

Germany; [13]Institute for Tumor Immunology, Center for Tumor Biology and Immunology (ZTI), Philipps University of Marburg, Marburg, Germany, Marburg, Germany

Introduction: The expanding EV field needs reproducible and sensitive methods to characterize single EVs and EV-mediated effects on single cells. Although single-EV techniques are now commonly applied in the field, high-dimensional single-cell analyses to elucidate the functional impact of EV uptake on recipient cells are lacking.
Here, we developed the novel EV labeling method TeLEV based on L-2-tellurienylalanine (TePhe), a phenylalanine mimic containing the heavy metal tellurium (Te). TePhe is applied as an amino acid substitute to EV-producing cells, resulting in the release of Te-labeled EVs (TeLEVs). TeLEV recipient cells can be easily identified by mass cytometry (MC). As MC and imaging MC (IMC) can discriminate over 52 heavy metal isotopes conjugated to antibodies, both emerged as technologies for the phenotypic dissection of heterogeneous cell populations and tissues amongst others for PBMCs. Combining TeLEV with MC, we became thus able to explore EV uptake at a single-cell level in over 30 different cell types in parallel and could also study the impact of EVs on various cellular functions such as apoptosis, DNA-damage response, migration, proliferation and tyrosine-kinase signaling.
Methods: In our studies, we separated TeLEVs from serum-free cultured cells of eight different cell lines (incl. HEK293T, HeLa, JVM-3) and from primary cells (incl. chronic lymphocytic leukemia cells) by combining 0.2 μm filtration, 10 kDa ultrafiltration and size-exclusion chromatography (qEV/35nm).
Results: Upon characterizing TeLEVs according to the MISEV2018 criteria and comparing them with non-labeled EVs of the same cells, we did not observe any differences in composition or morphology. Next, we analyzed the uptake of TeLEVs by PBMCs cultured in the presence of TeLEVs for 16 h. Our analyses revealed that independent of the EV source, classical, transitional and non-classical monocytes, as well as myeloid dendritic cells, were the main recipients of TeLEVs. Since we found differences in the individual label intensities of these EV incorporating cells depending on the EV source, we conclude that the differences emerged from different cellular uptake behaviors rather than being unspecific. Notably, although we analyzed T cells from 14 subsets (incl. Tregs and $\gamma\delta$ T), we did not identify EV uptake in any T cell.
Summary/Conclusion: In summary, TeLEV is a broadly applicable and easy-to-operate labeling method for EVs secreted by various cell types. Due to the advantages of MC and IMC, TeLEV will provide new avenues for studying the biodistribution of EVs and their functional impact on recipient cells.
Funding: This work was funded by the Deutsche Krebshilfe through a Mildred Scheel Nachwuchszentrum Grant (Grant number 70113307)

OF19.04 | Systematic evaluation of endogenous single EV labeling by membrane-localizing-peptide- and tetraspanin-based genetic tagging strategies

Molly L. Shen[1]; Emilie Solymoss[2]; Andreas Wallucks[1]; Sébastien Tabariès[2]; Matthew G. Annis[2]; Andy Ng[1]; Peter M. Siegel[2]; David Juncker[1]
[1]Department of Biomedical Engineering, Faculty of Medicine and Health Sciences, McGill University, Montreal, Canada; [2]Rosalind and Morris Goodman Cancer Research Centre, McGill University, Montreal, Canada

Introduction: Endogenous EV labeling via membrane-localizing peptides and EV-associated tetraspanins serve as an attractive solution for long-term in vivo EV analysis. In this work, we systematically evaluated the labeling efficiency of various EV labeling tags at the single EV level.
Methods: We generated a library of lentiviruses encoding a panel of fluorescent proteins (GFP, ZsGreen, Venus, tdTomato, or RFP) that are tagged with: 1) Src myristoylation sequence (myr-) ; 2) Lyn kinase myristoylation and palmitoylation sequence (myrpalm-) ; 3) full-length human CD63; or 4) full-length human CD9; as well as the GAP43 palmitoylation sequence (palm-) tagged with GFP or tdTomato (a kind gift from Professor Charles P. Lai). A panel of cell lines (293T, A431, T47D, BT459, and MDA-MB-231) were stably transfected. Their EVs were purified using IZON columns and analyzed using a 3 laser CytoFLEX flow cytometer and an in-house interferometric scattering microscope capable of label-free single EV imaging.
Results: All EV tags successfully labelled the plasma membrane of all cell lines screened. We also observed fluorescence signal in the cytosol of cells transfected with the myr-, myrpalm- and palm- tags, suggesting an accumulation of non-membrane bound fluorescent proteins. When benchmarked using GFP as a model fluorescent protein, palm- and the tetraspanin-based tags achieved EV labeling efficiencies of over 75%, as measured by both instruments. Meanwhile, myr- and myrpalm-GFP only labeled around 20∼30% of the total EV population. While some cell-type-dependent fluctuations in labeling efficiency was observed, the excitation profile of the fluorescent protein also influenced the measurable labeling efficiency, most notable on the CytoFLEX platform that lacks a laser optimal for orange/red fluorescent proteins. It recorded a labeling efficiency of 89.4% for palm-GFP but only 10.3% for palm-tdTomato. Similarly, myr-ZsGreen (21%) recorded a labeling efficiency much greater than myr-Venus and myr-RFP (1.4∼5%).

Summary/Conclusion: Our work demonstrated that single EV labeling efficiency can vary greatly depending on the tag and fluorescent protein of choice. Ongoing work entails proteomic profiling of the labelled EVs vs. their non-transfected counterparts to examine any potential alterations in the EV proteome caused by each EV labeling tags.

OF19.05 | Introducing ultra-correlative microscopy for single-EV analysis

Zubair Ahmed Nizamudeen[1]; Victoria James[2]; Robert Markus[2]; Rachael Xerri[2]; Kiran Suain[2]; Mark Platt[3]; Jason P. Webber[4]; David Scurr[2]; Christopher Parmenter[2]; Chris Denning[2]; Virginie Sottile[5]; Kenton Arkill[2]
[1]University of Nottingham, Oxford, United Kingdom; [2]University of Nottingham, Nottingham, United Kingdom; [3]Loughborough University, Loughborough, United Kingdom; [4]Swansea University, Swansea, United Kingdom; [5]University of Pavia, Pavia, Italy

Introduction: This presentation summarises our efforts towards ultra-correlative microscopy that can allow single-EV analysis with utmost clarity. We developed new approaches based on direct stochastic optical reconstruction microscopy (dSTORM) and correlative light-electron microscopy (CLEM). dSTORM uses multi-iterative localisation of fluorophores between light and dark states to provide a super resolution image that can visualise EVs ≥30nm. CLEM allows alignment of fluorescently labelled molecules under light microscopy with their ultrastructural details observed under electron microscopy, providing extensive information at the nanoscale. Ultra-correlation involves advanced CLEM that includes high-content compositional correlation.
Methods: For dSTORM (complemented by NTA, qNano, DotBlot-CD63,TSG101,GM130, TEM and cryoTEM analysis), cell uptake and sonication experiments, DiD-labelled-EVs were isolated using Qiagen exoEasy Maxi Kit (from primary mouse stem cells). For CLEM, Du145 prostate cancer derived EVs were isolated (ultracentrifugation) and labelled with AF488 C5-maleimide. For compositional correlation at 3D nanoscale, two approaches were developed: 1) 3D ORBISIMS using the HYBRIDSIMS platform and 2) Cryo-Biopsy approach using the 3D FIBSEM platform, for omics analysis of native-state biology.
Results: d-STORM approach can simultaneously provide size, shape and count analysis with high range and speed, and complements cryo-TEM data for detection of EV damage/changes due to low-power sonication. CLEM allowed structural correlation of single fluorescent EVs with their lipid bilayers. 3D cryo-ORBISIMS approach allowed identification of subcellular mass spec data (e.g. phosphatidylcholine, phosphatidylinositol) of DAPI correlated cells. Cryo-biopsy approach allowed detection of 18s RNA and transcriptomic analysis from fluorescence correlated subcellular microsurgeries (< 200μm2) as confirmed by PCR and RNA sequencing, respectively.
Summary/Conclusion: dSTORM and CLEM show high potential for ultra-correlative single-EV analysis allowing native-state structure-function-composition correlation, providing valuable information about EVs in situ and after isolation. Future work will focus on standardisation of protocols.
Funding: BBSRC, EPSRC, MIUR

OF19.06 | Imaging flow cytometry, a sensitive tool to measure urinary extracellular vesicles with a simple and isolation-free protocol

Liang Wu[1]; Wouter W. Woud[2]; Wenda Verschoor[3]; Ana M. Rodriguez[3]; Dennis Hesselink[2]; Carla Baan[2]; Karin Boer[2]
[1]Erasmus MC Transplant Institute, Department of Internal Medicine, University Medical Center Rotterdam Erasmus MC, Rotterdam, Netherlands; [2]Erasmus MC Transplant Institute, Department of Internal Medicine, University Medical Center Rotterdam, Rotterdam, The Netherlands, Rotterdam, Netherlands; [3]Erasmus MC Transplant Institute, Department of Internal Medicine, University Medical Center Rotterdam Erasmus MC, Doctor Molewaterplein 40, 3015 GD Rotterdam, the Netherlands;, Rotterdam, Netherlands

Introduction: The measurement of urinary extracellular vesicles (uEV) is still hindered by low sensitivity of detecting techniques, or bias induced by isolation. Imaging flow cytometry (IFCM) is a highly sensitive technique to detect small single uEV. Centrifugation, dithiothreitol (DTT) treatment, and dilution are commonly used to remove contaminants, increase uEV yield, or adjust urine pH. Here, we present a reliable, simple and isolation-free protocol to measure uEV by IFCM.
Methods: Urine from healthy volunteers (n=5) and kidney transplantation recipients (KTR) (n=5) were collected. Supernatant of a colorectal cancer cell line (COLO 205) were used as a positive control. Samples were labeled with CD63-Alxa488 and CD63-APC and isotype labeling was set as negative control. Detergent treatment identified biological structures. Coincidence events were excluded from analysis, double positive events represented EV.
Results: 14-times repeated IFCM detecting positive control showed a coefficient of variance of 4.6%. Centrifugation (10,000 g 10 min) removed autofluorescent particles in unstained urine, therefore optimizing the gating strategy enabling to distinguish uEV from autofluorescent background. In stained urine samples, this centrifugation removed 6.13 ± 4.15 × 10^6/ml uEV ($P < 0.05$), representing 20.8 ± 15.8% uEV ($P < 0.05$). Addition of DTT (25 mg/ml) did not affect uEV numbers in healthy urine,

but showed a 17.5 ± 25.8% higher concentration of uEV in KTR urine (P = 0.06). This might be associated with a higher total protein/creatinine ratio in patient urine (89.6 ± 44.4 g/mol) than healthy urine (4.0 ± 1.6 g/mol) (P < 0.01). Dilution resulted in a logarithmic decrease of uEV concentration with no significant effects on total uEV numbers.
Summary/Conclusion: In summary, IFCM is a sensitive and reliable tool to characterize single uEV in both urine from healthy as well as diseased individuals, with 25 mg/ml DTT incubation, and no need of centrifugation or uEV isolation.

OF20: Cell Biology Aspects

Chair: Deborah C. Goberdhan – Department of Physiology, Anatomy and Genetics, University of Oxford

Chair: Alissa M. Weaver – Vanderbilt University School of Medicine

OF20.01 | Vesicle induced receptor sequestration: a universal mechanism behind extracellular vesicle-based protein signaling?

Oskar Staufer[1]; Joachim Spatz[2]
[1]*University of Oxford, Oxford, United Kingdom;* [2]*Max Planck Institute for Medical Research, Heidelberg, Germany*

Introduction: Extracellular vesicles (EVs) are fundamental for proper physiological functioning of multicellular organisms. By shuttling nucleic acids and proteins between cells, EVs regulate a plethora of cellular processes. However, our mechanistic understanding concerning the biophysical principles underlying EV-based communication is still incomplete. Mechanisms explaining why and when cells apply EV-based communication and how EV might agument the signalling potency of proteins presented on their surface, are sought. In our study, we focuse on immune-modulatory protein ligands presented on EVs, aiming to eludciate any amplfying biophysical effects of the EV membrane on ligand-receptor interactions.
Methods: We apply an in vitro reconstitution approach to engineer fully-synthetic EVs based on vesicles released into T-cell immune synapses and between osteoblast and osteoclasts. This approach allows to prescicly control the biophysical properties of the EV mimetics. Based on this, we verify the proposed mechanism using natural EVs isolated from human primary T-cells and combine our analysis with in silico diffusion simulations.
Results: We identify vesicle-induced receptor sequestration (VIRS) as a potentially universal mechanism augmenting the signalling potency of proteins presented on EV-membranes. By bottom-up reconstitution of synthetic EVs, we show that immobilization of the receptor ligands FasL and RANK on EV-like vesicles, increases their signalling potential by more than 1000-fold compared to their soluble forms. Moreover, we perform diffusion simulations within immunological synapses to compare receptor activation between soluble and EV-presented proteins. By this we propose vesicle-triggered local clustering of membrane receptors as the principle structural mechanism underlying EV-based protein presentation.
Summary/Conclusion: We conclude that EVs act as extracellular templates promoting the local aggregation of membrane receptors at the EV contact site, thereby fostering inter-protein interactions. Our results uncover a potentially universal mechanism explaining the unique structural profit of EV-based intercellular signalling.

OF20.02 | Real-time single-molecule observation and analysis of the species-dependent interactions between extracellular vesicles (EVs) and recipient cells

Mette Malle[1]; Yan Yan[1]; Ping Song[1]; Philipp Löffler[2]; Jørgen Kjems[1]
[1]*Interdisciplinary Nanoscience Center, Aarhus University, Aarhus, Denmark;* [2]*Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Odense, Denmark*

Introduction: Extracellular vesicles (EVs) hold the potential as a native and multifunctional nanovesicle-mediated drug carrier. Both the drug loading strategy of EVs, and the single-EV level of characterization and tracking in live cells, are crucial for EV application in drug delivery. In this study, we optimized two different EV loading strategies: electroporation and liposome fusion, and characterized the difference of interactions between cross-species EVs and cells on a single EV level.
Methods: We isolate EVs from four different origins and three different species – mouse plasma, C2C12 cell culture, human plasma, human MSC cell culture, and bovine milk. The EVs were characterized following the MISEV 2018 guidelines. The siRNA loading efficiency was analyzed at the single-particle level using state-of-the-art TIRF microscopy. Then single-particle tracking of fluorescent-labeled EV in live cells (HEK293 cells, bEnd-3 cells, and ARPE-19 cells) was performed using HILO microscopy.

The data was analyzed by an in-house developed python script. The EVs were loaded with siRNAs against GADPH and the knockdown efficiency of GADPH in recipient cells was evaluated by QRT-PCR.
Results: In our results, milk EVs showed the highest loading efficiency of above 90% EVs loaded with siRNAs, while other EVs showed 70-80%. The interactions between individual EVs and cells are species/origin-dependent with different efficiency of EV co-localization with the cell membrane, uptake in the recipient cells, and co-localization with an endosome. Our data also supported that EVs can deliver siRNAs into recipient cells and knock down the gene expression with origin-dependency.
Summary/Conclusion: Here we demonstrate the origin/species dependency of EV interaction with recipient cells by our single EV tracking technique. The technique allows us to determine the mechanistic interspecies differences. We envision that this is an important step for specific EV-mediated therapeutic and native drug delivery.

OF20.03 | Syntenin-1 regulates the expression of adhesion proteins on small extracellular vesicles

Barnabas Irmer[1]; Antonio Egea-Jiménez[2]; Stéphane Audebert[2]; Luc Camoin[2]; Annalen Bleckmann[3]; Pascale Zimmermann[4]; Kerstin Menck[3]
[1]Dept. of Medicine A, Haematology, Oncology, and Pneumology, University Hospital Münster, Muenster, Germany; [2]Centre de Recherche en Cancérologie de Marseille (CRCM), Aix-Marseille Université, Marseille, France; [3]Dept. of Medicine A, Haematology, Oncology, and Pneumology, University Hospital Münster, Münster, Germany; [4]K. U. Leuven, Leuven, Belgium

Introduction: Tumour-derived small extracellular vesicles (sEVs) are known as potent promotors of tumour progression and metastasis. However, the attribution of such effects to specific sEV subpopulations remains challenging. Syntenin-1 (ST1) is a key regulator for the biogenesis of an endosomal-derived sEV subpopulation and overexpressed in many tumour entities. Moreover, ST1 contributes to the assembly of protein complexes at the membrane. Indeed, the PDZ domains of ST1 directly bind various transmembrane interactors like syndecans and tetraspanins supporting the formation of higher order signalling nexi. Therefore, this study aimed to characterize the ST1-dependent sEV protein signature and its functional relevance in breast cancer.
Methods: sEVs from murine wild type and ST1 knockout breast cancer cells (4T1) were isolated by serial ultracentrifugation and classified by proteomics and nanoparticle tracking analysis. Several differentially expressed proteins (DEPs) were selected for immunoblot validation, database screening and further functional analysis. Direct protein interaction was quantified via surface plasmon resonance (SPR) spectroscopy.
Results: Proteomic analysis revealed a subset of 52 ST1-dependent sEV marker proteins including direct ST1-binding partners like ALIX, CD63 and SDC4 as well as tumour-associated proteins with clinical impact on patient survival. Intriguingly, many of the downregulated DEPs on ST1-deficient sEVs were attributed to cell adhesion, extracellular matrix assembly and cell-cell contacts via pathway enrichment analysis. A ST1-dependent expression on sEVs was validated in 4T1 and human MCF 7 breast cancer cells for selected DEPs with such adhesive potential including the epithelial cell adhesion molecule (EpCAM). By SPR technology we show that EpCAM directly binds to ST1 via its PDZ-binding motif. Functionally, EpCAM seemed to act merely as a cargo protein without evident contribution to sEV biogenesis.
Summary/Conclusion: ST1 regulates the formation of a sEV subpopulation associated with cell adhesion. Thus, ST1-dependent sEVs might be potential drivers for enhanced tumour-derived sEV distribution and pre-metastatic niche formation in breast cancer by promoting the disappearance of adhesion complexes from cancer cells and their secretion in the matrix on sEVs.

OF20.04 | ER-membrane contact sites regulate biogenesis of RNA containing extracellular vesicles via VAP-A-CERT linkages

Bahnisikha Barman[1]; Bong Hwan Sung[1]; Evan Krystofiak[1]; Jie Ping[2]; Marisol Ramirez[2]; Bryan Millis[1]; Ryan Allen[2]; Nripesh Prasad[3]; Sergei Chetyrkin[1]; Wade Calcutt[1]; Kasey Vickers[1]; James G. Patton[1]; Qi Liu[2]; Alissa M. Weaver[4]
[1]Vanderbilt University, Nashville, USA; [2]Vanderbilt University Medical Center, Nashville, USA; [3]Discovery Life Sciences, Huntsville, USA; [4]Vanderbilt University School of Medicine, Nashville, USA

Introduction: Extracellular RNAs have emerged as a novel mechanism for cell-to-cell communication and drive many physiological and pathological processes including cancer. The major vehicle for transmission of these extracellular RNAs is extracellular vesicles (EVs). However, the underlying mechanisms by which these RNA-containing EVs are generated are poorly understood. Here, we identify VAP-A and CERT linkages at endoplasmic reticulum-endosome membrane contact sites (ER MCS) as key subcellular locations for the biogenesis of RNA-containing EVs.
Methods: We used bioinformatics, RNA-sequencing, lipidomic, confocal and transmission electron microscopy, tumor xenograft and various biochemical techniques to analyze EV biogenesis and cargo content in colon cancer cell lines.

Results: High-resolution imaging data indicated small RNA localization at ER-endosome membrane contact sites. RNA-Seq analysis revealed several small RNAs that are changed in VAP-A KD small and large EVs compared to control EVs. Density gradient fractionation revealed that VAP-A regulates a select subpopulation of small EVs that are enriched with RNA. Confocal microscopy data revealed that key RNAs are altered in multivesicular endosomes in VAP-A KD cells. Lipidomics analysis of small and large EVs revealed that VAP-A controls levels of ceramide, a key lipid involved in EV biogenesis. In addition, KD of the VAP-A binding ceramide transporter CERT led to a similar defect in EV biogenesis as that of VAP-A KD. Furthermore, imaging data revealed that the ceramide producing enzyme neutral sphingomyelinase 2 co-localizes with VAP-A positive ER membranes.
Summary/Conclusion: Altogether, we discovered that VAP-A-CERT linkages at ER membrane contact sites are critical for biogenesis of RNA-containing EVs. These data suggest a model in which ceramide transfer at ER MCS drives biogenesis of a select subpopulation of EVs containing RNA-RBP complexes. Beyond improving our understanding of EV biogenesis, we expect that these findings may be useful for exploring the functions of RNA-containing EVs.
Funding: Funding was provided by NIH grants U19CA179514 and PO1CA229123.

OF20.05 | Loading lysosomal enzyme, beta-glucocerebrosidase, onto exosomes via CD63

Biao Lu[1]; Annie Brown[2]
[1]*Department of Bioengineering, Santa Clara University, Santa Clara, USA;* [2]*Department of Bioengineering, Santa Clara, USA*

Introduction: Exosomes are cell-derived nano-sized extracellular vesicles that hold a great promise for targeted delivery of therapeutic proteins and enzymes. However, an exosome-based strategy to deliver preloaded enzymes into targeted intracellular action sites is still lacking. Here we describe a novel genetic method to produce enzyme-loaded exosomes for the targeted intracellular delivery and treatment of Gaucher disease, one of the most common lysosomal storage disorders.
Methods: We designed and synthesized a cohort of fusion genes composed of human β-glucocerebrosidase (GBA), green fluorescence protein, and tetraspanin CD63 in different configurations. We then cloned these fusion genes into mammalian vectors for the production of enzyme-loaded exosomes.
Results: Using transfection and confocal microscope, we show the successful incorporation of therapeutic GBA into pre-secreted exosomes in 293T cells. We also show that GBA-loaded exosomes are eventually released into culture medium. Isolation and characterizations of exosomes confirm the successful enzyme loading without significantly affecting vesicle size as compared to non-modified controls. Image study further demonstrates that addition of enzyme-loaded exosomes to the cultured human cells results in intracellular delivery of fusion enzymes to the endosomal compartments, the targeted therapeutic sites of GBA.
Summary/Conclusion: Genetic fusion of lysosomal enzyme with CD63 enables exosomal loading. This strategy is useful to produce exosome-based nano-medicine for the treatment of lysosomal storage disorders.
Funding: This work is supported by the National Institute of General Medical Sciences of the National Institute of Health under Award Number R15GM137449.

OF20.06 | EVs utilize a virus-like membrane fusion mechanism for cargo delivery

Mattia Morandi[1]; Petro Busko[1]; Efrat Ozer-Partuk[2]; Suman Khan[1]; Giulia Zarfati[3]; Yael Alon[1]; Neta Regev-Rudzki[1]; Ori Avinoam[1]
[1]*Weizmann Institute of Science, Rehovot, Israel;* [2]*Weizmann Institute of Science, rehovot, Israel;* [3]*Weizmann Istitute of Science, Rehovot, Israel*

Introduction: Extracellular vesicles (EVs) are essential in mediating cell – cell communication across physiological processes and pathologies, yet the mechanism by which EVs release their content into recipient cells remains a key open question. Enveloped viruses such as Influenza, HIV and VSV, utilize fusion between the viral- and cellular- membrane to deliver their genomes into the cytosol. This membrane fusion is mediated by glycoproteins on the viral enveloped known as fusogens, which are typically triggered by the low pH and environment of the endosome to mediate virus-to-host membrane fusion. Here, we tested the hypothesis that EVs release their cargo into the cytosol via pH-dependent membrane fusion and sought to identify the fusogens driving this process.
Methods: EVs samples were isolated from cell culture supernatant via density gradient ultracentrifugation. Membrane fusion of EVs and enveloped viruses was evaluated using an in vitro FRET-based membrane mixing assay. Transmission Electron Microscopy (Cryo-TEM) and Cryo Electron Tomography (Cryo – ET) were used to visualize the fusion process and resolve

the 3D ultrastructure of the EV-liposome interaction. CRISPR-Cas9 genome editing was used to delete CD9p-1, an EV protein from the immunoglobulin superfamily, to investigate its role in EV fusion.

Results: Our results show that EVs fuse with membranes mimicking the lipid composition of the late endosome in a pH dependent manner, reminiscent of enveloped viruses. Unlike most envelope viruses, exposure of EVs to low pH in the absence of target membranes did not result in their irreversible inactivation. Analysis of EVs mixed with liposomes by cryo-TEM and Cryo-ET revealed clear fusion intermediates only upon acidification. Finally, CRISPR-Cas9 mediated knockout (KO) of the immunoglobulin superfamily protein CD9-p1 in ovarian cancer cells resulted in up to 40% reduction in the EV fusion capability. CD9-p1 KO also results in lower EV biogenesis.

Summary/Conclusion: Our work suggests that EVs utilize a virus-like mechanism to transfer their cargo into the cytoplasm. Our results are consistent with a model wherein EV are produced in the low pH environment of the multivesicular bodies which would render them inactive. Upon secretion, exposure to neutral pH would prime the fusogen on the EV membrane. While the identity of the fusogen/s remain unknown, we identified a novel protein, CD9p-1, involved not only in the EVs cargo delivery process but also in EVs biogenesis, as it has been observed for virogenesis upon deletion of key viral proteins. These findings provide novel insight and shed a light on still obscure aspects of EVs biology and their interaction with recipient cells.

Funding: This research was supported by the Sergio Lombroso Program and by the Israel Science Foundation (ISF) Israel Precision Medicine Partnership program.

OF21: Therapies (ISEV-ISCT)

Chair: Sai Kiang Lim – Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore. Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

OF21.01 | EV proteomic signatures mirror the pro-inflammatory state of severe COVID-19 infection

Luisa Weiss[1]; Lara Boehm[1]; Paulina Szklanna[1]; Sarah cullivan[1]; Shane Comer[1]; Sarah Kelliher[2]; Caitriona Scaife[3]; Fionnuala Ní Áinle[2]; Barry Kevane[2]; Patricia Maguire[1]

[1]UCD Conway SPHERE Research Group, Conway Institute, University College Dublin, Ireland, Dublin, Ireland; [2]Department for Haematology, Mater Misericordiae University Hospital, Ireland, Dublin, Ireland; [3]Mass Spectrometry Core Facility, Conway Institute, University College Dublin, Ireland, Dublin, Ireland

Introduction: COVID-19 infection can present with a heightened inflammatory state and an increased thrombotic risk. Extracellular vesicles (EVs) are pro-coagulant and pro-inflammatory mediators of intercellular communication and frequently attributed to inflammation, however their role, if any, in COVID-19 remains poorly characterised. We hypothesised that circulating EV signatures differ between COVID-19 patients and healthy controls (HC) as well as COVID-negative (COVID-) general medicine (GM) patients.

Methods: Platelet poor plasma samples from COVID-19 positive (COVID+) patients requiring intensive care (severe) or hospital care (non-severe), as well COVID- general medical inpatients and healthy controls were enriched for EVs by ultrafiltration. Immunoblotting confirmed successful EV enrichment. Proteomic signatures were established by mass spectrometry.

Results: Quantitative proteomic profiling of circulating EV enrichments robustly quantified over 300 proteins. Strikingly, hierarchical clustering of EV protein levels distinctly separated all patient cohorts. Moreover, compared to HC, COVID+ patients displayed fundamentally increased expression of several highly proinflammatory proteins and complement factors. Intriguingly, EV protein levels clearly separated severe and non-severe COVID patients. Although GM patients were also hospitalised for pro-inflammatory conditions of the respiratory system, no differences in protein expression were observed between non-severe COVID+ and GM patients. Our data highlight that severe COVID-19 presents with a distinct inflammatory state and indicate that plasma EV protein signatures may be a useful tool in assessing the severity of COVID-19.

Summary/Conclusion: In conclusion, we have established that EV protein signatures mirror the pro-inflammatory state of COVID-19 patients and may be surrogate markers of COVID-19 severity. These findings may be of translational relevance towards characterising the underlying differences in severe and non-severe COVID-19 disease progression.

Funding: This study was funded by Science Foundation Ireland.

OF21.02 | Extracellular vesicles and soluble factors secreted by lung fibroblasts support alveolar epithelial organoid formation

Luke van der Koog[1]; Anika Nagelkerke[2]; Reinoud Gosens[1]

[1]Department of Molecular Pharmacology, University of Groningen, The Netherlands, Groningen, Netherlands; [2]Department of Pharmaceutical Analysis, University of Groningen, The Netherlands, Groningen, Netherlands

Introduction: COPD is one of the most common lung diseases, characterized by progressive and irreversible airflow limitation as a result of increased tissue destruction and defective tissue repair. Since current therapeutics do not alter disease progression, new therapies that pharmacologically reactivate lung repair are needed. Recently, the secretome of lung fibroblasts, consisting of Extracellular Vesicles (EVs) and other soluble factors (SF), has been associated with alveolar regeneration. We aimed to elucidate the supportive function of lung fibroblast (MRC5)-derived EVs and SF on the regenerative potential of alveolar epithelial progenitor cells in an organoid assay.

Methods: EVs and SF derived from MRC5 cells (~ 3.0 E+08 cells) were purified using ultrafiltration and size exclusion chromatography. EVs were characterized by a combination of nanoparticle tracking analysis, total protein analysis, dot blot analysis for different EV-markers (CD9, CD63, and CD81), and cryogenic transmission electron microscopy. Murine organoids were established by co-culturing 10,000 alveolar epithelial progenitor cells (EpCAM+/CD45-/CD31-) with 2,500 CCL206 lung fibroblasts in Matrigel. MRC5-derived EVs (1E+09 particles/ml) or SF (30 μg/ml) were added once on day 0 or consecutively for 14 days. On day 14 total number and size of the organoids formed was determined. Immunostainings were used to assess the number of differentiated alveolar organoids.

Results: Treatment with EVs or SF on day 0 significantly increased the number of organoids formed, i.e. a 29.50% ± 8.11% increase for EVs and 33.00% ± 20.34% for SF. Neither single treatment with EVs nor SF affected the size of organoids. Immunostaining for prosurfactant protein C revealed that the alveolar organoid count was significantly enhanced upon single treatment with EVs or SF (i.e. a 13.33% ± 5.01% increase for EVs and 13.50% ± 5.75% for SF). In addition, treatment with EVs or SF over the course of 14 days resulted in enhanced organoid count (i.e. a 58.17% ± 39.60% and 91.67% ± 33.28% increase respectively) and organoid size (i.e. a 36.50% ± 10.46% and 37.50% ± 27.02% increase respectively).

Summary/Conclusion: Both lung fibroblast-derived EVs and SF support the formation of alveolar epithelial organoids, making them an interesting potential regenerative treatment to further pursue for COPD.

OF21.03 | Development of bioengineered small extracellular vesicles as a novel vaccine against SARS-CoV-2

Hannah K. Jackson[1]; Heather M. Long[2]; Graham S. Taylor[2]; Lesley Young[3]; John Trowsdale[3]; Mathew Lo[3]; James R. Edgar[1]

[1]Department of Pathology, University of Cambridge, Cambridge. CB2 1QP. United Kingdom, Cambridge, United Kingdom; [2]Institue of Immunology and Immunotherapy, University of Birmingham, Birmingham. B15 2TT. United Kingdom, Birmingham, United Kingdom; [3]Exosis, Inc. Palm Beach, Florida. 33480. USA, Palm Beach, USA

Introduction: With the worldwide spread of emerging SARS-CoV-2 variants, it is critical to develop vaccines that can induce broadly protective responses with increased potency and durability. Small extracellular vesicles (sEVs), key intercellular communicators characterised by low toxicity and immunogenicity, offer a novel platform for effective SARS-CoV-2 vaccine development. sEVs containing multiple SARS-CoV-2 antigenic regions were tested for their ability to stimulate SARS-CoV-2 specific T cells.

Methods: FLAG tagged fusion constructs containing regions from SARS-CoV-2 proteins were cloned to scaffold proteins that are enriched in sEVs. Constructs were designed with multiple distinct sites on scaffolds accessible for inserts – allowing viral regions to be placed inside or outside sEVs. Constructs were confirmed by sequencing, and protein localisation verified by flow cytometry and immunofluorescence. sEVs were isolated by ultracentrifugation and characterised using western blotting, electron microscopy and nanoFCM. Immunogold electron microscopy confirmed antigen topology on sEVs. In vitro confirmation of sEV antigen processing was assessed by stimulation of T cells using sEV-exposed B cell lines.

Results: The expression of fusion proteins containing SARS-CoV-2 antigenic regions on or within sEVs, that are likely endosome-origin exosomes, did not alter steady-state trafficking of sEV proteins or yields. B cells exposed to bioengineered sEVs potently stimulated virus-specific T cell clones at significantly lower doses compared to control recombinant viral proteins.

Summary/Conclusion: We have developed a novel prototype exosomal vaccine against multiple SARS-CoV-2 antigens that is able to elicit potent T cell responses. The SARS-CoV-2 vaccine demonstrates the potential development of exosome-based vaccines to target multiple coronaviruses and novel vaccines to emerging pathogens.

Funding: Exosis, Inc

OF21.04 | Therapeutic effects of mesenchymal/stromal stem cells and their derived extracellular vesicles in rheumatoid arthritis

Dimitrios Tsiapalis[1]; Achilleas Floudas[2]; Tobias Tertel[3]; Verena Börger[3]; Bernd Giebel[3]; Ursula Fearon[2]; Lorraine O'Driscoll[4]
[1]School of Pharmacy and Pharmaceutical Sciences & Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland, Dublin, Ireland; [2]Molecular Rheumatology, Trinity College Dublin, Ireland, Dublin, Ireland; [3]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany; [4]School of Pharmacy and Pharmaceutical Sciences, Trinity Biomedical Sciences Institute, and Trinity St. James's Cancer Institute, Trinity College Dublin, Dublin 2, Ireland., Dublin, Ireland

Introduction: Rheumatoid arthritis (RA) is an autoimmune disease characterised by inflammation, progressive damage, and pain in joints. Currently available therapy is inadequate to alleviate the inflammation and reduce the joint damage. While the immune-regulatory effect of MSC-EVs has been tested in many diseases, little is known with respect to their effect on RA. Thus, we aimed to assess the effect of human MSCs and MSC-EVs on T cells and synovial fibroblasts (RASFs) of RA patients. The effect of EVs derived from MSCs primed with interferon beta (IFNb) was also assessed.
Methods: MSC-EVs were collected using a PEG precipitation, followed by ultracentrifugation-based protocol and were characterised via NTA, TEM, imaging flow cytometry (IFCM) and immunoblotting analysis. Immune-regulatory properties of MSCs and MSC-EVs were assessed on CD4+ T cells stimulated with CD3/CD28. Also, the effect of MSC-EVs on RASFs stimulated with TNFa was assessed.
Results: EVs from naïve and IFNb primed MSCs were prepared and all fulfilled MISEV2018 criteria as evaluated by NTA, TEM and immunoblotting. Additionally, IFCM confirmed the recovery of CD9+ and CD63+ small-sized EVs. Applied onto the stimulated CD4+ T cells, EV preparations from IFNb primed MSCs suppressed the expression of more inflammatory cytokines (GM-CSF, IL-2, IL-4 and TNFa; $p < 0.05$, in all cases) associated with the pathogenesis of RA. However, while MSCs suppressed T cell proliferation, all MSC-EVs had a tendency to increase numbers of T regulatory cells. Furthermore, MSC-EVs inhibited ($p < 0.05$) the migration of RASFs and reduced ($p < 0.05$) the expression of the RA surface markers HLA-DR and CD34.
Summary/Conclusion: Both MSCs and MSC-EVs exerted immune-regulatory effects on RA CD4+ T cells and MSC-EVs, but not the MSCs themselves, inhibited RASFs migration. The beneficial effect of MSC-EVs on RA derived T cells and RASFs was further enhanced by priming the MSCs with IFNb.
Funding: European Union's Horizon 2020 research and innovation action. Grant agreement No. 814495-EVPRO

OF21.05 | Mesenchymal stromal cell-derived extracellular vesicles attenuate metabolic changes in pre-clinical models of ARDS through mitochondrial transfer

Johnatas Silva[1]; Declan Doherty[1]; Catherine McClintock[1]; Daniel McAuley[1]; Cecilia O'Kane[1]; Anna D. Krasnodembskaya[2]
[1]Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Belfast, United Kingdom; [2]Wellcome-Wolfson Institute for Experimental medicine, Queen's Universiy Belfast, Belfast, United Kingdom

Introduction: Mesenchymal stromal cells (MSC)-derived extracellular vesicles (EVs) are being investigated as a therapy for acute respiratory distress syndrome (ARDS). Previously, we demonstrated that MSC EVs restore functional activity of the injured cells through mitochondrial transfer. However, the impact of this mechanism on the balance of oxidative phosphorylation and glycolysis is not known. Here we investigated how EV mitochondrial transfer modulates metabolic alterations in the primary human pulmonary cells exposed to LPS or ARDS plasma.
Methods: EVs were isolated from bone-marrow MSCs with normal or dysfunctional mitochondria by ultracentrifugation. Mitochondrial dysfunction in MSCs was induced by Rhodamine6G. EVs were characterized for number, size distribution, tetraspannin expression, and mitochondrial content. Primary human distal lung epithelial and endothelial cells, and monocyte derived macrophages were stimulated with LPS or plasma from ARDS patients and treated with EVs. Mitochondrial respiration and glycolytic flux were assessed by Seahorse metabolic analyser, barrier properties were assessed by xCELLigence, phagocytosis was assessed by flow cytometry. Also, single cell transcriptomic analysis was performed on mouse lungs in the in vivo LPS-induced lung injury model.
Results: Inflammatory stimulation resulted in pronounced reduction of mitochondrial respiration, increase in glycolysis and functional impairment in all cell types. MSC EVs isolated from normal MSCs inhibited glycolytic flux, restored mitochondrial respiration and cell function while mitochondria-depleted EVs were not effective. Analysis of the single cell seq data showed that MSC EVs administration regulates expression of essential genes involved in mitochondrial metabolism in vivo.
Summary/Conclusion: MSC EVs alleviate ARDS-induced metabolic alterations via transfer of healthy mitochondria.
Funding: Horizon-2020-MSCA-IF ALGORITM 895134 to JS, MRC UK MR/R025096/1 and MR/S009426/1 to AK

OF21.06 | Effects of GMP-grade, mesenchymal stromal cell-derived extracellular vesicles on the development of fibrosis and on pulmonary epithelial function in a rat model of bronchopulmonary dysplasia

PAOLA BISACCIA[1]; FABIO MAGAROTTO[1]; Stefania D'Agostino[2]; Marcin Jurga[3]; Eugenio Baraldi[2]; Andrea Porzionato[4]; Maurizio Muraca[5]; Michela Pozzobon[6]

[1]Department of Women and Children Health, Università di Padova, Italy, PADUA, Italy; [2]Department of Women and Children Health, Università di Padova, Italy, Padua, Italy; [3]ExoBiologics, Belgium, Niel, Belgium; [4]Department of Neuroscience-DNS, Università di Padova, Italy, PADUA, Italy; [5]Department of Women's and Children's Health, University of Padova, Padua, Italy., Padova, Italy; [6]Department of Women's and Children's Health, University of Padova, Padua, Italy. Fondazione Istituto di Ricerca Pediatrica Città della Speranza, Padua, Italy, Padova, Italy

Introduction: Mesenchymal stromal cells-derived extracellular vesicles (MSC-EVs) can prevent the development of Bronchopulmonary Dysplasia (BPD) in animal models and in preliminary clinical trials. We investigated the effects of MSC EVs on the development of fibrosis and on some functions of lung epithelial cells in an animal model of hyperoxia-induced BPD.
Methods: Good Manufacturing Practice-grade (GMP-grade) EVs were produced by human Wharton-Jelly derived MSCs (Exo Biologics, Belgium) isolated by tangential flow filtration (TFF) and characterized according to MISEV2018. Rat pups were divided in 3 groups: normoxia + PBS vehicle, hyperoxia with PBS, hyperoxia with MSC EVs in PBS. Both PBS and EVs were injected intratracheally (IT) on days 3, 7 and 10 and pups were sacrified on day 14. It was evaluated the expression of the genes involved in fibrosis pathways such as TGFβ1 and alpha-SMA. To evaluate epithelial secretory function, the expression of glycosaminoglycans (Alcian blue staining) and of surfactant protein C (SFTPC) was analyzed by immunohistochemistry and immunofluorescence. Collagen deposition was assessed by Sirius Red staining. Macrophages from bone marrow were cultured and analyzed for alpha-SMA and CD90 expression by flow cytometry after TGFβ1 treatment.
Results: Pups under hyperoxia exhibited an increase both in collagen deposition and in the expression of pro-fibrotic genes in the lungs. Both parameters were reduced by treatment with MSC EVs. Both Glycosaminoglycan and SFTPC lung content were significantly increased in MSC EV-treated rat pups compared to untreated animals. In vitro, MSC EVs suppressed the induction of alpha-SMA in macrophages.
Summary/Conclusion: Intratracheal administration of clinical-grade MSC-EVs counteracts the development of fibrosis and improve pulmonary epithelial function in a neonatal model of hyperoxia-induced lung injury. These results can contribute to unraveling the mechanism of action of these nanoparticles in preventing the development of BPD.

OF22: Microbe Human Host Communication

Chair: Ana Claudia Torrecilhas – Federal University of São Paulo

Chair: Antonio Marcilla – Universitat de Valencia

OF22.01 | Visualizing Transfer of Microbial Biomolecules by Outer Membrane Vesicles in Microbe-Host-Communication In Vivo

Stefan Momma
Goethe University Frankfurt, Frankfurt, Germany

Introduction: The intestinal microbiota influences mammalian host physiology in health and disease locally in the gut but also in organs devoid of direct contact with bacteria such as the liver and brain. Extracellular vesicles (EVs) or outer-membrane vesicles (OMVs) released by microbes are increasingly recognized for their potential role as biological shuttle-systems for inter-kingdom communication. However, physiologically relevant evidence for the transfer of functional biomolecules from the intestinal microbiota to individual host cells by OMVs in vivo is scarce.
Methods: By introducing Escherichia coli engineered to express Cre-recombinase (E. coliCre) into mice with a Rosa26.tdTomato-reporter background, we leveraged the Cre-LoxP system to report the transfer of bacterial OMVs to recipient cells in vivo.
Results: Colonizing the intestine of these mice with E. coliCre, resulted in Cre-recombinase induced reporter gene-expression in cells along the intestinal epithelium, including intestinal stem cells as well as mucosal immune cells such as macrophages. Furthermore, even far beyond the gut, bacterial-delivered Cre induced extended marker gene expression in a wide range of host tissues, including the heart, liver, kidney, spleen, and brain.
Summary/Conclusion: Together, our findings provide a method and proof of principle that OMVs can serve as a biological shuttle system for the horizontal transfer of functional biomolecules between bacteria and mammalian host cells.

OF22.02 | Extracellular weapons against bacterial infection: protective roles of antigen-encapsulating extracellular vesicles derived from Salmonella-infected macrophages

Lisa E. Emerson[1]; Winnie Hui[2]; Hailey Barker[1]; Carol Hoffman[1]; Mark Ou[1]; Beata Clapp[1]; Gustavo Maegawa[3]; David Pascual[1]; Mariola J. Edelmann[1]
[1]*University of Florida, Gainesville, USA;* [2]*Nanostring, Boston, USA;* [3]*Columbia University, New York, USA*

Introduction: Salmonella Typhimurium is a causative agent of nontyphoidal salmonellosis, for which there is a lack of a clinically approved vaccine in humans. As an intracellular pathogen, Salmonella impacts many cellular pathways, which may play a role in the immunity towards this pathogen. However, the intercellular communication mechanism facilitated by host-derived small extracellular vesicles (sEVs) is an overlooked aspect of the host responses to this infection.
Methods: We used a proteome-based network analysis of proteins associated with sEVs derived from Salmonella-infected macrophages. Next, we performed in vitro and in vivo studies using BALB/c mice to identify the role of sEVs in adaptive immunity against the challenge with Salmonella.
Results: Our bioinformatics-based analysis of vesicular proteins generated by infected macrophages predicted that the host-derived sEVs generated during macrophage infection stimulate macrophages and promote activation of T helper 1 (Th1) cell activation. We also identified that sEVs generated during infection contain Salmonella proteins, including specific antigens that were previously shown to stimulate protective immune responses against Salmonella in vivo. Furthermore, we showed that sEVs generated by infected macrophages stimulate a mucosal immune response against Salmonella infection when delivered intranasally to BALB/c mice, a route of antigen administration known to initiate mucosal immunity. Specifically, sEVs stimulated the production of anti-Salmonella IgG and IgA antibodies. These sEVs also lead to an antigen-specific cell-mediated immunity. In particular, splenic mononuclear cells isolated from mice administered with sEVs derived from Salmonella-infected macrophages increased CD4+ T cells secreting Th1-type cytokines if restimulated with Salmonella antigens. Moreover, sEVs led to a reduced bacterial burden in organs in the challenge study and increased survival of the animals.
Summary/Conclusion: Our results demonstrate that sEVs formed by macrophages during infection contribute to Th1 cell bias in the anti-Salmonella responses. Collectively, we unraveled the novel role of sEVs as vehicles transmitting antigens to induce Th1-type immunity against Gram-negative bacteria. Understanding the sEVs-mediated defense mechanisms will allow the development of future approaches to combat intracellular bacterial infections.
Funding: R03 AI135610-02

OF22.03 | Antiviral activity of human epithelial cell extracellular vesicles (EVs) is mediated by EVs-associated interferons

Tiziana Corsello-Gorgun; Yue Qu; Teodora Ivanciuc; Antonella Casola; Roberto Garofalo
Department of Pediatrics, Division of Clinical and Experimental Immunology and Infectious Diseases, Galveston, USA

Introduction: Respiratory syncytial virus (RSV) is a major cause of acute lower respiratory tract infections in children and elderly. Extracellular vesicles (EVs) are microvesicles known to carry biologically active molecules, including RNA, DNA and proteins. EVs have the potential to modulate cellular responses of recipient cells following a variety of stimuli. Viral infections can induce profound changes in EV composition, but EV contribution to the replication and pathogenesis of respiratory viruses is largely unknown. In this study, we investigated the composition/function of EVs released from airway epithelial cells infected with RSV and tested whether RSV- EVs affect the antiviral response of recipient cells.
Methods: EVs were isolated from uninfected (control-EVs) and RSV infected (RSV-EVs) A549, a human alveolar type II–like epithelial cell line, or from primary small airway epithelial (SAE) cells. EVs were purified using a two-step enrichment procedure and characterized using particle sizing (size and concentration) and Western blot for the EV markers. Recipient A549 and SAE cells were cultured for 24 hours in the presence or absence of control-EVs or RSV-EVs, then infected with RSV for 24 hours. Viral titers by plaque assay were measured in recipient infected cells. IFNs and cytokine assays were used to detect their levels in EVs and in recipient cells.
Results: Recipient cells, both A549 and SAE cells, treated with RSV-EVs showed significantly lower viral replication than control EV-treated cells in response to RSV infection, indicating that EVs released from A549 and SAE cells infected with RSV (RSV-EVs) can transfer antiviral activity to infected neighboring cells. In addition, significant amount of immuno-reactive interferon beta (IFN-β) and lambda (IFN-λ) were detected by ELISA in preparations of isolated RSV-EVs. To test the bioactivity of IFNs associated with RSV-EVs, recipient cells were treated with Cerdulatinib, an IFN receptor signaling inhibitor. Following RSV infection, increased viral replication was observed in recipient cells treated with Cerdulatinib and RSV-EVs compared to recipient cells treated with RSV-EVs alone, suggesting that IFNs carried by epithelial EVs are indeed biologically active.

Summary/Conclusion: No vaccine or effective treatment is currently available for RSV. Cellular pretreatment with RSV-EVs reduced the RSV replication in airway epithelial recipient cells, suggesting that could exert its antiviral activity in the context of RSV infection potentially through modulation of EV and IFNs mediated mechanism. Therefore, RSV-EVs could represent a future novel pharmacological approach for ameliorating virus-induced lung disease.

OF22.04 | Are microbiota-derived outer membrane vesicles promoters of inflammation and neurodegeneration in Parkinson's disease?

Tiana F. Koukoulis[1]; Leah C. Beauchamp[2]; Adityas Purnianto[1]; Victoria A. Lawson[3]; Neil M. O'Brien-Simpson[4]; Maria Kaparakis-Liaskos[5]; David I. Finkelstein[1]; Kevin J. Barnham[1]; Laura J. Vella[1]

[1]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Australia, Melbourne, Australia; [2]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, VIC, Australia, Parkville, Australia; [3]Department of Microbiology and Immunology, The Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Parkville, Australia, Melbourne, Australia; [4]Centre for Oral Health Research, Melbourne Dental School, The University of Melbourne, Parkville, Australia, Melbourne, Australia; [5]Department of Microbiology, La Trobe University, Bundoora, Australia, Melbourne, Australia

Introduction: The microbiota-gut-brain axis is beginning to take centre stage in Parkinson's disease (PD) research, as the spotlight becomes fixed on microbiotic dysbiosis as a facilitator and driver of PD pathogenesis. It is understood that the gut microbiota promotes local, systemic and neural inflammation, which can promote neurodegeneration in PD, however how bacteria influence these processes remains unclear. We propose that Outer membrane vesicles (OMVs) released from Gram-negative bacteria are a mediators of PD, due to their immune stimulatory composition, including containing potent lipopolysaccharides (LPS) and capability to impact gastrointestinal permeability and influence global inflammation.
Methods: OMVs isolated from bacterial culture or murine faeces were characterized by density, size, morphology, LPS content and ability to stimulate immune cells in vitro. OMVs from bacterial culture or an equivalent dose of LPS were orally administered to a PD model mouse to determine if OMVs contribute to the acceleration and exacerbation of PD inflammation, and gastrointestinal and neurological dysfunction.
Results: Escherichia coli OMVs exacerbated dysfunction in the gut of PD model mice evidenced by reduced faecal pellet output and increase of some proinflammatory cytokines. OMVs also promoted motor dysfunction in PD model mice and accelerated neurodegeneration. These results indicate OMVs can influence gastrointestinal and neuronal pathology and function.
Summary/Conclusion: This is the first study to demonstrate that orally administered OMVs promote inflammation, behavioural dysfunction and neurodegeneration in a PD mouse model, and thus could contribute to the pathogenesis of PD. The greater implications of this work include drawing functional connections between microbiotic dysbiosis, inflammation and the gut-brain axis, which is relevant to PD, but also many other neurodegenerative disorders.

OF22.05 | The human gut bacteria Bacteroides thetaiotaomicron releases extracellular vesicles containing proteins that influence host cell physiology and metabolism

Regis Stentz; Emily Jones; Rokas Juodeikis; Andrew Goldson Goldson; Arlaine Brion; Catherine Booth; Simon Carding

Quadram Institute, Norwich, United Kingdom

Introduction: It is increasingly apparent that bacterial extracellular vesicles (BEVs) produced by members of the intestinal microbiota contribute to microbe-host cell interactions. Unresolved questions are, what is the nature of the cargo packaged into BEVs and how do they impact on host cell function? Here we analyzed and compared the proteome of BEVs produced by the major human gut symbiont Bacteroides thetaiotaomicron (Bt) produced in vitro and in the mouse intestine and identified proteins that are exclusively enriched in BEVs produced in vivo suggesting that their increased abundance is induced by host-related factors.
Methods: In vitro conditions consisted of cultures of Bt grown in a complex medium and in vivo studies consisted of orally administering Bt to germfree mice and collecting cecal contents 3 days later. Vesicles from cultures and cecal contents were concentrated by crossflow filtration, separated by size exclusion chromatography (SEC) and recovered by ultracentrifugation followed by protein extraction. Differential expression of BEV proteins obtained under different conditions were explored using tandem mass tagging (TMT) combined with liquid chromatography mass spectrometry (LC-MS/MS). Data sets were analyzed using the Proteome Discoverer v2.1 software. Enzyme assays were performed using intact BEVs fractionated by SEC and obtained from the same conditions.

Results: We identified 102 proteins highly enriched in BEVs in vivo of which the majority (66/102) were enriched independently of their expression in parental cells. These abundantly BEV-secreted proteins included a dipeptidyl-peptidase IV (CD26) and an asparaginase and the highly efficient degradation of substrates by intact BEVs derived from the mouse caecum was demonstrated. The potential significance and impact of these activities on host physiology will be discussed.
Summary/Conclusion: These findings provide new insights into the role BEVs play in microbiota-host interactions with their contents capable of playing key roles in the maintenance of intestinal homeostasis and host metabolism.
Funding: This work was supported in part by the UK Biotechnology and Biological Sciences Research Council under grant numbers BB/J004529/1, BB/R012490/1, and BBS/E/F000PR10355 (SC).

OF22.06 | Pseudomonas aeruginosa extracellular vesicles upregulate NFkB inflammatory pathway via cystic fibrosis transmembrane conductance regulator protein

Andrea L. Hahn[1]; Aszia Burrell[2]; Kylie I. Krohmaly[3]; Claire Hoptay[2]; Robert J. Freishtat[1]
[1]Children's National Hospital, Washington, USA; [2]Children's National Research Institute, Washington, USA; [3]George Washington University, Washington, USA

Introduction: Cystic fibrosis (CF) is an autosomal recessive disease caused by dysfunction of the CF transmembrane conductance regulator (CFTR) gene. Persons with CF suffer from recurrent and chronic lung infections, with Pseudomonas aeruginosa (Pa) being one of the most impactful on lung inflammation and disease progression. One particular virulence factor carried by Pa extracellular vesicles (EVs), Cif, induces lung inflammation through modification of CFTR expression and induction of the NFkB signaling pathway. We hypothesized that Pa EVs would induce increased NFkB signaling in wild-type (WT) compared to CF human bronchial epithelial (HBE) cells due to increased presence of CFTR protein.
Methods: EVs were isolated from the sputum supernatants of persons with CF using precipitation and size exclusion chromatography (1 mL starting volume). Pa antibodies were attached to releasable magnetic beads to isolate Pa-specific EVs. Life extended WT and CF HBE cells (F508del/F508del) were grown to 80% confluency, and equivalent volumes of Pa EVs (250 uL) were added and incubated for 22 hours before cell harvesting. Cells were immediately lysed with TRIzol and RNA extracted with a DirectZol MiniPrep kit. RNAseq was performed using NextSeq and HiSeq. A Galaxy workflow incorporating HISAT2, Stringtie, Gffcompare, featureCounts, and DESeq2 was used to determine differential gene expression. Ingenuity pathway analysis (IPA) was used to identify differences in canonical pathways.
Results: RNA quality assessment showed RIN values near 10, with an average of 35 million reads per sample (range 14-55 million). A total of 246 transcripts were differentially abundant between CF and WT HBE cells after Pa EV exposure with an unadjusted p-value < 0.05, which were imported into IPA. Importantly, NFkB signaling was significantly upregulated (Z-score 2.12, genes BMP4, CSNK2B, FGFR3, IL1A, LTBR, MAP2k7, PRKACA, RELA), while interleukin (IL)-6 and IL-8 signaling pathways also trended toward upregulation in WT HBE cells compared to CF HBE cells.
Summary/Conclusion: NFkB, IL-6, and IL-8 inflammatory signaling pathways were increased in WT compared to CF HBE cells, suggesting CFTR expression is an important variable in Pa EV induced inflammation.
Funding: NIH NCATS UL1TR001876

Oral Presentation

OS23: Engineered EVs

Chair: Marie Morille – ICGM, Montpellier University, CNRS, ENSCM, Montpellier, France

Chair: Minh T.N Le – Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

OS23.01 | Nano-transfection for on demand cellular packaging of extracellular vesicles in therapeutic delivery

Nina Erwin[1]; Andrew Brock[1]; Thomas Schmittgen[1]; Mei He[2]
[1]Department of Pharmaceutics, College of Pharmacy, University of Florida, Gainesville, USA; [2]Department of Pharmaceutics, College of Pharmacy, University of Florida, GAINESVILLE, USA

Introduction: Extracellular vesicles (EVs) are the emerging alternative as the therapeutic delivery and vaccine platform. However, low efficiency on EV cargo loading and cellular secretion remains the biggest roadblock. Therefore, we developed a "smart"

polymeric micelles as the first nanoparticle-based cellular transfection tool for enhancing the site-specific packaging of EVs with desired cargoes.

Methods: The PCL-PEG functionalized polymeric micelles have been designed and synthesized for carrying therapeutic peptides/drugs via photo-cleavable chemical groups and self-assembled as nanoparticles. The human dendritic cells were used for fast uptaking which allows the internalization of carried cargos. Owing to the conjugated photo-cleavable group, the photo-controlled release of cargos are enabled on demand for releasing carried cargos into endocytic pathway specifically, which is subsequently the precursor for packing EVs and secreting into extracellular environment. The characterization of synthesized nanoparticles has been confirmed by NMR and Dynamic Light Scattering (DLS) analysis. The secreted EVs have been characterized by nanoparticle tracking analysis in terms of size, concentration, and zeta potential, NanoView surface marker profiling, as well as the SEM and TEM for morphological study.

Results: Compared to conventional transfection of cargo, the small hydrodynamic size and uniform spherical morphology has been observed from synthesized nanoparticles allowing for rapid cellular uptake and internationalization. Here we used the tumor antigenic peptide gp-100 as the model cargo. The time elapse confocal imaging of internalization and cargo packing demonstrated the cargo release into the endocytic pathway for forming the secreted EVs. Such highly efficient cellular transfection is specific to endocytic pathway, which provides a new solution for EV therapeutic cargo loading. The entire cellular transfection and EV loading protocol is simple by adding polymeric micelle nanoparticles into culture medium and treat with 365nm light at the incubation time of 30-60 minutes, which is compatible with conventional cell culture system for scaling up and GMP manufacturing without special facility setup. The further investigation on therapeutic function in vivo is underway.

Summary/Conclusion: We introduce a viable nanotransfection method for promoting cellular production of cargo loaded EVs via the endocytic pathway. The photo-controlled release strategy during the cell culture is simple and effective, which is desirable for large-scale EV cellular manufacturing.

Funding: NIH NIGMS MIRA award 1R35GM133794

OS23.02 | In situ EV engineering for potent biotherapeutics delivery in vivo

Dhanu Gupta[1]; Samir El Andaloussi[2]

[1]*Department of laboratory medicine, Karolinska Institutet, Stockholm, Sweden;* [2]*Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, Huddinge, Sweden*

Introduction: The unique properties of extracellular vesicles (EVs), such as biocompatibility and the ability to cross biological barriers, render EVs a next-generation drug delivery tool. Therapeutic EV research has seen tremendous development in the past decade, from in vitro studies towards pre-clinical models to various clinical trials. Even so, the road towards successful clinical translation has various hurdles such as manufacturing and fast plasma clearance of exogenously delivered EVs. Here we have utilised gene therapy modalities to engineer cells in vivo to utilise endogenously secreted EVs to deliver biotherapeutics.

Methods: For the generation of in situ engineered EVs in vivo, the EV engineering transgenes were delivered either as an mRNA or DNA using LNPs or in adeno-associated viruses. Importantly, these delivery vectors have tropism to the liver and in part to the spleen. Therefore the in situ produced engineered EVs primarily have a hepatic origin. For tracking and assessing pharmacokinetics in situ engineered EVs in vivo, endogenous bioluminescent (BL) EV labelling strategies were used. The therapeutic application of this platform was evaluated in an in vivo intestinal inflammation model which mimics Crohn's disease.

Results: BL tagging of in situ EVs showed a body-wide distribution of EVs to all major organs, including hard-to-reach tissues such as CNS and muscle. This system could achieve a sustained release of engineered EVs in circulation and showed a dramatic increase in pharmacokinetic profile over exogenously administered EVs. For therapeutic applications, using the liver as a bio factory, in situ engineered EVs potently delivered an anti-inflammatory protein to alleviate intestinal inflammation in an in vivo disease model.

Summary/Conclusion: By harnessing the latest innovations in nucleic acid therapy, this approach utilises the power of both synthetic and natural drug delivery vectors for efficient biotherapeutics delivery to hard-to-reach organs.

OS23.03 | YAP/TAZ facilitates extracellular vesicle release upon mechanical stimulation

René Weiss[1]; Felix Nägele[2]; Tanja Eichhorn[1]; Viktoria Weber[1]; Johannes Holfeld[2]

[1]*Department for Biomedical Research, Center for Biomedical Technology, Danube University Krems, Krems, Austria, Krems, Austria;* [2]*Department of Cardiac Surgery, Medical University of Innsbruck, Austria, Innsbruck, Austria*

Introduction: The physical stimulus of shockwave therapy (SWT) has a pro-angiogenic impact on ischemic tissue, representing a promising regenerative approach. The Hippo signaling pathway YAP/TAZ plays a key role in angiogenesis and can be regulated

by mechanical signals. Both SWT stimulation and YAP/TAZ activation cause a release of extracellular vesicles (EVs). We aim to substantiate the mechanotransduction of SWT via YAP/TAZ facilitated EV release and subsequent angiogenic response.
Methods: Human umbilical vein endothelial cells (HUVECs) were stimulated with 300 impulses at a frequency of 3 Hz and an energy flux density of 0,1mJ/mm2. 4h thereafter, mRNA expression of YAP/TAZ target genes (ANKRD1, CYR61) was measured and the nuclear localization of YAP/TAZ was examined by immunofluorescence. The culture supernatant was collected. EV release was characterized by flow cytometry (Cytoflex LX calibrated with fluorescent silica beads) using the membrane dye Cell Mask Orange (CMO) and tetraspanin markers CD63 and CD81. Furthermore, EVs were analyzed by a bead-based flow cytometry assay with CD63-coupled magnetic beads.
Results: SWT of HUVECs resulted in a higher concentration of CMO+ EVs (9,953±2,039 vs.6,092±1,503 EVs/μl) in the culture supernatant as compared to the untreated control. This was confirmed by a higher percentage of EV-decorated beads after SWT, accompanied by higher mRNA expression of YAP/TAZ target genes ANKRD1 ($p=0.0005$, respectively) and CYR61 ($p=0.0006$, respectively). Immunofluorescence staining showed nuclear translocation of YAP/TAZ upon SWT compared to untreated controls. These effects were abolished and the EV concentration was decreased upon pharmacological inhibition of YAP/TAZ nuclear translocation.
Summary/Conclusion: The mechanical stimulus of SWT activates the Hippo-Pathway YAP/TAZ and causes EV release. Our study provides substantial insight into cellular mechanotransduction and serves as the basis for translation into clinical practice.

OS23.04 | Engineering extracellular vesicles for embryo implantation

Qi Hui Poh; Alin Rai; David W. Greening
Baker Heart and Diabetes Institute, Melbourne, Australia

Introduction: A major cause of infertility is failure of the embryo implanting to the maternal endometrium. This process of implantation requires precise coordination between the endometrium and embryo and is mediated, in part, by extracellular vesicles (EVs) released by the outer layer of the embryo (trophectoderm). Hence, engineering nanovesicles that recapitulate trophectoderm EV function is an attractive therapeutic strategy to improve embryo implantation and reproductive success.
Methods: Human stem cell-derived trophectoderm cells (Tsc) were serially extruded (10, 5, 1 μm) to generate nanovesicles (NVs), purified using density-based separation (6.5 x 105 cells, 1.13g/cm2), characterized using cryo-electron microscopy, single particle tracking analysis, and quantitative proteomics, and functionally assessed for attachment of human trophectoderm spheroids and mouse embryo outgrowth post-hatching from the zona pellucida.
Results: Tsc-NVs were spherical in morphology, ~150 nm in diameter, transferred to endometrial cells (24 h), and significantly improved trophectoderm spheroid attachment to low receptive human endometrium (HEC1A) cells ($p< 0.05$, $n=5$) and mouse embryo outgrowth on fibronectin matrix, similar to native trophectoderm EVs. Using quantitative proteomics, nanovesicle transfer reprogrammed low receptive endometrial cell proteome landscape, upregulating expression of factors (ALPP, CD44, ITGA2, ITGB1, PHB, TNC) and molecular pathways (non-integrin membrane-ECM interactions, ECM proteoglycans) involved in embryo implantation and endometrial remodeling and receptivity. To enhance therapeutic efficacy, engineered nanovesicles can also be modified to encapsulate pro-implantation factors, including epidermal growth factor (EGF).
Summary/Conclusion: Therefore, this strategy of nanovesicle generation is potentially a highly scalable and modifiable therapeutic approach for personalized reproductive medicine.
Funding: This research was supported by National Health and Medical Research Council Project Future Fund grant 1201805 (to D.W.G). We note further support by Helen Amelia Hains Fellowship (to D.W.G), and by the Victorian State Government Operational Infrastructure funding to the Baker Institute. QHP is supported by a joint Baker Institute-La Trobe University Research Training Program Scholarship.

OS23.05 | Increase the anti-inflammatory efficacy of small extracellular vesicles through glycoengineering

Wenyi Zheng[1]; Xiuming Liang[1]; Rui He[2]; Jeremy Bost[3]; Samir El Andaloussi[1]
[1]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, Huddinge, Sweden; [2]Karolinska Institutet, Huddinge, Sweden; [3]Department of laboratory medicine, Karolinska Institutet, Stockholm, Sweden

Introduction: Small extracellular vesicles (EVs) from immunomodulatory cells, such as mesenchymal stem cells, are potential therapeutics for inflammatory disorders, while the efficacy thereof is limited by fast elimination from circulation as well as low abundance at target tissues. Endothelial cells at inflammatory sites typically upregulate E-selectin and other adhesion molecules

to facilitate leukocytes infiltration via binding to the surface glycan sialy Lewis-X (sLeX).In this study, we aim to boost the anti-inflammatory efficacy of small EVs by displaying the glycan ligand sLeX.

Methods: EV source cells, primarily HEK-293T cells, were genetically engineered in a modular manner to display the glycan sLeX on EV surface. Small sLeX-EVs were harvested and characterized in terms of ligand expression by single-vesicle flow cytometry, size distribution, affinity to recombinant E-selectin protein, cellular uptake by endothelial cells, and circulation time in mouse.

Results: Through simultaneous expression of fucosyltransferase 7 and a modified glycoprotein based on the EV scaffold protein CD63, sLeX was successfully displayed on source cells as well as on small EVs. The small sLeX-EVs had typic spheric shape and size distribution as exosome, like their counterpart without glycoengineering. In difference, only small sLeX-EVs displayed increased uptake in activated murine as well as human endothelial cells. Moreover, displaying the glycan sLeX extended the circulation time of small EVs in multiple mouse models.

Summary/Conclusion: Displaying the glycan sLeX on small EVs through genetic engineering achieved higher uptake by endothelial cells in inflammatory state and retention in mouse plasma. The next steps involve using immunomodulatory mesenchymal stem cell as the source cell and examining the anti-inflammatory functions of sLeX-EV.

Funding: S.E.-A. is supported by H2020 EXPERT, SSF-IRC, ERC CoG, and SSMF. S.E.-A. is a founder, consultant, and stock holder of EVOX Therapeutics.

OS24: Biomarkers of Neuronal Diseases

Chair: Christian Neri – Institute of Biology Paris-Seine (IBPS), Sorbonne University, CNRS UMR 8256, ERL INSERM U1164

Chair: Julie A. Saugstad – Oregon Health & Science University

OS24.01 | The effect of exercise on the plasma extracellular vesicle proteome of myalgic encephalomyelitis/chronic fatigue syndrome

Ludovic Giloteaux; Maureen Hanson
Cornell University, Ithaca, USA

Introduction: ME/CFS is a debilitating disease of unknown etiology lasting for 6 months or more, featuring fatigue, cognitive impairment, myalgias, and immune system dysfunction. These symptoms are exacerbated upon physical or mental activity, a phenomenon known as post-exertional malaise. EVs are known to be released following exercise and their cargo could be involved in cell-to-cell signaling that results in abnormalities in patients' immune function and metabolism at baseline, but particularly after exertion. We aimed to characterize the content of EVs in ME/CFS and controls before and after exercise.

Methods: Blood samples were collected from 18 female with ME/CFS and 18 female controls before, 15 minutes and 24h after a cardiopulmonary exercise test. EVs were isolated from plasma by SEC and characterized by NTA,TEM and immunoblotting. An LC-MS/MS based shotgun proteomics analysis strategy was used to perform longitudinal proteomic profiling of EVs. Linear mixed models assessed differences between groups and changes in response to exercise at each time point along with gene ontology (GO), pathway enrichment and protein-protein interaction network analysis. Linear regressions were applied to find analytes associated with physiological parameters.

Results: Morphological analysis showed a homogeneous population of vesicles in both groups with no significant differences of size at the 3 time points. ME/CFS individuals had significantly higher levels of EVs at baseline and post exercise. There was a significant increase of EVs in the control group post exercise not observed in patients. EV proteomics analysis identified of 886 proteins, including 164 and 95 unique to the controls and ME/CFS subjects respectively. Several proteins were found to be significantly and consistently dysregulated at the 3 time points, while other were only dysregulated during recovery (15min. and 24h). GO and pathway enrichment analysis of differentially expressed proteins evidenced redox imbalance in ME/CFS, disruption of the ephrin signaling pathway and dysregulation of carbon metabolism. Furthermore, we found EV proteins that were solely associated with peak oxygen consumption in the ME/CFS group post-exercise.

Summary/Conclusion: The abundance of EVs changes following exercise and the protein cargo of EVs differs at each time point and between cases and controls. This work demonstrates that patients and controls differ in their response to exercise with regard to EV proteins involved in immune response, energy metabolism, muscle contraction, and axon guidance. The findings of this study may open new windows to reveal ME/CFS pathogenic mechanisms and may aid in the development of better biomarkers.

Funding: This project was funded by NIH NNINDS/OD/NIDA/NHLBI/NHGRI through NINDS U54NS105541 to the Cornell Center for Enervating Neuroimmune Disease.

OS24.02 | Single-vesicle analyses validate L1CAM as a marker of blood-borne neuron-derived extracellular vesicles

Carlos J. Nogueras-Ortiz[1]; Pamela Yao[1]; Liz Calzada[1]; Erden Eren[1]; Christopher Dunn[2]; Olga Volpert[3]; Francheska Delgado-Peraza[1]; Maja Mustapic[1]; Christopher Ramsden[1]; Erez Eitan[3]; Dimitrios Kapogiannis[4]
[1]Laboratory of Clinical Investigation, National Institute on Aging, Baltimore, USA; [2]Flow Cytometry Unit, National Institute on Aging, Baltimore, USA; [3]NeuroDex Inc., Natick, USA; [4]National Institutes of Health, Baltimore, USA

Introduction: The neuronal cell adhesion molecule L1CAM has been considered until recently as an extracellular membrane marker of blood-borne neuron-derived extracellular vesicles (NDEVs) and hence targeted via immunoprecipitation (IP) to derive EV biomarkers of brain pathology. Although cumulative evidence showing enrichment of neuronal cargo after L1CAM IP supports this rationale, recent publications claimed that L1CAM is not EV-associated. To address this challenge, we performed single-vesicle studies showing L1CAM in plasma-derived EVs in association with bona fide neuronal markers.
Methods: The existence of L1CAM+ NDEVs was evaluated by measuring both EV-associated and soluble L1CAM in human plasma fractionated via size-exclusion chromatography (SEC; Izon® qEV10/70nm columns) using a previously validated Simoa® assay and an intact EV Luminex assay designed to detect L1CAM in pan-tetraspanin positive EVs. The detection of EV-associated L1CAM in SEC EV fractions as well as in the L1CAM IP eluate was confirmed using high-resolution confocal microscopy, immune-electron microscopy and high sensitivity nanoscale multiplex flow cytometry analysis (FCA) under non-swarming conditions.
Results: L1CAM Simoa® detected L1CAM in early SEC EV fractions devoid of soluble plasma proteins. Findings were confirmed by: 1) intact EV Luminex assay showing EV-associated L1CAM in early SEC fractions; 2) immune electron and confocal microscopy allowing visualization of single L1CAM/Alix and L1CAM/VAMP2 double-positive nanoparticles; and 3) FCA demonstrating a sub-population of L1CAM+ EVs among total plasma EVs. These methods also showed that L1CAM IP results in the capture of L1CAM-bearing EVs double-positive for bona fide EV markers, as well as neuronal markers VAMP2 and GAP43, and a 25-fold enrichment of L1CAM-positive EVs over SEC EV fractions.
Summary/Conclusion: Multiple EV analyses at the single-vesicle level confirmed the presence of plasma NDEVs bearing surface L1CAM, thus validating L1CAM as a target for NDEV immunocapture.

OS24.03 | Identification of neuron-specific extracellular vesicle markers

Dmitry Ter-Ovanesyan[1]; Emma J. K. Kowal[2]; Wendy Trieu[3]; Bogdan Budnik[3]; Siddharth Iyer[3]; Aviv Regev[4]; George Church[3]; David Walt[3]
[1]Harvard University, Cambridge, USA; [2]MIT, Cambridge, USA; [3]Wyss Institute for Biologically Inspired Engineering, Boston, USA; [4]Genentech, San Francisco, USA

Introduction: Extracellular vesicles (EV) are an exciting class of biomarkers since they contain RNAs and proteins from the cell of origin. Isolating neuron-specific EVs from human biofluids such as cerebrospinal fluid (CSF) or plasma represents a promising approach for understanding the state of neurons and diagnosing neurological disease. Previous attempts to isolate neuron-derived EVs have relied on immuno-isolation of L1CAM, which we have previously found to be a free protein in CSF and plasma. Thus, new candidate markers are needed.
Methods: We developed an unbiased pipeline for the identification of cell-type specific EV markers based on gene expression and EV proteomics data and applied it to prioritizing candidates for the isolation of neuron EVs. We generated high quality data for the EV proteome of human CSF and plasma based on a novel EV purification technique involving multimodal chromatography resin. We then analyzed transmembrane proteins to assess their cell type of origin based on organ-level and cell-level RNA-Seq datasets.
Results: We have characterized the EV proteome of human CSF and plasma to higher depth than previous studies after purifying EVs to exceptionally high purity. Using this data, we suggest several new candidate markers that found in EV biofluids as well as displaying neuron specific expression within the brain and brain-specific expression within the body.
Summary/Conclusion: Our work represents a framework for the identification of cell type-specific EV markers and an important step towards the isolation of neuron-specific EVs for diagnostics of neurological disease.
Funding: Chan Zuckerberg Initiative; Open Philanthropy Project; Wyss Institute

OS24.04 | Tau, antioxidant proteins, and cellular markers are altered in brain tissue extracellular vesicles in Alzheimer's disease

Yiyao Huang[1]; Tom A. P. Driedonks[1]; Lesley Cheng[2]; Harinda Rajapaksha[2]; David A. Routenberg[3]; Javier Redding[1]; Tanina Arab[1]; Bonita H. Powell[1]; Olga Pletniková[1]; Juan C. Troncoso[1]; Laura Vella[4]; Lei Zheng[5]; Andrew F. Hill[6]; Vasiliki Mahairaki[7]; Kenneth W. Witwer[1]

[1]Johns Hopkins University School of Medicine, Baltimore, USA; [2]La Trobe University, Bundoora, Australia; [3]MesoScale Diagnostics, LLC, Rockville, MD, USA, Rockville, USA; [4]The University of Melbourne, Parkville, Australia; [5]Southern Medical University, Guangzhou, China (People's Republic); [6]Victoria University, Melbourne, Australia; [7]Department of Genetics, Johns Hopkins University School of Medicine, Baltimore, MD, USA. The Richman Family Precision Medicine Center of Excellence in Alzheimer's Disease, Johns Hopkins University School of Medicine, Baltimore, MD, US., Baltimore, USA

Introduction: Alzheimer's disease (AD) is a slowly progressive neurodegenerative disease characterized by intracellular or extracellular protein accumulation and associated neuroinflammation. Extracellular vesicles (EVs) have emerged as important participants in AD pathophysiology. Brain tissue derived EVs (bdEVs) act locally at the source and also leave the brain to betray the state of the central nervous system (CNS). Recent advances allow us to separate bdEVs and reveal bdEV proteome changes in AD brains. To find additional candidates for AD detection and peripheral bdEV capture, selected AD pathogenesis-related proteins and CNS cellular origin markers were tested on bdEVs from AD and controls.

Methods: Post-mortem human AD (n=23) and control (n=7) brains were obtained from the Johns Hopkins Alzheimer's Disease Research Center. Brain tissue-derived EVs were separated as previously described (Huang, et al., JEV, 2020; Vella, et al., JEV, 2017) and characterized in accordance with the recommendations of the Minimal Information for Studies of EVs (MISEV). Lysed and intact bdEVs were then subjected to electrochemiluminescence-linked (ECL) immunoassay for detection of AD pathogenesis related proteins and selected cellular of origin markers.

Results: Levels of total tau, phosphorylated tau, and antioxidant proteins peroxiredoxin 1 (PRDX) 1 and 6 were significantly elevated in AD bdEVs compared with controls. CNS cell marker detection indicated bdEVs from different cell types, including astrocytes, endothelia, microglia, and neurons. Moreover, several EV cellular origin surface markers were elevated in AD patients.

Summary/Conclusion: Elevation of several EV markers was observed, indicating cell activation and/or increased release of these EVs in AD. The dysregulated molecules may be involved in AD mechanisms, constitute new biomarkers for disease monitoring, and, in the case of surface molecules, serve as targets for bdEV capture.

Funding: This work was supported in part by grants AI144997, MH118164, and UG3CA241694 to Kenneth W. Witwer.

OS24.05 | Fluorescence tagged Small extracellular vesicles role in early diagnosis of Parkinson's disease

Simran Rastogi[1]; Fredrik Nikolajeff[2]; Saroj Kumar[1]

[1]All India Institute of Medical Sciences, New Delhi, New Delhi, India; [2]Lulea University of Technology, Lulea, Sweden

Introduction: The earlier studies of our group proved the link between saliva-derived small-sized extracellular vesicles (sEVs) and Parkinson's disease (PD). PD, a manageable condition although remains asymptomatic in the early stages. The current study used Fluor NTA (nanoparticle tracking) and explores how it could be a potent biomarker for PD.

Methods: The salivary sEVs of the subjects (PD=70, healthy controls=26) were isolated by PEG-based chemical precipitation followed by antibody-based validation through CD63 (universal surface marker) and confirmed neuronal origin by CD171 as well morphologically characterized through cryo-EM. The sEVs quantification via fluorescence-tagged sEVs NTA and antibody-based NTA using CD63. The α-syntotal in sEVs cargo was determined by ELISA. The confirmation of the disease severity staging was done by 99mTc-TRODAT-SPECT.

Results: We observed a significant increase of fluorescence-tagged sEVs in PD ($p < 0.0001$) than the HC via NTA (sensitivity of 94.34%) as well it was in line with the antibody-tagged sEV $p=0.006$ (sensitivity of 94.12%). A significant increase of α-syntotal concentration in the sEVs of PD when compared to HC (sensitivity of 88.24%). The fluorescence-tagged sEV depicted a positive correlation with the hallmark protein of PD α-syntotal $r=0.4709$, $p=0.0486$. The striatal binding ratios in the 99mTc-TRODAT-SPECT shown to have a positive correlation with the fluorescent sEVs concentration $r=0.3000$, α-syntotal concentration $r=0.8000$. The findings suggested that the salivary sEV concentration and the expression level of α-syn are not only higher in PD patients than HC but also followed the results of the TRODAT scan.

Summary/Conclusion: This study is the first to address that the fluorescence tagged sEVs can screen the progression of the disease with clinically acceptable sensitivity and can be a potent early detection method for PD.

Funding: The funding for this study was provided by Lulea University of Technology, Sweden, Department of Health Research (DHR) and Indian Council of Medical Research (ICMR), India for extramural and AIIMS intramural research grants.

OS24.06 | Identification of neural cell type-specific molecules in the extracellular vesicles enriched from human cerebrospinal fluid and their application to Alzheimer's disease

Tsuneya Ikezu[1]; Satoshi Muraoka[2]; Mark P. Jedrychowski[3]; Yang You[1]; Zhengrong Zhang[1]; Seiko Ikezu[4]
[1]*Mayo Clinic Florida, Jacksonville, USA;* [2]*National Institutes of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan;* [3]*Department of Cell Biology, Harvard Medical School, Boston, USA;* [4]*Department of Neuroscience, Mayo Clinic Florida, Jacksonville, USA*

Introduction: Extracellular vesicles (EVs) are emerging vehicles in studying neurodegenerative diseases including Alzheimer's disease (AD). Almost every cell type in the central nervous system including neurons and glia are known to shed EVs, which can be detected in the cerebrospinal fluid (CSF). Monitoring cell type-specific EVs from patient-derived CSF and profiling their contents by transcriptomic or proteomic analyses provide a useful method to study the pathophysiology of AD. We have recently identified EV unique molecules to human neurons, astrocytes, microglia-like cells, and oligodendrocytes. We applied these datasets for the characterization of CSF EV proteins and their changes in mild cognitive impairment (MCI) and AD cases.
Methods: We performed combined label-free and tandem mass tag-labeling based quantitative mass-spectrometry of EVs isolated from human induced pluripotent stem cells (hiPSCs) by using size exclusion column method and CSF by MagCapture method to conduct a comprehensive EV proteomics study on controls, AD and MCI risk cases. The enrichment of EV was determined by quantitative mass-spectrometry and immunoblotting of EV and non-EV markers. The changes in the cellular composition of the EV profiles were assessed for their correlation with disease status, pathway analysis and correlation with cognitive function.
Results: Novel cell type-specific EV protein markers were identified from induced pluripotent stem cell-derived excitatory neurons (e.g., NCAM1, ATP1A3), astrocytes (e.g., LRP1, ITGA6), microglia-like cells (e.g., LCP1, ITGAM) and oligodendrocytes (e.g., LAMP2, FTH1). Furthermore, cell type-specific EV molecule analysis revealed significant enrichment of astrocyte and microglia-derived EVs in AD CSF compared to control or MCI EV samples. Moreover, Quantitative proteomics analysis showed that EVs expressed 1284 unique proteins in AD, MCI and control groups. Statistical analysis identified three proteins (HSPA1A, NPEPPS, and PTGFRN) involved in AD progression. Finally, the PTGFRN showed a correlation with amyloid plaque (rho = 0.404, p = 0.027) and tangle scores (rho = 0.500, p = 0.005) in AD, MCI cases.
Summary/Conclusion: Our study presents novel human neural cell type-specific EV markers, highlights the key role of glia-derived EVs in AD CSF. The study also indicate that three proteins, HSPA1A, NPEPPS and PTGFRN, may be used to monitor the progression of MCI to AD.
Funding: Abbvie, Inc. (TI), Alzheimer's Association AARF-9550302678 (SM), Alzheimer's Research UK (TI), Cure Alzheimer's Fund (TI), NIH RF1 AG054199 (TI), NIH R01 AG054672 (TI), NIH R01 AG066429 (TI), NIH R01 AG072719 (TI), NIH R01 AG067763 (TI)

OS25: EV Characterization

Chair: An Hendrix – Department of Urology, Ghent University Hospital, Ghent, Belgium; Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Ghent University, Ghent, Belgium; Cancer Research Institute, Ghent, Belgium

Chair: Céline Elie-Caille – Institut FEMTO-ST, UMR 6174 CNRS, 25030 Besancon cedex, France

OS25.01 | Rapid and sensitive extracellular vesicle detection by fluorescent lateral flow immunoassay

Baihui Wang[1]; José María Duque[2]; Guillermo García-Santos[3]; María Fernández-Hevia[3]; Luis sánchez[2]; Luis García-Flórez[4]; Esther Serrano-Pertierra[1]; María Carmen Blanco-López[5]
[1]*Universidad de Oviedo, Dpto. Química Física y Analítica, Oviedo, Spain;* [2]*Hospital Universitario San Agustín, Avilés, Spain;* [3]*Hospital Universitario Central de Asturias, Oviedo, Spain;* [4]*Hospital Universitario Central de Asturias, oviedo, Spain;* [5]*Universidad de Oviedo, Dpto Química Física y Analítica, Oviedo, Spain*

Introduction: Extracellular vesicles (EV) are small membrane structures released by cells that act as potent mediators of intercellular communication. In the field of biomedicine they have been studied as a novel source of biomarkers and drug delivery vehicles. Detection and potential characterization of freshly isolated EV is of great interest for subsequent EV analysis and studies. Thus, lateral flow immunoassay is a potential, low-cost, and user-friendly tool for rapid EV characterization.

Methods: Fluorescent nanoparticles (EuNPs) were functionalized with an antibody against tetraspanin CD63 (detection antibody). For LFIA assays, antibodies anti-CD9 and anti-IgG were dispensed on a nitrocellulose membrane as test line and control line, respectively.
Plasma-derived EV were isolated by using a precipitation reagent or by size exlusion cromatography (SEC). Size characterization and concentration were determined by nanoparticle tracking analysis (NTA) at Nanovex (Spain). Protein content in the obtained fractions was measured by BCA assay. Fluorescent LFIA was then performed to enable rapid on-site detection of EV.
Results: The high sensitive fluorescent LFIA system can detect CD63+CD9+ EVs is freshly isolated plasma-derived EV, by SEC or by using a precipitation reagent. EV could be clearly detected in both cases, even in high diluted SEC fractions, and employing a only minimum volume of the sample. The limit of detection (LOD) of our system was 5.27×104 EVs /μl.
Summary/Conclusion: This study shows a fluorescent LFIA may be used for rapid on-site detection of plasma-derived EVs isolated by SEC or by using a precipitation reagent. This system achieved low LOD. In addition, our system may be adapted to other surface markers to characterize further freshly isolated EV fractions according to the field of interest.
Funding: This research was funded by the Ministerio de Ciencia e Innovación (Spain), grant number: PID2020-119087RB-I00. This study was also financed by European Union through the European Regional Development Fund (Principado de Asturias, FICYT, PCTI 2018-2022), under the grant number: IDI/2021/000112.

OS25.02 | Raman spectroscopy for the quality assessment of extracellular vesicles as cell-free products in regenerative medicine

Alice Gualerzi; Silvia Picciolini; Francesca Rodà; Valentina Mangolini; Marzia Bedoni
Laboraotry of Nanomedicine and Clinical Biophotonics, IRCCS Fondazione Don Carlo Gnocchi, Milan, Italy, Milan, Italy

Introduction: Extracellular vesicles (EVs) from mesenchymal/stromal cells (MSC) are one of the emerging strategies for cell-free regeneration therapies. Problems of purification, yield, and reproducibility of the isolation procedure are some of the hurdles in their translation to clinics. Differently from their cellular counterpart, MSC-derived EVs cannot rely for their quality control only on the superficial markers because EV-markers are more difficult to be detected, require dedicated batches and sophisticated technologies. Raman spectroscopy (RS) can be used as quality control as it provides the label free biochemical characterization of the sample; it is fast, cost-effective and sensitive, it does not focus on a specific component, but rather looks at the true complexity of their composition.
Methods: The RS bulk characterization of EVs was performed on preparations from different research laboratories and small industries . The reproducibility of different isolation methods, the purity of the preparations and the quality of the cell sources were assessed by calculating the spectroscopic protein-to-lipid ratio and performing the multivariate statistical analysis of the collected spectra.
Results: The results obtained by the Laboratory of Nanomedicine and Clinical Biophotonics demonstrate that the Raman approach is effective in the quality control of both EVs and conditioned media obtained from MSC cultures, demonstrating that RS can be a valuable tool in the reproducibility assessment of cell-free products to be used in the pipeline of stem cell-derived preparations for regenerative medicine.
Summary/Conclusion: The RS approach to the massive production of EVs can help in the assessment of the proper isolation procedure for a downstream application, in the evaluation of reproducibility among batches and operators and in the comparison of data obtained between laboratories by the generation of online databases that can merge Raman data of EVs from various sources to foster transparency of data.
Funding: The project was supported by the Italian Ministry of Health.

OS25.03 | Advance Molecular Tools for Detection and Characterization of Extracellular Vesicles

Ehsan Manouchehri Doulabi[1]; Tanya Kumar Sinha[1]; Radiosa Gallini[1]; Liza Löf[1]; Masood Kamali-Moghaddam[2]
[1]*Dept. of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden, Uppsala, Sweden;*
[2]*Dept. of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden., Uppsala, Sweden*

Introduction: DNA-assisted proximity assays are powerful and versatile tools for sensitive, specific and high throughput detection of macromolecules such as DNA, RNA and proteins as well as posttranslational modifications in in situ and in liquid biopsies.
Methods: Commonly, in these assays the target molecules are recognized by several proximity probes, each equipped with a DNA oligonucleotide. Upon binding of the target molecules, the DNA oligonucleotides are brought in proximity, subjected to enzymatic ligation or polymerization, which results in formation of an amplifiable reporter molecule. The use of

multiple recognition events in combination with signal amplification allows highly specific and sensitive detection of the target molecules.

Results: We have developed a large number of affinity-based proximity assays for single- and multiplex detection of large complexes. Several of these technologies, such as proximity ligation assay (PLA) combined with flow cytometry readout, multiplex proximity extension assays (PEA) and proximity barcoding assays (PBA) are used for sensitive detection and characterization of extracellular vesicles in bulk or at single molecule level. In addition, we have applied these technologies in combination with mass spectrometry for identification of exosomal surface proteins, allowing establishment of assays for detection of organ-specific extracellular vesicles in liquid biopsies.

Summary/Conclusion: Here, we discuss the application of proximity assays for screening, characterization and sensitive detection of extracellular vesicles in body fluids.

OS25.04 | Machine learning approaches for label-free nanoplasmonic classification of placental extracellular vesicles

Mohammadrahim Kazemzadeh[1]; Colin L. Hisey[2]; Miguel Martinez-Calderon[3]; Song Paek[4]; Neil Broderick[1]; Lawrence W. Chamley[1]; Weiliang Xu[1]

[1]University of Auckland, Auckland, New Zealand; [2]The Ohio State University, Columbus, USA; [3]Department of physics University of Auckland, Auckland, New Zealand; [4]Department of Obstetrics and Gynaecology, University of Auckland, New Zealand, Auckalnd, New Zealand

Introduction: Surface Enhanced Raman Spectroscopy (SERS) has been extensively applied for both labeled and label-free detection and classification of extracellular vesicles (EVs). However, the heterogeneity of EVs and their diverse biochemical compositions have restricted the clinical translation of EV SERS. Novel machine learning techniques are capable of dealing with this heterogeneity and mitigating the disadvantages of labeled methods. In this research, we developed bottleneck classifiers (BC) that can extract the unique differences between highly heterogeneous placental EV SERS data, enabling the label-free classification and visualization of preeclampsia.

Methods: Placental extracellular vesicles (EVs) were harvested from donated placentae by culturing 400 mg explants in netwell inserts. EVs were isolated from conditioned media using differential ultracentrifugation and size exclusion chromatography. Following validation using transmission electron microscopy and nanoparticle tracking analysis, EV SERS spectra were obtained using EVs from both healthy and preeclamptic placentae. We then constructed an autoencoder-like network by changing the decoder to a classifier to achieve the desired BC. Encoders with both linear and nonlinear activations were considered, as linear activation achieves highly interpretable results nonlinear achieves maximum classification accuracy.

Results: We show that the linearly activated encoder leads to a classification accuracy of 92%, which is greater than any other conventional machine learning, while also producing interpretable results. In fact, Raman bands of 1745 cm-1 and 1330 cm-1 play the most vital roles in classification between Normotensive (NT) and Preeclamptic (PE) EVs. Both of these bands are associated with phospholipids and this finding is consistent with previous biological research in this area.

Summary/Conclusion: We have developed novel bottleneck classifiers for both the visualization and classification of EV SERS data. The superiority of the presented technique over classical techniques is shown via interpretable classification and visualization of placentae NT and PE EVs.

Funding: HRC New Zealand, AMRF, and Goodfellow

OS25.05 | Genetically engineered MRI-trackable extracellular vesicles as SARS-CoV-2 mimetics for examination of viral binding

Andrea Galisova; Jiri Zahradnik; Hyla Allouche-Arnon; Mattia Morandi; Ori Avinoam; Neta Regev-Rudzki; Gideon Schreiber; Amnon Bar-Shir

Weizmann Institute of Science, Rehovot, Israel

Introduction: Understanding viral-induced pathologies is of great interest, particularly nowadays with the prolonged COVID19 pandemic. In this regard, extracellular vesicles (EVs) offer a unique platform for studying viral infection paths and mechanisms due to similar properties as viruses and possibility of visualization by imaging methods (Han Z. et al, JEVs 2021, e12054). Here, we show the development of genetically engineered EVs displaying the receptor-binding domain (RBD) of SARS-CoV-2 on their surface as corona-virus-like mimetics that can be labeled both magnetically or fluorescently.

Methods: HEK293 cells were genetically modified to express the RBD of SARS-CoV-2 on their surface. Then, these cells were incubated with SPIO nanoparticles (2.5-40 μg/mL) and the secreted labeled EVs (EVsRBD as corona-mimetics or EVsnoRBD

as controls) were isolated by differential centrifugation (400 g, 2000 g, 10 000 g, 100 000 g) and characterized by several methods (MRI, NanoSight, Western blot, flow cytometry and transmission electron microscopy - TEM). For in vivo examination, two types of subcutaneous tumors were induced in nude mice (HEK293T as control or HEK293T overexpressing ACE2) to study in vivo targeting of EVsRBD to the ACE2 receptor. EVs (3x10E11) were intravenously injected in mice followed by in vivo MRI and ex vivo fluorescence imaging.

Results: Cryo-TEM analysis showed that the secreted genetically engineered EVs have typical EVs size (~100 nm) and shape. Binding to the ACE2 receptors was confirmed by a higher uptake of EVsRBD into ACE2-expressing cells compared to control EVsnoRBD. MRI of isolated EVs showed concentration-dependent accumulation of SPIONs as depicted from the lower MRI signal on T2-weighted images. In vivo targetability of EVsRBD was demonstrated by MRI and fluorescence imaging of mice following EVs injection. The higher accumulation of the EVsRBD in ACE2-expressing cells as compared to control cells was confirmed by the lower T2/T2* MRI signal. Similarly, ex vivo fluorescence imaging showed higher accumulation of EVsRBD in the ACE2 expressing cells as compared to EVsnoRBD.

Summary/Conclusion: Here, we show a multimodal imaging platform for mapping the binding of coronavirus mimetics to the ACE2 receptors both in vivo and in vitro by genetically engineered EVs expressing the RBD of SARS-CoV-2. The proposed platform can be implemented to study other viruses by engineering a tailored peptide on the EVs surface and thus to explore the role of receptors in a wide spectrum of viral pathologies and to evaluate antiviral treatments.

Funding: This study was supported by the Ben B. and Joyce E. Eisenberg Foundation.

OS25.06 | High-throughput nanomechanical differentiation of lipoproteins and extracellular vesicles

Andrea Ridolfi[1]; Laura Conti[1]; Marco Brucale[1]; Roberto Frigerio[2]; Alessandro Gori[3]; Jacopo Cardellini[4]; Costanza Montis[5]; Debora Berti[6]; Paolo Bergese[7]; Marina Cretich[3]; Francesco Valle[1]

[1]Italian National Research Council (CNR), Institute for the Study of Nanostructured Materials (ISMN), Bologna, Italy, Bologna, Italy; [2]Consiglio Nazionale delle Ricerche, Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC), Milan, Italy, Milano, Italy; [3]Consiglio Nazionale delle Ricerche, Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC), Milan, Italy, Milano, Italy; [4]Università degli Studi di Firenze, Florence, Italy; [5]University of Florence, Italy, Firenze, Italy; [6]University of Florence, Italy, Florence, Italy; [7]Università degli studi di Brescia, Brescia, Italy

Introduction: Proper isolation of Extracellular vesicles (EVs) and lipoproteins (LPs) from plasma and serum samples remains challenging. Due to their similar size and density distributions, EVs and LPs often co-isolate when using physical-based enrichment methods; detecting reciprocal contamination is in itself nontrivial. We apply a recently developed single-particle nanomechanical screening method to EVs and LPs, showing that it can be used to detect co-isolated subpopulations, obtain their size distributions and estimate their relative abundance.

Methods: EVs were obtained through tangential flow filtration from a culture of human cardiac progenitor cells (CPCs) and characterized via cryo-Transmission Electron Microscopy, Atomic Force Microscopy (AFM), Nanoparticle Tracking Analysis, Western Blot. LPs (VLDL, IDL, LDL, HDL, chylomicrons) were purchased from commercial sources. All samples were characterized via a nanomechanical screening method we recently developed (Ridolfi et al 2020, doi:10.1021/acs.analchem.9b05716; Frigerio et al 2021, doi:10.3390/cells10030544)

Results: Each LP subtype is found to display a specific mechanical fingerprint, which is different from that of EVs and of other LPs. Only Very-Low Density Lipoproteins (VLDL) and chylomicrons exhibit substantial reciprocal overlap in term of size and mechanical stiffness. While VLDL and chylomicrons exhibit a nanomechanical behavior associated with pressurized elastic vessels, other LPs do not - suggesting major (but still unclear) structural differences.

Summary/Conclusion: To the best of our knowledge, these results represent the first single-particle mechanical investigation of lipoproteins. Our AFM-based method can resolve the nanomechanical properties of co-isolated EVs and LPs, hence providing a useful tool for assessing the purity of plasma- and serum- derived preparations.

Funding: We acknowledge the grants H2020 FETOPEN-801367 "evFOUNDRY", H2020 FETPROACT 952183 "BOW", H2020-FETPROACT-951768 "MARVEL".

OS26: Novel EV Cargo

Chair: David W. Greening – Baker Heart and Diabetes Institute

Chair: Ryan C. Pink – Oxford Brookes University

OS26.01 | Application of phage-displayed random peptide library technology for identification of extracellular vesicles in patients with brain tumors

Wenbo Zhou[1]; Julia Craft[2]; Alex Ojemann[1]; Luke Bergen[1]; Arin Graner[1]; Aitana Gonzales[1]; Anthony Fringuello[1]; Qianbin He[1]; Michael W. Graner[1]; Xiaoli Yu[1]
[1]*University of Colorado Anschutz Medical Campus, Aurora, USA;* [2]*University of Colorado Denver, Aurora, USA*

Introduction: Extracellular vesicles (EVs) mediate cell–cell communication through activation of receptors at the surface of recipient cells. EVs released by tumor cells are instrumental in affecting tumor surrounding cells to favor their own growth and dissemination. Protein markers and non-protein constituents such as lipids and glycoconjugates have been shown to be highly specific as EV markers, therefore understanding the mechanisms of EV surface recognition would provide insight for tumor pathogenesis. Phage-displayed random peptide libraries provide an unbiased approach for identification EV surface markers.
Methods: We purified EVs from plasma and tumor cell lines in patients with glioblastoma and meningioma. We used ExoEasy (Qiagen), ExoQuick® (SBI), and ultracentrifugation methods for EV isolation. EVs were evaluated by NTA and Western blots. We applied two phage-display peptide libraries (7-mer &12-mer) for identification of peptides specific to brain tumor EVs. Phage peptides selected were evaluated for their binding specificities to EVs using ELISA and peptide immunoprecipitation. Synthetic peptides were used for inhibition assays for tumor EV cytotoxicity in neurons.
Results: We identified a total of 45 (36 unique) phage peptides that were specific to brain tumor EVs derived from pooled and individual Glioblastoma (GBM) and Meningioma (MMA) plasma and tumor cell lines. We showed that these peptides bind to brain tumor EVs in a dose-dependent manner. Furthermore, we demonstrated that EV specific peptides inhibited GBM EV induced cytotoxicity in neurons.
Summary/Conclusion: Phage peptide technologies can be used for enrichment, isolation, and characterization of EVs derived brain tumor plasma and cell lines, and these EVs could provide biomarker potentials and insight into brain tumor pathogenesis.
Funding: NIH/NIMH

OS26.02 | Hemodynamics control tumor EVs uptake, trafficking and function

Benjamin Mary[1]; Nandini Asokan[2]; Annabel Larnicol[3]; Olivier Lefebvre[2]; Jacky G. Goetz[2]; Vincent Hyenne[4]
[1]*INSERM U1109, Strasbourg, France;* [2]*Inserm U1109, Strasbourg, France;* [3]*Inserm U1109, strasbourg, France;* [4]*INSERM / CNRS, Strasbourg, France*

Introduction: Tumor extracellular vesicles (tEVs) escape the primary tumor and reach distant organs where they can alter the microenvironment and eventually promote the formation of pre-metastatic niches. To do so, tEVs transit through body fluids including the blood circulation where they are subjected to various hemodynamic forces. Despite the importance of this transit, and the massive amounts of EVs present in the blood circulation of cancer patients, very little is known about the intravascular behavior of EVs. In particular, how tEVs cope with hemodynamics and how the latter impact their delivery in endothelial cells and their function remain(s) elusive.
Methods: In this study, we combine an in vitro (microfluidics) and in vivo (zebrafish) approaches to simultaneously tune flow forces and track fluorescently labelled tEVs. We exploit multi-scale imaging to follow internalized EVs, and transcriptomics to assess the impact of EVs on receiving endothelial cells.
Results: We found that a moderate flow velocity (400 μm/s, mimicking hemodynamics of small capillaries in mammals) promotes the uptake of tEVs by endothelial cells. Interestingly, we observed that such flow redirects the trafficking of a small portion of internalized EVs to non-degrative endosomes suggesting that endothelial mechanosensing could favor EV escape from the degradation machinery. As a consequence, circulating tEVs induce a strong pro-angiogenic response in flow-stimulated endothelial cells, notably by promoting the expression of pro-angiogenic transcription factors.
Summary/Conclusion: Our work shows that hemodynamics affect the uptake, fate and function of circulating EVs and demonstrates the importance of studying EVs in realistic environmental conditions. We propose that tEVs contribute to pre-metastatic niche formation by acting on specific vascular regions with permissive flow profiles.

OS26.03 | Extracellular vesicles are enriched in metals and transfer metals intercellularly

Adityas Purnianto[1]; Celeste H. Mawal[1]; Huaqi Su[1]; Leah C. Beauchamp[1]; Tiana F. Koukoulis[2]; Mark A. Greenough[1]; Krista A. Dent[1]; Catriona A. McLean[3]; Scott Ayton[1]; David I. Finkelstein[2]; Ashley I. Bush[4]; Kevin J. Barnham[2]; Laura J. Vella[2]

[1]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, VIC, Australia, Parkville, Australia; [2]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Australia, Melbourne, Australia; [3]Department of Anatomical Pathology, Alfred Hospital, Melbourne, VIC, Australia, Melbourne, Australia; [4]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Australia, Parkville, Australia

Introduction: EVs are of interest as a potential source of biomarkers and mediator of intercellular communication. Trace metals are essential elements involved in various physiological and pathological processes, however there is limited information on the metal content of EVs or their potential role in metal homeostasis. This study aims to comprehensively examine the metal content of EVs, isolated from a variety of in vitro and in vivo sources, and the capacity of EVs for intercellular metal transfer.

Methods: 15 biologically important metals were measured in sEVs from 3 cell lines and human nasal secretions, frontal cortex or plasma using inductively coupled plasma mass spectrometry. sEVs were isolated by differential centrifugation followed by ultracentrifugation (UC), size exclusion chromatography (SEC), density gradient-UC (DGUC) or combination of DGUC and SEC. Immunoblotting and transmission electron micrography were used for validation. SH-SY5Y cells treated with Fe57 isotope or Pb were used to determine metal packaging into EVs and transfer to recipient cells.

Results: Relative to cells or neat clinical samples, sEVs from in vitro and in vivo sources, were enriched in essential trace metals, particularly Fe and Cu. While Al and Pb were undetectable in neat plasma, these non-physiological metals were detected in sEVs, implying a role for sEVs in removal of toxic metals. In vitro, Fe 57 and Pb were enriched in sEVs and in recipient cells after loading donor cells with these rare metals, confirming the intracellular packaging of metals into sEVs and the capacity of sEVs for intercellular metal transfer.

Summary/Conclusion: This is the first study to comprehensively characterize the metal content of EVs and demonstrate transfer of essential trace and heavy metals via EVs. This study highlights the potential roles of EVs in trace metal homeostasis and cellular defense against toxic metals and has implications for biomarker and therapeutic target discovery of diseases with metal dysregulation.

OS26.04 | Supermeres, a small secreted nanoparticle, represent a new functional carrier of cellular information reflecting diverse extracellular heterogeneity

Jeff Franklin[1]; Qin Zhang[2]; Dennis Jeppesen[3]; James Higginbotham[2]; Mark Castleberry[2]; Clark Massick[2]; Marisol Ramirez[2]; Qi Liu[2]; Kasey Vickers[2]; Al Charest[4]; Robert Coffey[2]

[1]Vanderbilt University Medical Center/Vanderbilt University, Nashville, USA; [2]Vanderbilt University Medical Center, Nashville, USA; [3]Vanderbilt Univeristy Medical Center, Nashville, USA; [4]Dana-Farber / Harvard Cancer Center, Boston, USA

Introduction: The heterogeneity of the cellular secretome has been rapidly expanding demonstrating a diverse population of extracellular vesicles (EVs) but also populations of non-membranous nanoparticles. One of these, exomeres, identified by the Lyden lab (Zhang et al.., … Lyden. Nat Cell Biol. 2018;20(3):332-43), are around 35nm and have distinct cargo that are different from EVs and are functional, carrying active ST6gal1 as one example (Zhang et al… Coffey RJ. Cell Rep. 2019;27(3):940-54). We have characterized even smaller lipid-poor nanoparticles termed supermeres (Zhang et al., … Coffey RJ Nat Cell Biol. 2021;23(12):1240-54).

Methods: Supermeres, EVs and exomeres are purified using a combination of methods including differential ultracentrifugation, density-gradient purification and size exclusion chromatography. Because producing EVs and nanoparticles can be labor intensive using a traditional 2D cell culture method, a hollow fiber bioreactor is used to produce these in high concentration. Proteomic and RNA analysis of the hollow fiber secretome was compared with the same cells grown on tissue culture dishes.

Results: Supermeres have consistent protein cargos within DiFi cells grown in 2D and in the bioreactor; small RNA cargo differs somewhat between the two growth conditions. Supermeres contain various RNA binding proteins as well as proteolytically cleaved membrane proteins, including proteins associated with neurodegeneration. A major RNA cargo in supermeres is miR-1246, derived from the RNU2 snRNA. Functionally, supermeres have the ability to traverse diverse tissues including crossing the blood brain barrier, transfer drug resistance and can metabolically reprogram hepatocytes in vivo.

Summary/Conclusion: Supermeres are a new class of secreted nanoparticle replete with disease biomarkers and therapeutic targets.

Funding: NCI grant nos R35 CA197570, UG3 241685, P01 CA229123 and P50 236733 to R.J.C. We acknowledge the generous support of the Nicholas Tierney GI Cancer Memorial Fund

OS26.05 | Can the oncomolecules carried by small EVs help to diagnose pancreatic cancer early?

Marco Falasca[1]; Arunima Panda[1]; Melissa Rhodes[1]; Pratibha Malhotra[1]; Peter Meikle[2]; David W. Greening[2]; Krish Ragunath[1]; Marcello Manfredi[3]; Ilaria Casari[1]
[1]*Curtin University, Perth, Australia;* [2]*Baker Heart and Diabetes Institute, Melbourne, Australia;* [3]*University of Piemonte Orientale, Novara, Italy*

Introduction: Pancreatic cancer (PC), is one of the deadlier and most aggressive tumours, increasing in prevalence and death rate. Its lack of early symptoms, and consequent late diagnosis, result in fast spread and resistance to chemotherapy, making it a significant challenge for the majority of patients. Finding a way to detect PC earlier and a successful cure is a high priority. Small extracellular vesicles (sEVs) are used by cancer cells to communicate and aid in cancer progression. Molecules transported by sEVs unique to PC could be used as markers to detect PC in its early stage.
Methods: We characterised sEVs from human non-malignant pancreatic duct cells (HPDE, hTERT–HPNE), PC cells (AsPC-1, BxPC-3 and MIA PaCa-2) and patient-derived xenograft (PDX) PC cell lines. Plasma samples (60 PC patients and 30 healthy controls) have been also collected. sEVs have been isolated following MISEV 2018 guidelines. Comprehensive proteomic and lipidomic analyses of sEVs have been performed. Ethics have been approved for use of human blood samples from PC patients and age-matched healthy controls (Royal Perth Hospital RGS4208).
Results: Our data revealed that specific oncoproteins and lipids are only present in PC-derived sEVs and not in non-malignant pancreatic duct cells. In addition, we have found that sEVs from human PC cell lines and PDX cells contained metastatic regulatory factors and signalling molecules fundamental to PC progression as cargo unique to PC sEVs. Interestingly, proteomic analysis of sEVs-derived from human PC cell lines reveals enrichment of key enzymes involved in inositol synthesis and metabolism such as the transporter SLC5A3. SLC5A3 is involved in inositol transport and the formation of key signalling molecules such as phosphatidylinositol 4,5-bisphosphate. SLC5A3 is an unfavourable prognostic factor for PC and correlates with a lower survival probability. Our findings have been validated in sEVs obtained from blood samples from PC patients.
Summary/Conclusion: We propose that the combination of lipids and proteins could be a potential PC biomarker, which offers the opportunity of an early PC diagnosis. In addition, we identify how sEV-derived lipids and proteins mediate signalling between PC and recipient cells (e.g., other PC cells, cancer-associated fibroblasts, endothelial cells).
Funding: This research is funded by the Australian Pancreatic Cancer Foundation

OS26.06 | Tetraspanins are unevenly distributed across single extracellular vesicles and bias sensitivity to multiplexed cancer biomarkers

Rachel R. Mizenko[1]; Terza Brostoff[2]; Tatu Rojalin[3]; Hanna J. Koster[3]; Hila S. Swindell[4]; Gary S. Leiserowitz[5]; Aijun Wang[6]; Randy P. Carney[7]
[1]*Biomedical Engineering Department, University of California Davis, Davis, USA;* [2]*Department of Pathology, University of California San Diego, San Diego, USA;* [3]*Department of Biomedical Engineering, University of California Davis, Davis, USA;* [4]*Department of Surgery, University of California Davis, Davis, USA;* [5]*Division of Gynecologic Oncology, University of California Davis Medical Center, Sacramento, USA;* [6]*Department of Surgery, University of California Davis, Sacramento, USA;* [7]*UC Davis, Davis, USA*

Introduction: Development of extracellular vesicle (EV) based diagnostics is of great interest due to EVs' representation of the proteome and transcriptome of their parent cell. Most diagnostics rely on capture by a single tetraspanin; however, there is growing evidence that these tetraspanins are expressed on subpopulations of EVs and little work has scrutinized co-expression of tetraspanins and relevant markers. For this reason, we sought to determine i) if tetraspanin colocalization was unique to specific biofluid sources and ii) if enrichment by a single tetraspanin impacted identification of diagnostically relevant markers in ovarian cancer (OvCa) EVs.
Methods: EVs were isolated by differential ultracentrifugation from OvCa cells, placental mesenchymal stem cells (PMSCs), and serum. Single EVs were interrogated for tetraspanin(CD9, CD63, CD81) or OvCa marker(CD24, EpCAM, Her2) expression by immunocapture/immunofluorescence (ExoView R100). OvCa EVs were also non-specifically biotinylated and captured by anti-biotin capture spots.
Results: EVs from all sources had heterogeneous colocalization of tetraspanins that remained consistent across successive isolations. Yet these tetraspanin profiles were distinct for EVs isolated from serum, OvCa cells, and PMSCs. When labeling for OvCa markers, each marker was colocalized most frequently with a specific tetraspanin. After biotinylation, as many or more OvCa marker+ EVs were captured non-specifically than by any single tetraspanin.
Summary/Conclusion: This work suggests that tetraspanin colocalization is consistent for EVs from a given source but that it in general varies by source. Importantly, we found that capture by a single tetraspanin biased the frequency of multiplexed proteins

and dramatically impacted the sensitivity to a given OvCa marker. This suggests that use of a single tetraspanin for capture in a diagnostic device should be vetted to confirm high colocalization with a given marker to maximize sensitivity.
Funding: R.P.C was supported by NIH NCI (1R01CA241666).

OS27: Natural and Engineering EVs as Therapeutics

Chair: Kathrin Gärtner, Helmholtz Center Munich German Research Center for Environmental Health, Research Unit Gene Vectors, Munich, Germany

Chair: Farrukh Aqil – University of Louisville

OS27.01 | Extracellular Vesicles carrying silencing sequences alleviate Machado-Joseph Disease (MJD)

David -. Rufino-Ramos[1]; Kevin Leandro[2]; Vitor Carmona[3]; Inês M. Martins[2]; Patrícia Albuquerque[3]; Rosário Faro[3]; Rita Perfeito[3]; Rui Jorge J. Nobre[3]; Luís Pereira de Almeida[3]
[1]CNC - Coimbra, Portugal, Coimbra, Portugal; [2]CNC - Coimbra, Coimbra, Portugal; [3]CNC, Coimbra, Portugal

Introduction: Extracellular vesicles (EVs) are membrane-based structures produced by cells, with capacity to carry miRNAs, non-coding RNAs with around 22 nucleotides. MiRNA enrichment into EVs is promoted by specific motifs, designated as ExoMotifs.
Therefore, the aim of this work was to investigate whether ExoMotifs would promote packaging of engineered miRNA-based silencing sequences into EVs to use them as therapeutic vehicles to treat Machado-Joseph Disease (MJD). MJD is a neurodegenerative disorder caused by abnormal over-repetition of a CAG tract within the ataxin-3 (ATXN3) gene, conferring toxic properties to the ATXN3 protein.
Methods: An ExoMotif signal was associated with silencing sequences targeting mutant ATXN3 (mutATXN3) to promote its packaging into EVs, which was evaluated by qRT-PCR. Additionally, neuronal targeting proteins were expressed at EVs surface, and their neuronal targeting efficiency was evaluated in vitro and in vivo by immunocytochemistry and flow cytometry. To evaluate target engagement, engineered EVs were administered by daily intranasal administration to mice expressing a mutATXN3 dual luciferase reporter system.
Results: We found that silencing sequences with the ExoMotif retained the capacity to silence mutant ataxin-3 and were effectively incorporated into EVs. Furthermore, the bioengineered EVs significantly decreased mutATXN3 mRNA levels in neuronal cells. Importantly, continuous intranasal administration of therapeutic EVs significantly decreased the brain luminescence associated with mutATXN3 dual luciferase reporter system.
Summary/Conclusion: MutATXN3 silencing sequences enriched in EVs reach the brain via intranasal route and are therapeutically active reducing in vivo the disease-causing ataxin-3.
Funding: This work was funded by Competitiveness Factors Operational Program (COMPETE 2020) and National Funds through FCT (Foundation for Science and Technology) (SFRH/BD/132618/2017), ViraVector (CENTRO-01-0145-FEDER-022095), SpreadSilencing POCI-01-0145-FEDER-029716, by National Ataxia Foundation (USA), the American Portuguese Biomedical Research Fund (APBRF) and the Richard Chin and Lily Lock Machado-Joseph Disease Research Fund.

OS27.02 | Protective Antibody Responses Produced by Extracellular Vesicles isolated from Salmonella Infected Macrophages

Lisa E. Emerson; Hailey Barker; Terri Tran; Samantha Barker; Samantha Enslow; Mark Ou; Carol Hoffman; David Pascual; Mariola J. Edelmann
University of Florida, Gainesville, USA

Introduction: Non-Typhoidal Salmonella (NTS) causes over 95 million infections each year, and despite the severity and disease burden there is no approved vaccine to combat infection. A potential vaccine strategy that has not been deeply explored, are extracellular vesicles (EVs) produced during NTS infection.
Methods: To study the role of EVs isolated after NTS infection, we infected macrophages with Salmonella and isolated EVs using ultracentrifugation. Next, we treated 35 mice with either PBS control, live Salmonella vaccine (ΔaroA) control, EVs isolated from NTS infection of macrophages, or disrupted EVs; and collected blood and stool samples to assess the effect of EVs on mucosal immunity. We performed ELISAs to measure IgG in serum and IgA in stool. Finally, we challenged mice orally with NTS.

Results: Our analysis of serum IgG and fecal IgA showed a significant increase in Salmonella-specific antibody responses in the EV treated mouse group. IgG and IgA increased over time for 12 weeks in both live vaccine and EV treatment groups. Challenge results showed that mice with protective IgA and IgG responses had a lower bacterial burden and increased survival compared to PBS control mice and mice given disrupted vesicles.
Summary/Conclusion: Our results display the ability of EVs isolated during Salmonella infection to generate Salmonella-specific protective antibody responses. Our findings demonstrate a previously unknown role of EVs in bacterial infection and their potential as a vaccine strategy. Understanding the full immune potential of EVs will aid in EV vaccine approaches to fight the bacterial infection.
Funding: R03 AI135610-02

OS27.03 | Small extracellular vesicles from metabolically reprogrammed mesenchymal stem cells as a potential acellular therapy for osteoarthritis

Patricia Luz-Crawford[1]; Eliana Lara-Barba[2]; Felipe A. Bustamante-Barrientos[2]; Cynthia García[3]; Yeimi Herrera-Luna[2]; Carolina Pradenas[2]; Noymar Luque-Campos[2]; Gabriela Maita[4]; Roberto Elizondo-Vega[5]; Farida Djouad[6]; Ana María Vega-Letter[3]
[1]Laboratorio de Inmunología Celular y Molecular, Centro de Investigación Biomédica, Facultad de Medicina, Universidad de Los Andes, Santiago, Chile; IMPACT, Center of Interventional Medicine for Precision and Advanced Cellular Therapy, Santiago, Chile, Santiago, Chile; [2]Laboratorio de Inmunología Celular y Molecular, Centro de Investigación Biomédica, Facultad de Medicina, Universidad de Los Andes, Santiago, Chile, Santiago, Chile; [3]Laboratory of Molecular and Cellular Immunology, Faculty of Medicine, Universidad de los Andes, Santiago, Chile, Santiago, Chile; [4]Laboratorio de Inmunología Celular y Molecular, Centro de Investigación Biomédica, Facultad de Medicina, Universidad de Los Andes, Santiago, Chile;Department of Physiology, Pontificia Universidad Católica de Chile, Santiago, Chile, Santiago, Chile; [5]Laboratorio de Biología Celular, Departamento de Biología Celular, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile, Concepcion, Chile; [6]Institute for Regenerative Medicine and Biotherapy (IRMB), Univ Montpellier, Institut national de la santé et de la recherche médicale (INSERM), Montpellier, France, Montpellier, France

Introduction: Osteoarthritis (OA) is a degenerative disease in which there is a loss of articular cartilage and chronic inflammation. Currently, only palliative treatments are available. In this context, mesenchymal stem cells (MSC) and their derived small extracellular vesicles (sEVs) appear as the candidate of choice for the development of new therapies for OA due to their chondroprotective and immunomodulatory properties.
Methods: sEVs were isolated from metabolically reprogrammed MSC through ultracentrifugation. sEVs were then quantified by nanoparticle tracking analysis and characterized by flow cytometry. Then, sEVs were added to the culture media of either chondrocytes for regeneration or macrophages for inflammation evaluation, respectively. After 24 hours, chondrocytes were collected to measure classical OA markers through RT-qPCR and macrophage polarization was assessed through flow cytometry.
Results: sEVs derived from MSC with an induced metabolic reprogramming promote the polarization of macrophages towards an anti-inflammatory phenotype by increasing expression of M2 marker CD206+. On the other hand, we observed that the sEVs were able to protect the key components of cartilage tissue by increasing the ratio of COL2/COL1 expression and decreasing the expression of aggrecan degrading enzyme ADAMTS4.
Summary/Conclusion: Our results show that the metabolic reprogramming of MSC improves the therapeutic properties of their sEVs. Indeed, we demonstrate that these sEVs have an anti-inflammatory effect, evidenced by the polarization of macrophages towards an M2 phenotype. Furthermore, sEVs from metabolically modulated MSC showed a chondroprotective effect, evidenced by the increase of the COL2/COL1 expression ratio and a decrease of ADAMTS4, indicating a recovery of the hyaline cartilage phenotype.
Funding: This research was supported by the Agencia Nacional de Investigación y Desarrollo (ANID) from Chile through the grants FONDECYT regular n°1211353; FONDECYT iniciación n°11220549; FONDEF ID: 21I10194.

OS27.04 | Small EVs from inflammation-primed adipose-derived stem cells facilitate tendon healing in a preclinical mouse Achilles tendon injury and repair model

Hua Shen[1]; Ryan Lane[2]; Ratna B. Ray[3]
[1]Washington University School of Medicine, St. Louis, USA; [2]Washington University School of Medicine, Saint Louis, USA; [3]Saint Louis University, Saint Louis, USA

Introduction: Full restoration of tendon structure and function after injury has been a clinical challenge. This is mainly due to profound inflammation and insufficient regeneration after injury. We found EVs from inflammation-primed adipose-derived stem cells (iEVs) reduce inflammation after injury. However, if iEVs may improve tendon structural and functional recovery after injury and the active subgroup and mechanism of iEVs in these actions remain elusive.
Methods: Bulk iEVs were prepared from conditioned medium of IFNγ-primed mouse ASC culture containing 2% EV-free FBS via differential centrifugation, separated by tetraspanin markers, and analyzed via TEM, western blot, DLS, NTA, and flow cytometry. With IACUC approval, the dose-effect of iEVs (0, 1e+09, or 5e+09 iEVs/repair) on tendon healing was assessed in a preclinical mouse Achilles tendon injury and repair model. The active subgroup and the mechanism of action of iEVs were determined in vitro.
Results: iEVs dose-dependently reduced injury-site NF-κB activity in the first week after injury and led to over 2-fold increases in Arg1, Il13, and Il1rn expression and dramatic improvements in collogen deposition and organization at 4 weeks after injury. iEVs also improved mouse ankle movement by up to 45% and 64% at 3 and 4 weeks after injury and reduced the incidence of postoperative tendon rupture/gap formation by 35% at 4 weeks after injury. In vitro studies revealed that iEVs blocked TLR4/NF-κB signaling in IFNγ+LPS activated macrophages and promoted tendon cell proliferation, anabolic genes Postn, Scx, Tnmd, and Igf1 expression, and type I collagen release. The effects of CD9+/CD63+/CD81+ small iEVs were more dramatic than those of bulk iEVs.
Summary/Conclusion: iEVs reduced inflammation and improved tendon structural and functional recovery after injury via modulating macrophage and tendon cell functions. It is to be determined if the identified active subgroup is more effective than bulk iEVs in enhancing tendon repair.
Funding: NIH R21AR075274, WU ICTS/CTRFP4805.

OS27.05 | The therapeutic effect of umbilical cord mesenchymal stromal cell extracellular vesicles in inflammatory arthritis

Rebecca L. Davies[1]; Mairead Hyland[1]; Claire Mennan[1]; Karina Wright[1]; Mark Platt[2]; Oksana Kehoe[1]
[1]Keele University, Oswestry, United Kingdom; [2]Loughborough University, Loughborough, United Kingdom

Introduction: Rheumatoid arthritis (RA) is an inflammatory disease of which there is no cure. Cell therapies using umbilical cord mesenchymal stromal cells (UC-MSCs) are becoming a popular possibility for treatment due to their ability to promote immunosuppression. Extracellular vesicles (EVs) mimic this but offer the advantage of safety (no malignant transformation or rejection), being easily stored and an extended shelf life. Hence, we sought to determine the ability of UC-MSCs, and their derived EVs, to treat RA using an antigen induced model of arthritis (AIA).
Methods: Mice were injected with 5x105 UC-MSCs, or equivalent EVs from the same cell number, 24 hours post-arthritis induction. Cells were characterised by flow cytometry and EVs by Tunable Resistive Pulse Sensing (IZON) and cryo-electron microscopy following isolation using ultracentrifugation with a 30% sucrose cushion. Joint swelling was assessed over 72 hours, after which histological analysis was performed.
Results: Cells displayed the expected phenotype markers for MSCs. EVs from 5x105 cells yielded 2.15x109 particles/ml with a mean size of 166±70nm, of which most particles displayed an EV-like morphology of a double lipid membrane bilayer. Joint swelling decreased for both treatments, in comparison to control, but only MSC-EV treated mice achieved significance ($P < 0.01$) at the 72-hour timepoint. EV superiority over UC-MSCs was reflected in the histological scoring, with EVs achieving a consistently lower mean score in comparison to controls (improved clinical outcome), whilst cells showed higher mean scores (worse clinical outcome).
Summary/Conclusion: In this study, EVs appear to be superior to their cell counterparts when treating an arthritis model. This supports the argument that MSC-EVs may be a suitable therapy to treat RA.
Funding: EPSRC/MRC DTC in Regenerative Medicine, The James Richardson Studentship, Institute of Orthopaedics Ltd., Oswestry and ACORN funding, Keele University.

OS27.06 | Facilitate by-stander effects by EV-mRNA cargo in AAV gene replacement therapy for treating MPS IIIC

Tierra A. Bobo[1]; Michael Robinson[2]; Marina Sokolski-Papkov[2]; Haiyan Fu[2]
[1]UNC-CH, Mebane, USA; [2]UNC-CH, chapel hill, USA

Introduction: Mucopolysaccharidosis (MPS) IIIC is a devastating neuropathic lysosomal storage disease, with no treatment currently available. MPS IIIC is caused by autosomal recessive defects in heparan-α-glucosaminide-N-acetyltransferase (HGSNAT)

gene. HGSNAT is a non-secretive, exclusively trans-lysosomal-membrane enzyme. AAV gene therapy offers a great tool for treating neurogenetic diseases. All cells are known to continuously release extracellular vesicles (EVs) and communicate by exchanging large molecules via EV trafficking. Targeting the unmet need, we developed a vector with an EV-mRNA packaging signal to engender by-stander effects for the normally non-secreted HGSNAT protein.

Methods: We constructed a rAAV vector with an EV-mRNA packaging zip code (ZC) signal linked to human HGSNAT cDNA. We tested it in human MPS IIIC skin fibroblasts to determine rHGSNAT expression, EV packaging of hHGSNAT-mRNA and the correction of lysosomal GAG storage. The conditioned media from transduced MPS IIIC cells were processed for EV purification and characterization. Purified EVs were incubated with non-infected MPS IIIC cells to assess the EV-facilitated hHGSNAT expression and the cross-correction of lysosomal GAG storage.

Results: rAAV-hHGSNATzc vector mediated not only efficient expression of functional HGSNAT protein, but also the release of abundant EVs containing hHGSNAT mRNA to the media. Importantly, incubation with purified EVs from transduced cells resulted in the expression of functional HGSNAT protein and the clearance of GAG contents in non-treated recipient cells.

Summary/Conclusion: This study demonstrates that the incorporation of the ZC signal sequence in rAAV-hHGSNAT vector mediates EV packaging of hHGSNAT-mRNA, which can be transported to recipient cells and translated into functional rHGSNAT protein. Our data strongly support the therapeutic potential of rAAV gene therapy with EV-mRNA-cargo facilitated by-stander effects for treating MPS IIIC. It may therefore reduce the vector dose and potential risks. Further, this technology may also be applicable to broad disease targets involving transcription factors and proteins that are not secreted.

Funding: R21AI146653
NC Biotech Center #2020-GTF-6903

OS28 Late-Breaking Session: EV Methodology Advances

The other abstracts in this session were late breaking and will be included in a late breaking journal addendum after the conference.

OS28.01 | Therapeutic potential of extracellular vesicles from stromal cells produced by turbulence stimulation for regenerative medicine: proof-of-concept in 5 animal models

Amanda Silva[1]; Arthur Berger[2]; Elise Coffin[3]; Max Piffoux[4]; Julien Branchu[4]; Claire Wilhelm[5]; Florence Gazeau[6]; Gabriel Rahmi[3]

[1]Laboratoire Matière et Systèmes Complexes, Paris, France; [2]Université de Paris, Paris, France; [3]HEGP, Paris, France; [4]EVerZom, Paris, France; [5]Institut Curie, Paris, France; [6]CNRS Université de Paris, Paris, France

Introduction: Inspired by extracellular vesicle (EV) released by blood flow, we propose to boost EV release by exerting a controlled turbulence shear stress on adipose stromal cells (ADSCs) during their culture in bioreactors in an unprecedented approach. We evidenced that turbulent flow tuning elicited massive EV release (10 times more and 10 times faster than the classical starvation method). We identified turbulence micro-vortex size as a physical parameter that ensures consistent and massive EV shedding and process scalability. The aim of this study was to investigate the regenerative effect of turbulence EVs.

Methods: The therapeutic potential of turbulence EVs for regenerative medicine was investigated in 5 animal models: (i) a myocardial infarction in mice; (ii) an inflammatory perianal fistula in rats; (iii) a post-surgical colo-cutaneous fistula in rats; (iv) a post-surgical gastro-cutaneous fistula in pigs and (v) an esophageal stricture in pigs.

Results: Our results in the myocardial infarction model in mice evidenced that, when compared to state-of-the-art EVs at the same dose, our turbulence EVs produced the same therapeutic effect. A healing therapeutic effect was also observed in an inflammatory perianal fistula model in rats. Our investigation in a post-surgical colo-cutaneous fistula model in rats demonstrated that turbulence EV biodistribution was improved when EV administration was performed locally combined to a thermo-responsive hydrogel. The therapeutic healing effect of this combined therapy was demonstrated in this colo-cutaneous fistula rat model as well as in the gastro-cutaneous fistula model and the esophageal stricture model, both in pigs.

Summary/Conclusion: The proposed turbulence method is expected to be advantageous in terms of high yield, cost-effectiveness, and time-saving. The therapeutic effect of turbulence EVs featuring the markers and potency of bona fide EVs was demonstrated in 5 animal models indicating the robustness and versatility of the turbulence production method.

Funding: ERC Exocyther (number 852791), ANR Fisther

OS29: EVs and Kidney

Chair: Benedetta Bussolati – Department of Medical Sciences, University of Turin, 10126 Turin, Italy

OS29.01 | Biofluid-derived extracellular vesicles are effectors of immune system dysfunction in children with Idiopathic Nephrotic Syndrome

Giulia Cricrì[1]; Linda Bellucci[1]; Stefania Bruno[2]; Federico Caicci[3]; Stefano Turolo[1]; William Morello[4]; Giovanni Montini[1]; Federica Collino[5]

[1]Laboratory of Translational Research in Paediatric Nephro-urology, Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico, Milan, Italy., Milano, Italy; [2]Department of medical science, University of Turin, Turin, Italy; [3]Department of Biomedical Sciences, University of Padova, Padua, Italy., Padua, Italy; [4]Pediatric Nephrology, Dialysis and Transplant Unit, Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico, Milan, Italy., Milano, Italy; [5]Laboratory of Translational Research in Paediatric Nephro-urology, Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico, Milan, Italy., Milan, Italy

Introduction: Idiopathic Nephrotic Syndrome (NS) is the most common glomerular disease in children with immune-related pathogenesis. Corticosteroids are the first-line treatment, but patients not responsive may progress to end-stage renal disease, requiring dialysis or kidney transplant. In this context, biofluid-derived extracellular vesicles (EVs) can function as carriers of biomarkers for monitoring immune activation and kidney damage in NS.

Methods: PBMCs, sera and urines were obtained from thirty NS patients (steroid-sensitive and resistant) and seven healthy subjects (HS) (approval by local ethical committee). Immune cell and EV (serum and urine) profile was conducted by multicolour flow cytometry. EVs concentration, size and structure were analyzed by NTA and transmission electron microscopy. Filtration/Ultracentrifugation followed by size exclusion chromatography were used to separate NS-EVs for molecular analysis.

Results: Total CD19+, naïve, and switched memory B cells in NS patients were increased compared to HS. Moreover, decreased transitional B cells and increased plasmablasts and plasma cells were found in NS with active proteinuria compared to therapy-responsive patients (remission). We also observed a reduction of Th1 and Th17, an increase of Th2 cells in NS in respect to HS, and reduced levels of Foxp3+ Treg during remission. NTA analysis revealed no differences in EV concentrations among groups, with a slight increase of NS-EV diameter (mode distribution). Biofluid NS-EVs expressed Tetraspanins with a heterogeneous distribution among the patients' subgroups; the highest expression was observed in serum EVs from proteinuria patients. Similarly to the cell compartment, serum NS-EVs showed abnormal lymphocyte markers (CD4, CD25, CD19 and HLA-DR). Interestingly, B/T cell markers were already present in serum EVs at the disease onset. Urine NS-EVs were enriched in adhesion, epithelial and leucocyte molecules, and their number positively correlated with proteinuria levels.

Summary/Conclusion: Change in the biofluid NS-EV profile might represent the bloodstream NS immune dysfunctions, and EVs can act as facilitators of kidney damage in NS children.

Funding: This research was supported by Grant P-0038 from IMPACTsim S.p.A. and by Fondazione La Nuova Speranza ONLUS -lotta alla sclerosi focale.

OS29.02 | Extracellular vesicles rescue Alport glomerular endothelial lipid dysfunction

Hasmik Soloyan[1]; Matthew Thornton[2]; Paolo Cravedi[3]; Andrea Angeletti[4]; Laura Perin[5]; Sargis Sedrakyan[6]

[1]Children's Hospital Los Angeles, Los Angeles, USA; [2]University of Southern California, Los Angeles, USA; [3]Icahn School of Medicine at Mount Sinai, New York, NY, New York, USA; [4]IRCCS Istituto Giannina Gaslini, Genoa, Italy, Genoa, Italy; [5]Children's Hospital Los Angeles-University of Southern California, Los Angeles, USA; [6]Children's Hospital Los Angeles - University of Southern California, Los Angeles, USA

Introduction: Glomerular endothelial dysfunction plays a key role in the development of chronic kidney disease (CKD). We have previously shown that glomerular endothelial cells (GEC) are damaged in Alport syndrome mice (AS, characterized by mutations in collagen IVα3α4α5), manifested by enlarged fenestrations and damaged glycocalix in the early stage of the disease. In the present study we report on the role of altered fatty acid utilization pathways leading to GEC dysfunction in AS, and the role of extracellular vesicles derived from amniotic fluid stem cells (AFSC-EVs) in re-establish lipid homeostasis.

Methods: GEC were isolated from tdTomato-reporter AS and WT mice at 4 months of age by FACS and transcriptome was analyzed and compared by bulk RNA-seq. Tissue samples from patients with AS were used to confirm our findings by immunohistochemistry. In vitro, silencing experiments using human primary GEC were performed to study the role of decreased fatty acid synthase (FASN) in GEC dysfunction, and AFSC-EVs (which contain FASN in their cargo) were applied as a rescue strategy to normalize FASN level and restore lipid homeostasis. Data were confirmed using AFSC-EV FASN-/-.

Results: AS GEC were highly enriched for differentially expressed genes (DEG) associated with cellular metabolism, and lipid metabolism in particular. Genes associated with fatty acid transport (CD36, FATP-1, FATP-2, Fabp3) and synthesis (FASN) among others were downregulated, which was further associated with glomerular accumulation of lipid droplets in mice. We observed similar findings in human biopsy samples from AS patients by histology. In vitro, AFSC-EVs were able to rescue FASN deficiency and improve GEC function, unlike AFSC-EV FASN-/-.
Summary/Conclusion: We report for the first time a lipid metabolic dysfunction in Alport GEC, and the ability of AFSC-EVs to rescue this phenotype. Therefore, better understanding of the functional role of GEC in AS could lead to the development of targeted new therapies for the treatment of this and other forms of CKD.

OS29.03 | Large and small urinary extracellular vesicles (uEV): a source of progressive biomarkers for diabetic nephropathy

Elisabetta AM Verderio[1]; Elisa Tonoli[1]; Melanie Tepus[1]; Maria Pia Savoca[1]; Clare Coveney[1]; David Boocock[1]; Stephen Watson[2]; Dimitris Goumenos[3]; Timothy Johnson[2]
[1]Nottingham Trent University, Nottingham, United Kingdom; [2]The University of Sheffield, Sheffield, United Kingdom; [3]University hospital of Patras, Patras, Greece

Introduction: Diagnosis of chronic kidney disease (CKD) progression through urinary markers could be of great potential to complement renal biopsies and imaging approaches. We aimed at identifying proteome changes of urinary extracellular vesicles (uEV) in progressive versus stable diabetic nephropathy (DN).
Methods: Large and small uEV were isolated by differential (ultra-)centrifugation (1). DN patients were stratified based on stable (n=17) or progressive pathophysiology (n=19) (mean GFR change of 1.9±1.8 and -9.4±1.2 mL/min per year, respectively), and cell-free urines sourced from two CKD-biorepositories upon ethical approval. Tryptic peptides were subjected to RP-HPLC-ESI-MS/MS using a TripleTOF6600+ mass spectrometer, in data dependent (DDA) mode and SWATH®2.0-data independent acquisition (DIA) (SCIEX-OneOmics) mode.
Results: Analysis of uEV via atomic force microscopy, scanning EM and nanoparticle tracking confirmed size (50-250 nm), positive EV-markers (alix, flotillin, TSG101) and absence of negative markers (TOM20, GS28). Urinary EV proteomics versus full urine proteomics was firstly investigated by shot gun proteomics (DDA-MS/MS): the uEV displayed 4-5 more protein identities (at 1% false discovery rate) compared to the full urine depleted of abundant serum proteins, showing the advantage of uEV. Next, cohorts of stable and progressive patients (12 each) were selected for large and small uEV isolation. Qualitative proteomics of small uEVs led to 1439 protein identities, of which 30% were uniquely detected in the progressive cohort. However, only a fraction (12%) were displayed consistently in at least 4-10/12 progressive patients. Similarly, large uEV revealed 1323 protein identities, of which 37% were solely present in progressive patients. Comparative quantitative SWATH-MS showed that a lysosomal membrane component and a nephrotic syndrome-linked lipoprotein are significantly associated with large uEV of progressive patients (N=11 progressive; N=10 stable; >60% confidence), whereas proteins regulating filaments dynamics and plasma membrane protrusions are associated with small progressive uEV (N=12 progressive; N=10 stable; >60% confidence). Validation of three of these progression markers by immune-blotting confirmed their differential expression in the progressive cohort, both in large and small uEV.
Summary/Conclusion: Urinary EV (large and small) have a great potential as a source of progression biomarkers in alternative to full urine, with possible application in clinical trials or clinical routine in the future, if their reproducibility can be ascertained.
Funding: Nottingham Trent University external engagement award and Vice-Chancellor studentship.
(1) Furini et al (2018) J Am Soc Nephrol. 29:880-905.

OS29.04 | Extracellular vesicle subsets released during normothermic machine perfusion are associated with human kidney characteristics

Wouter W. Woud[1]; Asel Arykbaeva[2]; Ian Alwayn[2]; Carla Baan[1]; Robert Minee[3]; Martin Hoogduijn[1]; Karin Boer[1]
[1]Erasmus MC Transplant Institute, Department of Internal Medicine, University Medical Center Rotterdam, Rotterdam, The Netherlands, Rotterdam, Netherlands; [2]Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands, Leiden, Netherlands; [3]Erasmus MC Transplant Institute, Department of Surgery, Division of Hepato-Pancreato-Biliary and Transplant Surgery, Erasmus MC, University Medical Center Rotterdam, The Netherlands, Rotterdam, Netherlands

Introduction: Extracellular Vesicles (EVs) represent stable, tissue specific nano-sized particles that reflect the conditional state of their tissue of origin. Normothermic Machine Perfusion (NMP), aimed at restoration of cellular metabolism and function to organs, offers the possibility to assess graft status prior to transplantation through analysis of biomarkers in the perfusion fluids.

Here, the dynamic release and phenotype of kidney EVs released during NMP were analyzed to examine whether EVs could function as a potential biomarker for assessing kidney quality before transplantation.

Methods: Eight discarded kidneys (~13 ± 5 hours of cold ischemia, age 68 ± 7 (mean ± standard deviation), all male) were perfused in a closed system at 37C for 6 hours. Perfusates were taken before and at 1, 3 and 6 hours and examined with Nanoparticle Tracking Analysis (NTA) and Imaging Flow Cytometry (IFCM). For IFCM, perfusates were stained with the tetraspanin EV markers CD9, CD63 or CD81, or a mix of the three markers in combination with CFSE to identify, quantify and characterize EVs.

Results: Analysis of perfusates with NTA revealed that the majority of nanoparticles present in the perfusates are < 300 nm. Using IFCM, we selectively studied these small nanoparticles. For CFSE and the mix of tetraspanin double-positive EVs, we observed a ~700 / 740 / 560 fold increase compared to EV levels before perfusion at 1, 3 and 6 hours of NMP, respectively. Especially after 1 hour of NMP, double-positive EV levels were found to be positively correlated with donor age whilst negative correlations were found for cold ischemia time. Furthermore, tetraspanin CD81 was found to represent the majority (~70%) of the excreted double-positive EV (CD9: ~15% / CD63 < 10%).

Summary/Conclusion: EVs are excreted during NMP with highest excretion levels during the first hour of perfusion. Tetraspanin CD81 is predominantly present on these EVs. The characterization of the excreted EVs as well as their correlation with clinical parameters provides a starting point to study their role as potential biomarkers of kidney quality.

Funding: The authors declare no funding-related or other conflicts of interest.

OS29.05 | Novel avenue of allograft monitoring: direct measurement of donor-derived extracellular vesicles in human plasma

Wouter W. Woud; Dennis Hesselink; Martin Hoogduijn; Carla Baan; Karin Boer

Erasmus MC Transplant Institute, Department of Internal Medicine, University Medical Center Rotterdam, Rotterdam, The Netherlands, Rotterdam, Netherlands

Introduction: Extracellular Vesicles (EV) - regarded as "snapshots" of their cell of origin - represent promising liquid biomarkers to monitor allograft function post transplantation. Recently, we developed an imaging flow cytometry (IFCM) based protocol to identify and characterize EV ≤ 400 nm in molecularly complex samples such as human plasma without prior isolation of EV. Using this protocol, we measure allograft derived EV based on HLA phenotype as a first step to detect allograft specific EV in the circulation of kidney transplant (KTx) recipients.

Methods: EDTA blood samples from kidney transplant donors (HLA-A2+, n=21) and recipients (HLA-A2-, n=33) were collected before transplantation as well as 3 days, 7 days, 6 months and during 'for-cause' biopsies (recipients only) after transplantation. Platelet-poor plasma (PPP) was stained with a donor-specific HLA antibody (HLA-A2) in combination with a common EV marker (tetraspanin CD9) and measured using standardized IFCM.

Results: Quantification and comparison of CD9+/HLA-A2+ double-positive EV showed 1.1E7 ± 8.9E6 vs 3.5E5 ± 2.5E5 objects/mL for donor and recipient (pre-KTx) EV respectively, with recipients A2- EV concentrations representing background level of the machine. CV values for inter- and intra-assay variability were 16% and 11%, respectively. Serial dilution of A2+ PPP in A2- PPP (n=5) showed a linear reduction in the numbers of CD9+/HLA-A2+ EV according to the dilution rate whilst total CD9+ EV levels remained unchanged. The lower limit of detection of our protocol was defined as the dilution at which point CD9+/HLA-A2+ EV dropped below baseline (A2- PPP), and was determined to be ~1% of the concentrations measured in undiluted A2+ PPP. Measurement of longitudinally collected recipient samples revealed the detection of allograft derived EV as soon as 3 days – but up to at least 6 months – after KTx.

Summary/Conclusion: Here we demonstrate for the first time the detection of allograft derived EV in the circulation of KTx recipients in unprocessed human plasma samples. Identification, quantification and characterization of these EV opens up the possibility to monitor these EV over time after transplantation, and may prove to be a minimally-invasive biomarker.

Funding: The authors declare no funding-related or other conflicts of interest.

OS30: EVs as Early Biomarkers of Tumors

Chair: Susana Garcia-Silva – Spanish National Cancer Research Centre (CNIO)

Chair: Irina Nazarenko – Institute for Infection Prevention and Hospital Epidemiology, Faculty of Medicine, University Medical Center Freiburg, Freiburg, Germany

OS30.01 | Small extracellular vesicles (sEVs) as biomarkers in head and neck squamous cell carcinoma (HNSCC)

My Mahoney[1]; Brianna L. Hill[1]; Joseph P. Flemming[1]; Sydney L. Gilmore[1]; Alban Linnenbach[2]; Larry A. Harshyne[1]; Andrew South[1]; Joseph Curry[1]; Adam Luginbuhl[1]
[1]Thomas Jefferson University, Philadelphia, USA; [2]Thomas Jefferson University, philadelphia, USA

Introduction: HNSCC is the sixth most common cancer worldwide, often caused by chronic alcohol and tobacco use or human papilloma virus (HPV) infection. Despite this prevalence, there are no clinical biomarkers to delineate early disease, HPV status, and immune checkpoint inhibitor (ICI) response. sEVs are emerging as potential cancer biomarkers due to their critical roles in cell-to-cell communication and cancer development.
Methods: Circulating plasma of HNSCC patients and control volunteers was used to isolate CD9, CD63, or CD81 positive sEVs by the immunoaffinity ExoRelease method. The sEVs were characterized by Western blot and quantified by Nanoparticle Tracking Analysis. sEV samples were subjected to bead-based Multiplex profiling of 12 cancer-specific antigens (Luminex) as sEV surface markers, and results were compared to corresponding total plasma. Machine learning (Weka Explorer) and pathway analysis (miRNet) were then performed.
Results: Analysis of the Luminex data demonstrated that several known cancer biomarkers were detected on the surface of HNSCC sEVs, with statistically significant differences between HPV- and HPV+ patients. Weka analysis demonstrated that multiple cancer antigens had a moderate correlation with HPV status, including HE4, b-HCG, AFP, SCF, CEA, CA124, and HGF. Of interest, HE4, a serine protease inhibitor that may protect sEVs from degradation, was elevated in HPV- patients. miRNet target analysis revealed that miR-155-5p, miR-374a-5p, miR-1343-3p, and miR-146a-5p target HE4. KEGG pathway analysis determined that these miRNAs had the highest number of shared targets in pathways in cancer and focal adhesion. Additional GO:BP pathway analysis showed that these miRNAs also had numerous shared targets in the regulation of DNA binding. Using qPCR, it has been demonstrated that sEVs from HPV- patients have lower levels of miR-146a-5p, as compared to normal healthy donors. As HPV- patients appear to have high HE4 and low miR146a while HPV+ patients have less HE4 and more miR146a, this data may demonstrate a correlation between HE4 levels and the aggressiveness of HPV- HNSCC.
Summary/Conclusion: These combined studies suggest the potential for identifiable cancer biomarkers, such as HE4, in HNSCC that could provide improvements in diagnosis and treatment.
Funding: This work was supported by a NIAMS-R01-AR074314 to MGM, T32-AA007463 to BLH (trainee), and the Sidney Kimmel Cancer Center (Thomas Jefferson University) Support grant 5P30CA056036-20.

OS30.02 | Plasma extracellular vesicles-based liquid biopsy for Glioblastoma diagnosis and personalization of care

Elena Zaccheroni[1]; Sofia Fallo[2]; Stefania Faletti[2]; Daniela Osti[2]; Massimiliano Del Bene[3]; Francesco DiMeco[3]; Giuliana Pelicci[2]
[1]1. Department of Experimental Oncology, European Institute of Oncology (IEO), IRCCS, Milan, Italy, Milano, Italy; [2]1. Department of Experimental Oncology, European Institute of Oncology (IEO), IRCCS, Milan, Italy, Milan, Italy; [3]2. Department of Neurosurgery, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy, Milan, Italy

Introduction: Glioblastoma (GBM) is a lethal tumor of the Central Nervous System. Diagnosis relies on MRI and brain biopsy which are challenged by low specificity, sensitivity, invasive nature, and high cost. The molecular dynamism of GBM hampers longitudinal monitoring of patients that can't face continual brain surgery to tailor the therapy to the different stages of the disease.
Liquid biopsy is a promising bedside-friendly procedure for early cancer diagnostics and routine evaluation of the tumor status, and blood carries a plethora of tumor-derived molecules of which EVs hold potential as circulating biomarkers.
Methods: We separate EVs from soluble plasma components (proteins and HDL) by Size Exclusion Chromatography (SEC), starting from 1ml of pre-operative, platelet-free plasma.
EVs are characterized by (i) immunoblot and FACS for the presence of known EV markers; (ii) TEM for morphology and size; (iii) Tunable Resistive Pulse Sensing (TRPS) for size, concentration, and surface charge; (iv) RNAseq for RNA cargo profiling.

Results: SEC-derived EVs express CD9, CD81, and Flotillin1 as EV-related markers and lack Cal-nexin as a non-EV marker. We probed ApoA1 and Albumin to assess contamination of plasma proteins and found a different degree of positivity for the two contamination markers in EVs from different patients.
EVs quantification by TRPS confirms their enrichment in the plasma from GBM patients.
We extracted EV-RNA: its size is < 200nt and the total yield ranges between 1 and 10 ng among different samples, which is suitable for RNAseq to identify mutations, splicing isoforms, translocations, fused mRNAs, and levels of GBM-specific mRNAs.
Summary/Conclusion: Increased plasma-EVs concentration is a reliable biomarker for GBM presence and can be assessed by a clinically applicable protocol that pairs SEC and TRPS.
The study of EV-associated RNA will permit tumor profiling with a focus on the actionable targets for a personalized approach.
Funding: This project is funded by the ERAPerMed grant.

OS30.04 | Identification of an inflammatory biomarker signature in plasma-derived extracellular vesicles of glioblastoma patients

Chiara Cilibrasi[1]; Thomas Simon[2]; Marian Vintu[1]; Christos Tolias[1]; Mark Samuels[1]; Nektarios K. Mazarakis[3]; Murat Eravci[1]; Nicolas Stewart[4]; Giles Critchley[5]; Georgios Giamas[1]
[1]Department of Biochemistry and Biomedicine, School of Life Sciences, University of Sussex, Brighton, BN1 9QG, UK, Brighton, United Kingdom; [2]Department of Biochemistry and Biomedicine, School of Life Sciences, University of Sussex, Brighton, BN1 9QG, UK, Los Angeles, USA; [3]Department of Neurosurgery, Beaumont Hospital, Dublin, D09 V2N0, Ireland, Dublin, Ireland; [4]Centre for Stress and Age Related Diseases, School of Applied Sciences, University of Brighton, Brighton, BN2 4GJ, UK, Brighton, United Kingdom; [5]Department of Neurosurgery, University Hospitals Sussex, Brighton, BN2 5BE, UK, Brighton, United Kingdom

Introduction: Diagnosis of Glioblastoma (GBM), an aggressive type of brain tumour, remains a clinical challenge, currently relying on symptomatic presentation of the tumour, brain imaging and invasive biopsy. Description of effective biomarkers in biofluids could therefore prove invaluable in GBM diagnosis. Extracellular vesicles (EVs) are essential to intercellular crosstalk in the tumour bulk and circulating EVs have been described as a potential reservoir of GBM biomarkers. Therefore, EV-based liquid biopsies have been suggested as a promising tool for GBM diagnosis and follow up.
Methods: Healthy donors and consenting GBM patients were enrolled at the Royal Sussex County Hospital (Brighton, UK) and an ethical approval was obtained for a prospective laboratory study. To identify GBM specific proteins, small EVs (sEVs) were isolated from plasma samples using differential ultracentrifugation and validated through Nanoparticles tracking analysis, transmission electron microscopy and detection of known sEVs markers such as CD9, CD63, CD81 and HSP70. sEVs content was characterised through mass spectrometry and bioinformatic tools.
Results: Our data indicate the presence of a GBM inflammatory biomarker signature comprising members of the complement and regulators of inflammation and coagulation including VWF, FCGBP, C3, PROS1, and SERPINA1. Bioinformatic analysis highlighted that all potential markers exclusively identified in patient samples had already been linked with either GBM diagnosis, prognosis or associated signalling, suggesting that sEVs protein cargo could mirror the landscape of the original tumour and that selective circulating sEV-derived proteins might be used as hallmarks for GBM patients.
Summary/Conclusion: Overall, this study is a step forward in the development of a non-invasive liquid biopsy approach for the identification of valuable biomarkers that could significantly improve GBM diagnosis and, consequently, patients' prognosis and quality of life.
Funding: This research was funded by Action Against Cancer, grant number ID6758/G1867

OS30.06 | Vesicular IL-8 and miR-146a Correlate with Response to Immunotherapy in Head and Neck Squamous Cell Carcinoma

Brianna L. Hill[1]; Alyssa N. Calder[2]; Joseph Flemming[2]; Larry Harshyne[2]; Sydney Gilmore[2]; Melissa Trofa[2]; Sean Daniels[3]; Alban Linnenbach[3]; Ulbaldo Martinez-Outshoorn[3]; Andrew South[2]; Joseph Curry[2]; Adam Luginbuhl[2]; My Mahoney[2]
[1]Thomas Jefferson University, Mohrsville, USA; [2]Thomas Jefferson University, Philadelphia, USA; [3]Thomas Jefferson University, philadelphia, USA

Introduction: HNSCC tumors harbor distinct genetic, immunogenic, and clinical characteristics. Anti-programmed-death-1 (aPD1) immune checkpoint inhibition (ICI) has triggered a recent paradigm shift in treatment; yet, only a fraction of patients respond and there is a critical lack of biomarkers. Small extracellular vesicles (sEVs) are emerging as important sources of biomarkers; however, the isolation and purification of sEVs from patient plasma poses a major challenge.

Methods: The Immunoaffinity ExoRelease™ method was used to isolate CD9, CD63 or CD81 positive sEVs from the pretreatment plasma of previously untreated HNSCC patients undergoing neoadjuvant aPD1 ICI with nivolumab in a clinical trial. These sEVs were characterized using Nanoparticle Tracking Analysis and Western blot. The miRNA content of sEVs was analyzed by miRNAseq (Aligned to Genome Reference Consortium Human Build 38 (GRCh38)) and machine learning (Weka Explorer) was used to identify miRNAs including miR-146a, that strongly correlated with response to treatment. To investigate the impact of miR-146a in vitro, HNSCC UMSCC1 cells were transduced using the XMIRXpress system to overexpress miR-146a and target them for loading into sEVs. Proliferation was assessed by WST-1 assay.
Results: Profiling by miRNAseq and analysis by Weka Explorer revealed that 12 miRNAs were very strongly correlated with response prior to treatment. Reactome Pathway analysis of the targets of the miRNAs that were correlated with response were involved in immune-related pathways. While the levels of miRNAs did not change in response to treatment, there was a statistically higher level of anti-inflammatory miRNAs, including miR-146a, which secondarily targets the pro-inflammatory cytokine IL-8 in responders vs non-responders. Circulating IL-8 was also down-regulated in HPV(+) responders. In response to mir-146a, UMSCC1 cells showed decreased proliferation and down-regulation of the miR-146a target IRAK1, a kinase that leads to the NFkB-mediated expression of IL-8. UMSCC1 cells treated with miR-146a-loaded sEVs lead to a dramatic reduction in IL-8.
Summary/Conclusion: Pretreatment peripheral blood sEV miR-146a correlated with clinical and pathologic response to neoadjuvant aPD1 ICI in HNSCC patients. This novel finding may suggest promise for both mechanistic understanding of therapeutic response and biomarker potential.
Funding: This work was supported by a NIAMS-R01-AR074314 to MGM and T32- AA007463 to BLH (trainee).

Poster Presentations

PT01: Physiology and Pathology: Stem Cells and Tissue Injury Repair

Chair: Dirk Strunk – Cell Therapy Institute, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI - TReCS), Paracelsus Medical University (PMU), Salzburg, Austria

Chair: Daniel W Hagey – Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

PT01.01 | A comparison of anti-inflammatory properties of different preparations of IL-1β primed mesenchymal stromal cells conditioned medium

Marianne DEDIER[1]; Muriel NIVET[2]; Juliette Peltzer[2]; Sylvie Goulinet[3]; Philippe Mauduit[3]; Sébastien Banzet[2]; Marina Trouillas[2]
[1]Institut de Recherche Biomédicale des Armées / INSERM UMR-MD 1197, CLAMART, France; [2]Institut de Recherche Biomédicale des Armées / INSERM UMR-MD 1197, Clamart, France; [3]Inserm UMR-MD 1197, Villejuif, France

Introduction: Mesenchymal Stromal Cells (MSC) have become an emerging therapeutic option in the field of tissue repair and especially for wound healing through their immunomodulatory and remodeling properties. These last years, these properties seem to be closely linked to their secretory products, also called conditioned medium (CM), composed of free bioactive molecules but also extracellular vesicles (EV) containing lipids, proteins and small RNAs. The discovery of their plasticity with regard to various environmental stimuli has more recently opened up new therapeutic perspectives, such as priming strategies. Our team previously showed that IL-1β primed MSCs CM can improve wound healing, but entities which carry MSC's potency are not clearly established. The aim of the present study was to compare the anti-inflammatory efficacy of free bioactive molecules or EV concentrated from MSC secretome with an in vitro test.
Methods: CM were collected after 48h of IL-1β primed MSC secretion in MEMα medium. Tangential flow filtration was used to isolate and concentrate interesting products contained in CM. 500 or 10 KD cut-off mPES filter allowed to respectively isolate EV, EV and soluble molecules or soluble molecules alone when associated with a successive 10KD filtration. CM were characterized by NTA, protein quantification and ELISA analysis before and after filtration. These different preparations were added to LPS-challenged THP-1 cell line at different concentration of protein or EV. After 24H, TNFα and IL1-RA secretions were analyzed by ELISA.
Results: These different preparations reduced similarly the inflammatory response of LPS-challenged THP-1 cell line as represented by a strong decrease of TNF-α and an increase of IL-1RA secretions at the minimal dose of 10 μg/ml of proteins or 1x108 particles/ml.

Summary/Conclusion: Overall, this study underlines the importance of the process on product's efficacy and would benefit for future clinical wound healing applications.
Funding: PhD scholarship is funded by "Agence Innovation Défense" and "Direction Générale de l'Armement".

PT01.02 | ADSC-EVs and macrophage polarisation in fat grafting for post-mastectomy breast reconstruction

Emma Symonds[1]; Kathryn Hally[1]; Alexander Brown[2]; Margaret Currie[3]; Elizabeth Dennett[1]; Ineke Meredith[4]; Kirsty M. Danielson[1]
[1]Department of Surgery and Anaesthesia, University of Otago, Wellington, New Zealand, Wellington, New Zealand; [2]Department of Surgery, Wellington Regional Hospital, Wellington, New Zealand, Wellington, New Zealand; [3]Mackenzie Cancer Research Group, University of Otago, Christchurch, New Zealand, Wellington, New Zealand; [4]Department of General Surgery, Wakefield Hospital, Wellington, New Zealand, Wellington, New Zealand

Introduction: Despite autologous fat grafting (AFG) being a favourable post-mastectomy breast reconstruction option, graft retention rates are variable. Enriching the grafted tissue with extracellular vesicles (EVs) released from adipose derived stem cells (ADSCs) may promote retention. However, we need to understand the immunogenic effect of ADSC-EVs on resident cells in the breast cavity, such as macrophages. We aimed to characterise the immune effect of ADSC-EVs on macrophages in vitro.
Methods: Following written informed consent, fat samples were collected from three women undergoing AFG in Wellington, New Zealand. ADSCs were isolated enzymatically and cultured in EV-depleted media. ADSC-EVs were isolated from media using size exclusion columns on an automated fraction collector (qEV35, Izon) and characterised by qNano, Cryo-TEM and Western Blotting. Monocytes were isolated from whole blood of two healthy volunteers and cultured towards M0 (unpolarised), M1-like (pro-inflammatory) or M2-like (anti-inflammatory) phenotypes. ADSC-EVs were added to cultures for 48hrs to achieve a "high" (10:1 of ADSC-EVs:macrophages) or "low" (1:1) exposure. Macrophages±EVs were examined using a flow cytometry panel interrogating antigen presentation (CD86, CD80, HLA-DR), adhesion (CD11b), pattern recognition (TLR4, CD14) and scavenger receptor expression (CD36, CD163).
Results: High exposure of ADSC-EVs significantly reduced expression of markers associated with antigen presentation, adhesion and scavenging on all macrophage phenotypes. M0 and M2-like phenotypes were unaltered by low EV exposure although, M1-like macrophages increased expression of CD86, HLA-DR and CD36 (20-64% increase in marker expression, $p < 0.05$). Pattern recognition receptor expression was unaffected by ADSC-EVs at either exposure.
Summary/Conclusion: ADSC-EVs appear to be complex regulators of macrophage function that, dependant on exposure, potentially dampen traditional pro-inflammatory pathways.
Funding: This study was funded by the Marsden Fund and HRC NZ.

PT01.03 | An organoid-based kidney-liver Multi-Organ-on-a-Chip model to study the therapeutic effects and bio-distribution of mesenchymal stromal cell-derived extracellular vesicles

Vivian V. Nguyen[1]; Shicheng Ye[2]; Vasiliki Gkouzioti[1]; Dennis Melkert[1]; Sofia Siti[1]; Bart de Jong[1]; Bart Spee[2]; Reyk Horland[3]; Luc van der Laan[4]; Marianne Verhaar[1]; Bas W. van Balkom[1]
[1]UMC Utrecht, Utrecht, Netherlands; [2]Utrecht University, Utrecht, Netherlands; [3]TissUse GmbH, Berlin, Germany; [4]Erasmus Medical Center, Rotterdan, Netherlands

Introduction: Mesenchymal stromal cell (MSC)-derived small EVs (sEVs) show therapeutic potential in multiple disease models, including kidney injury. Clinical translation of sEVs requires further preclinical and regulatory developments, like elucidation of the mode of action (MoA) and formulation of safety and release criteria. sEVs tend to accumulate in the liver and at sites of injury. Biodistribution knowledge is crucial to assess MoA, efficacy and safety, and can be obtained using labelled sEVs in animal models, which come with ethical concerns, are time-consuming and expensive, and do not represent all human physiological processes equally well. We hypothesized that, based on developments in microfluidics and human organoid biology, in vitro multi-organ-on-a-chip (MOC) models allow to study effects of sEVs in human organs in a semi-systemic manner.
Methods: Human kidney- and liver organoids were combined by microfluidics. Barrier integrity, transport (kidney) and transport and glycogen storage (liver) were assessed. A kidney injury model was established. MSC-sEVs were isolated via sequential ultracentrifugation and characterized by sucrose density gradient analysis, immunoblotting and nanoparticle tracking analysis. PKH67-labelled sEVs were applied in the kidney injury model and recovery and biodistribution were analyzed.
Results: In our MOC, kidney- and liver- organoids maintained physiological functions. The renal epithelium showed barrier and transport function, and liver organoids showed transport and glycogen storage capability. Upon H2O2 treatment, renal barrier

function declined, and recovered when MSC-sEVs were applied. Microscopic analysis show increased sEV accumulation in liver- and kidney cells after injury versus healthy control condition.

Summary/Conclusion: Our MOC model recapitulates the efficacy and biodistribution of MSC-sEVs as observed in animal models. Its human background allows for in-depth analysis of the MoA and identification of potential side effects.

Funding: This research was funded by Health~Holland TKI-LSH, the Dutch Heart Foundation, the EU H2020 research and innovation programme under Marie S. Curie Cofund RESCUE grant agreement No 801540 (to M.C.V.), Gravitation Program "Materials Driven Regeneration", funded by the Netherlands Organization for Scientific Research, the Dutch Kidney Foundation, the Dutch Society for the Replacement of Animal Testing, ZonMW/SGF

PT01.04 | Bioderived therapeutics in chronic liver diseases

Aymar A. Ganguin[1]; Cristina Zivko[2]; Paola Luciani[3]
[1]University of Bern, Bern, Switzerland; [2]Johns Hopkins, Baltimore, USA; [3]University of Bern DCBP, Bern, Switzerland

Introduction: Hepatic stellate cells (HSCs) play a key role in liver fibrosis, a pathological phenomenon often associated with the progression of various chronic liver diseases, including viral hepatitis, alcoholic liver disease, and metabolic associated fatty liver disease (MAFLD), conditions which together account for >1.3 million worldwide yearly deaths. Following liver injury, HSCs may transdifferentiate from a quiescent (anti-fibrotic) into a myofibroblast-like pro-fibrotic (activated) state, which, if unresolved, leads to fibrosis.1 C. Zivko et al. showed that extracellular vesicles (EVs) shed by quiescent LX-2 (human HSC cell line) cells can trigger an anti-fibrotic effect on naïve LX-2 cells.2 Therefore, we hypothesised that LX-2 produced cell- derived nanovesicles (cdNVs) will preserve the fibrosis-resolving features of EVs and additionally increase the therapeutic applicability due to higher yield and better scaling potential compared to standard EVs.

Methods: CdNVs were produced by serial extrusion3 of confluent LX-2 and purified by an iodixanol gradient, followed either by size exclusion chromatography (SEC) and ultracentrifugation (UC), or by UC only. LX-2 cells were either treated with polyenylphosphatidylcholines-rich lipid S80 (anti-fibrotic) or with transforming growth factor β1 (TGF-β1, pro-fibrotic) prior to cdNV production. The formed particles were characterized by concentration and size using nanoparticle tracking analysis, as well as by their protein content using a micro bicinchoninic acid (BCA) assay. The effect of cdNVs from differently treated LX-2 cells on seeded LX-2 (untreated) cells was investigated with fluorescent microscopy4, AdipoRedTM assay and qPCR.

Results: CdNVs from differently treated LX-2 yielded similar yields, protein amounts, size distribution, and zeta potential values. The yield for UC purified cdNVs was 2.20 to 8.20-fold higher than that of EVs, while purification via SEC + UC cdNVs led to a 1.04 to 6.10-fold increase. Fluorescent microscopy as well as AdipoRed assay results revealed an increase in cytosolic lipid droplets in LX-2 upon cdNV administration, which is directly correlated with fibrosis resolution.

Summary/Conclusion: Our results suggest an anti-fibrotic potential in cdNVs which could lead to the development of cell-derived therapeutics against liver fibrosis.

Funding: C.Z. and P.L. gratefully acknowledge the Phospholipid Research Centre for the financial support (Grant ID: PLU-2017-056/2-1).

PT01.05 | Effect of extracellular vesicles derived from platelets and from mesenchymal stromal cells on an in vitro model of osteoarthritis-induced cartilage explants

Maria Antònia Forteza-Genestra[1]; Miquel Antich-Rosselló[2]; Guillem Ramis-Munar[3]; Javier Calvo[4]; Antoni Gayà[4]; Marta Monjo[5]; Joana Maria Ramis[5]
[1]Institut d'investigació Sanitària de les Illes Balears, Palma, Spain; [2]Institut d'investigació Sanitària de les Illes Balears, Palma De Mallorca, Spain; [3]Cellomics Unit, Institut Universitari d'Investigació en Ciències de la Salut (IUNICS), University of Balearic Islands, Palma, Spain; [4]Banc de Sang i Teixits de les Illes Balears, Palma, Spain; [5]Universitat de les Illes Balears, Palma, Spain

Introduction: Extracellular vesicles (EVs) are being explored for regenerative medicine, and one of the target diseases is osteoarthritis (OA), whose current treatments present secondary effects and limited efficacy. Since platelet rich plasma (PRP) has shown promising effects in OA and platelet lysate (PL) is being studied as its alternative since it presents some advantages compared to PRP, specifically EVs present in PL seem to be responsible for its regenerative effect. However, EVs from other sources such as mesenchymal stromal cells (MSCs) are also being studied as a possible treatment for OA. Thus, we aimed at evaluating the effectiveness of PL-derived EVs (pEVs) as a new treatment for OA in an in vitro OA-induced model using human cartilage explants compared to EVs derived from conditioned media of human umbilical cord MSC (hUC-MSCs).

Methods: EVs isolated by size exclusion chromatography from PL or conditioned media of hUC-MSCs were characterized in terms of size and particle concentration by transmission electron microscopy, nanoparticle tracking analysis and in terms of

protein content by total protein quantification and western blot. After the inflammation of human cartilage explants was established (OA-induced in vitro model), the explants were treated with PL-derived EVs (pEVs) or conditioned media of hUC-MSCs derived EVs (cEVs) for 14 days. Then, DNA, glycosaminoglycans and collagen content was quantified. Moreover, an histological study was performed with toluidine blue and Sirius red staining. Finally, EVs uptake was monitored through a 5-hour study using PKH-26 labelled EVs.

Results: pEVs and cEVs presented typical tetraspanin markers and no differences were found in terms of size and particle concentration. pEVs treated cartilage explants presented statistically significant higher content of collagen compared to control and cEVs treated groups. Higher DNA content was also found for the pEVs treated group compared to the OA group without treatment. However, no differences were found in GAG quantification nor in the in vitro uptake within any group.

Summary/Conclusion: Higher collagen content on the extracellular matrix of inflamed cartilage explants was observed when treatment with pEVs was used, showing better results than cEVs. However, further studies using different models are needed in order to verify if pEVs are good candidates for OA treatment.

Funding: This research was funded by Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, co-funded by the ESF European Social Fund and the ERDF European Regional Development Fund (MS16/00124; CP16/00124), PROGRAMA JUNIOR del proyecto TALENT PLUS, construyendo SALUD, generando VALOR (JUNIOR01/18), financed by the sustainable tourism tax of the Balearic Islands, and the Direcció General d'Investigació and Conselleria d'Investigació, Govern Balear (FPI/2046/2017).

PT01.06 | Extracellular Vesicles: Novel Mediators of Peritoneal Adhesions Formation?

Miriam Sandanusová[1]; Anna Kocurková[1]; Michaela Kerberová[1]; Kristýna Turková[2]; Lukáš Kubala[1]; Gabriela Ambrožová[3]
[1]Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic, Brno, Czech Republic; [2]Institute of Biophysics of the Czech Academy of Sciences, Kralovopolska 135, 61265 Brno, Czech Republic, Brno, Czech Republic; [3]Institute of Biophysics, Academy of Sciences of the Czech Republic, Brno, Czech Republic, Brno, Czech Republic

Introduction: Peritoneal adhesions (PAs) represent one of the major complications following abdominal surgery leading to symptoms such as chronic pain and infertility. PA development is primarily caused by activation of the mesothelial layer in the peritoneal membrane, resulting in the transition of mesothelial cells and fibroblasts to pro-fibrotic phenotype. PA formation is an intricate process where inflammation, hypoxia, and deregulated fibrinolysis take place, all accompanied by extensive intercellular communication. Here, we aimed to provide a novel insight into the mechanism of PA formation by describing the involvement of EVs in the process.

Methods: Two subpopulations of EVs (small and large) were isolated by multistep ultracentrifugation from peritoneal fluid of healthy C57Bl/6J mice and mice with induced PAs. Changes in EVs production were evaluated 1, 3 and 10 days after PA induction. EVs analyses included determination of total protein concentration, characterization of protein expression by western blotting, particle number assessment by MADLS®, and visualization by cryo-EM.

Results: The production of EVs was increased in the peritoneum of fibrotic mice, especially 1 and 3 days after PA induction. Besides, the pro-fibrotic marker TGF-beta was detected in the EVs fractions isolated from mice 1 and 3 days after PA induction; the increase being statistically significant in the 1-day PA group when compared to healthy controls. Moreover, mesothelin was present in the peritoneal EVs, suggesting mesothelial origin of the EVs.

Summary/Conclusion: Our results provide the first evidence that EVs play role in PA formation. Understanding the exact involvement of EVs in the peritoneal fibrosis development may advance therapeutic strategies targeting the protection of physiological functions of the peritoneal membrane.

Funding: European Regional Development Fund - Project INBIO (CZ.02.1.01/0.0/0.0/16_026/0008451); GAMU (MUNI/C/0023/2020).

PT01.07 | Extracellular vesicle and modulation of miR-93 in kidney disease

Charmi Dedhia[1]; Paolo Neviani[1]; Hasmik Soloyan[1]; Benedetta Bussolati[2]; Sargis Sedrakyan[3]; Laura Perin[4]
[1]Children's Hospital Los Angeles, Los Angeles, USA; [2]Department of Medical Sciences, University of Turin, 10126 Turin, Italy, Torino, Italy; [3]Children's Hospital Los Angeles - University of Southern California, Los Angeles, USA; [4]Children's Hospital Los Angeles-University of Southern California, Los Angeles, USA

Introduction: Modulation of miRNAs in cells of the glomerular filtration barrier is associated with renal diseases. Our data indicate that miR-93, a potent regulator of glomerular damage, is down-regulated in glomeruli of mice with Alport syndrome (AS, our model of the renal disease characterized by a mutation in coll4a5) as well as in glomeruli of AS patients. Here, we

investigated the role of hEVs derived from human amniotic fluid stem cells (hAFSC) in disease-modifying activity in vitro and in vivo by regulation of miR-93.

Methods: hEVs were isolated by differential ultracentrifugation (from multiple consented donors), characterized by Guava easyCyte cytometry, Exoview, immune-modulatory activity (Mixed Lymphocyte Culture), RNA-seq, and proteomics. KO hEVs for miR-93 (hEVsmir-93-/-) were generated; transfer and modulation of miR-93 (and its targets) in damaged human glomerular was evaluated in vitro. hEV therapeutic effect was also evaluated in Alport mice by biodistribution, RNA-seq, spatial transcriptomics, renal function, and survival.

Results: hEV (~170nm) yield is ~2.8x1010EVs/1x106cells/24hrs. hEVs express CD9, CD63 and CD81, VEGFR1, CD73, CD44, and CD90. hEVs do not initiate activation PBMC and downregulate their response after activation. Proteomics identified 675 proteins and RNA-seq 2,535 miRs, with miR-93 higher expressed. In vitro, hEVsmir-93-/- did not rescue damage in glomerular cells vs hEVsmir-93+/+. The rescue was confirmed by direct transfer of miR-93 by hEVs to the targeted cells. When injected in AS mice, hEVs localized in the kidney, corrected proteinuria and prolong the lifespan. No side effects were noted. RNA-seq of glomeruli from injected mice showed similar gene expression patterns to WT, by cluster analysis. Spatial transcriptomics data indicated that hEVs modulated pathways involved glomerular cell survival and remodeling (such as) VEGF, FGF, TNF, and preserved glomerular cell structure and function.

Summary/Conclusion: hAFSC-EVs modulated pathways that are central to glomerular homeostasis and preserved glomeruli structure with improved kidney function through the transfer of miR-93. This suggests the possibility of using hAFSC-EVs as a new therapeutic option for treating AS.

Funding: NIH R01 R01DK121037

PT01.08 | Large-scale expansion of induced pluripotent stem cell (iPSC) using bioreactors to produce therapeutically active extracellular vesicles (EVs)

André Cronemberger Andrade[1]; Martin Wolf[1]; Heide-Marie Binder[1]; Fausto Gueths Gomes[1]; Felix Manstein[2]; Patrica Ebner Peking[1]; Rodolphe W. Poupardin[1]; Robert Zweigerd[2]; Katharina Schllmoser[3]; Dirk Strunk[1]

[1]Cell Therapy Institute, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI - TReCS), Paracelsus Medical University (PMU), Salzburg, Austria, Salzburg, Austria; [2]Department of Cardiac, Thoracic, Transplantation and Vascular surgery, Leibniz Research Laboratories for Biotechnology and Artificial Organs (LEBAO), Hannover Medical School, Hannover, Germany, Hannover, Germany; [3]Department of Transfusion Medicine and SCI-TReCS, PMU, Salzburg, Austria, Salzburg, Austria

Introduction: Improvement of protocols and methods using bioreactors for extracellular vesicles isolation is essential to produce vesicles with good quality and quantity. We choose to obtain vesicles from stem cells that secrete several paracrine factors including EVs that are important in cellular communication and can support the regeneration of injured tissues. A strategy to improve the function of the EVs derived from steam cells was preconditioning them using reduced oxygen conditions (hypoxia). As hypoxia is a key regulator in development and regeneration it may be an important factor influencing cellular communication via EVs. Here we investigated whether hypoxic pre-conditioning and cultivation in bioreactors can influence iPSC-EV EV quantity, quality and, EV-based angiogenic potential.

Methods: We cultivated iPSC in bioreactor or cell factories to produce iPSC EVs isolating using tangential flow filtration (TFF) from iPSC conditioned media from different oxygen level conditions, with further subsequent concentration by ultracentrifugation (TUCF). We quantified EVs by tunable resistive pulse sensing (TRPS). We characterized the EVs by immunoblotting for EV markers and using super-resolution microscopy (dSTORM). To check the functionality of the EVs derived from different oxygen conditions we performed an ECFC network formation assay on matrigel.

Results: EV quantity did not differ significantly at different oxygen conditions, but we also observed a higher yield of EVs when the cells were cultivated in bioreactors. In immunoblots, we found enrichment of the tetraspanins and Alix for the Ev purifications and not for Calnexin. We observed an elevated angiogenic potential on iPSC-EVs derived from 1% oxygen culture by TFF compared with iPSC-EVs from 5% and 18% conditions and soluble factors. An extended purification method to further concentrate the EVs by ultracentrifugation (TUCF) after TFF reduced the pro-angiogenic effect and showed a reduced amount of growth factor (VEGF) on the EVs.

Summary/Conclusion: We conclude that selecting a good strategy together with an improved protocol method can increase the production and function of iPSC-EVs from large-scale iPSC cultures (bioreactor) by TFF and concentrated by TUCF.

Funding: This research was funded by the European Union's Horizon 2020 research and innovation program and by Land Salzburg in the framework of EV-TT, EV-TT – Bpro, and Cancer Cluster Salzburg. R.Z. received funding from the German Research Foundation, the German Ministry for Education and Science, and the European Union H2020 project TECHNOBEAT.

PT01.09 | MSCs can restore the extracellular vesicles regenerative potential after in vitro damage

Nataliya Basalova[1]; Olga Grigorieva[1]; Maxim Vigovskiy[2]; Uliana Dyachkova[3]; Natalia Aleksandrushkina[1]; Mikhail Arbatskiy[3]; Anastasia Efimenko[2]
[1]Institute for Regenerative Medicine, Medical Research and Education Center, Lomonosov Moscow State University, Moscow, Russia, Moscow, Russia; [2]Institute for Regenerative medicine, Medical Research and Education Center, Lomonosov Moscow State University, Moscow, Russia; Faculty of Medicine, Lomonosov Moscow State University, Moscow, Russia, Moscow, Russia; [3]Faculty of Medicine, Lomonosov Moscow State University, Moscow, Russia, Moscow, Russia

Introduction: MSCs are one of the most important cell types that regulate tissue regeneration. Extracellular vesicles (EV) secretion are one of the key process mediating this MSC effects. However, MSC may change secretory phenotype in response to damage. Are these changes preserved when the normal tissue structure is restored? Using several in vitro models of damage, we have evaluated the changing and recovery of the EV regenerative potential.
Methods: To creating damaging conditions, we used a models of cellular aging (under the influence of free radicals from H2O2), chronic inflammation (using M2-macrophages conditioned medium) or profibrotic conditions (using decellularized fibroblasts extracellular matrix with TGFβ). EV were isolated from MSCs conditioned medium immediately after influence of damaging stimuli and 2 weeks later to assess the recovery of the regenerative potential. The regenerative potential was evaluated using an in vitro models of angiogenesis, TGFb-induced transdifferentiation fibroblasts to myofibroblasts and the ability of EV to maintain cells (spermatogonial stem cells) stemness.
Results: Thus, we have shown that immediately after injury, there is a tendency to a decrease in the angiogenic potential of EV. The antifibrotic and stemness-supporting potentials of EV dramatically decreases to the level of the corresponding controls. However, after removal of the action of matrix and TGFb or H2O2, MSC are able to restore the EV antifibrotic potential to the level of EV from normal MSC. Only EV, after exposure to M2 conditioned medium, restore their stemness-supporting potential.
Summary/Conclusion: Thus, we have shown that after various damaging effects, EV lose their regenerative potential. This potential can be partisially restored when cells return to normal conditions. Apparently, the possibility and degree of the EV-MSC regenerative potential restoration largely depends on the type of damaging effect. Further studies are needed to understand the dynamics of this process in vitro and in vivo.
Funding: The study was supported by RFBR # 19-29-04172.

PT01.10 | Mesenchymal Stem/Stromal Cells-derived EVs neuroprotection: which is the responsible cargo?

Elisa Tonoli; Maria Pia Savoca; Elvina Chrysanthou; Gareth Cave; Elisabetta AM Verderio
Nottingham Trent University, Nottingham, United Kingdom

Introduction: Mesenchymal Stem/Stromal Cells (MSCs) are multipotent progenitor cells able to mediate anti-inflammatory and cytoprotective effects in several pathologies, such as Alzheimer's Disease (AD). An important fraction of the MSCs secretome is carried by Extracellular Vesicles (EVs), which act as shuttles to deliver neuroprotective molecules to the brain. However, the mechanism leading to MSCs-EVs neuroprotection is poorly known. The aim of this study is to identify neuroprotective EVs-cargo, using an in vitro AD model as initial validation system.
Methods: MSCs were purchased (ATCC, n=1) or established from human cord tissues (n=3) provided by the Anthony Nolan Cell Therapy Centre following approval from their Ethical Committee. MSCs at passage 3 (p3) were characterized for specific markers by Flow cytometry and small EVs isolated by serial centrifugation after 24 h incubation in serum-free medium (p4-8). EVs were characterized by western blotting (positive markers: ALIX, FLOT-2, CD63; negative markers: TOM20, GS28), ZetaView, ExoView and TEM. EVs cargo from MSCs and control human dermal fibroblasts (HDF) was analyzed by comparative SWATH-MS proteomics and digital transcriptomics (nanoString). EVs were also evaluated for cytokine content by 27-plex ELISA.
Results: SWATH-MS led to the identification of 47 proteins consistently changed in MSCs-EVs compared to HDF (confidence $\geq$ 65%, n=4), of which 9 were enriched (log2FC$\geq$1). Gene Ontology revealed that the majority has cell adhesion functions or is involved in adhesion/survival signaling. Transcriptomic analysis by nanoString led to the quantification of ~351 miRNA, of which 97 were increased in MSCs-EVs compared to HDF (FC$\geq$1.5), including miRNAs related to anti-inflammatory processes. Multi-plex ELISA showed an enrichment of immunoregulatory cytokines ($p \leq 0.05$).
Summary/Conclusion: Adhesion-related signaling and delivery of anti-inflammatory miRNAs might concur to the mechanism mediating MSCs-EVs neuroprotection. The neuroprotective capability of relevant molecules is under validation using an in vitro AD model based on primary mouse hippocampal neurons treated with amyloid-β.
Funding: Marmont Foundation and the Health and Wellbeing Talent Fellowship (Nottingham Trent University).

PT01.11 | Scalable production of small extracellular vesicles (sEV) for immune therapy: integrating management of cellular stress in upstream processing

Laurence De Beaurepaire[1]; Thibaud Dauphin[2]; Claire Boursier[2]; Claire Nouguier[2]; Mathilde Laubert[2]; Mailys Le Devehat[2]; Lucie Grare[2]; Quentin Le Yondre[2]; Quentin Pruvost[2]; Eugénie Lahet[2]; Dominique Jegou[2]; Philippe Courcoux[3]; Aurélien Dupont[4]; Julien Pichon[5]; Laurence Dubreil[5]; Mayeul Collot[6]; Blandine Lieubeau[2]; Grégoire Mignot[2]; Jean-Marie Bach[2]; Steffi Bosch[2]; Mathilde Mosser[2]

[1]IECM, ONIRIS, INRAE, USC1383, Nantes, France, Nantes, France; [2]IECM, Oniris, INRAE, USC1383, Nantes, France, Nantes, France; [3]StatSC, Oniris, INRAE, USC 1381, Nantes, France, Nantes, France; [4]MRic, Biosit, UMS3480 CNRS, University of Rennes 1, Rennes, France, Rennes, France; [5]PAnTher, INRAE, Oniris, F-44307 Nantes France, Nantes, France; [6]Laboratoire de Biophotonique et Pharmacologie, UMR CNRS 7213, Université de Strasbourg, Illkirch, France, Strasbourg, France

Introduction: Current advances in the development of sEV-based therapeutics highlight the need for scalable production. Stirred tank bioreactors allow culturing adherent cells on microcarriers or in aggregates at large scale, but potentially engender cellular stress responses due to shearing forces and serum starvation that may affect sEV immune properties. Hence, alleviating cellular stress upstream is key to streamline robust manufacturing of therapeutic EV.
Methods: We here describe a generic framework for the identification and management of critical sources of stress in culture including the medium formulation (w/ or w/o serum, glucose control), the mode of culture (monolayer/ aggregates) and the process parameters (stirring, duration, cell density). The response variables were cell viability (Trypan blue, LDH), stress markers (CHOP, GRP94, XBP1), cell function (metabolic response) and sEV production. sEV were isolated by a method combing differential centrifugation, tangential flow filtration and size exclusion chromatography and compared for yield and purity.
Results: First, our data show the potential of aggregate cultures to promote cell maturation, without changing the quantity of sEV produced or their size distribution. However, both stirring and serum starvation significantly increase the expression of cellular stress markers and decrease cell viability. As these conditions are both required to design a GMP scalable process, a Response Surface Methodology was conducted to minimize cellular stress by tuning the impellor speed, the seeding cell density and the duration of culture. Interestingly, this optimization step in stirred tank, allowed reaching a similar level of the cell stress that was measured in the static two-dimensional standard culture.
Summary/Conclusion: The proposed workflow should instruct rational management of cellular stress upstream in sEV-manufacturing bioprocesses for therapy.
Funding: This work has been funded by the French National Agency of the Research AAPG2021 JCJC Bioprod-sEVforT1D.

PT01.13 | Secretion of small extracellular vesicles by myogenic cells: altered secretory rate and function in different cellular states

Prabhavathy Devan[1]; Ananga Ghosh[2]; Himadri D[3]; Pallavi Kshetrapal[3]; Jyotsna Dhawan[2]

[1]CSIR - Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad -500007, India., Hyderabad, India; [2]CSIR - Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad -500007, India. Academy of Scientific and Innovative Research (AcSIR), Ghaziabad - 201002, India., Hyderabad, India; [3]Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad, 121001, India., Gurugram, India

Introduction: Quiescence is an actively maintained, reversible cell cycle arrest from which adult skeletal muscle stem cells (MuSCs) are activated to facilitate muscle regeneration and homeostasis. Signals that activate proliferation and differentiation of MuSCs are well studied, but the mechanisms used to attain and maintain the quiescent state are still poorly known. We have used a synchronized myoblast culture model that can be induced to enter either reversible or terminal cell cycle arrest, characteristic of quiescent MuSC vs differentiated myofibers. In this study, we have determined the secretory rates and signaling functions of small extracellular vesicles (sEVs) from different cellular states.
Methods: sEVs were isolated from conditioned media of cultured C2C12 cells using differential ultracentrifugation. Validation of sEVs from the quiescent, proliferating and differentiated cells were performed using bonafide sEV marker proteins by western blot, for size and number by NTA and TEM. The uptake of PKH26 labeled sEVs from donor cells was evaluated by confocal microscopy of recipient cells cultured in different conditions, and uptake pathways defined by inhibitor analysis.
Results: Equivalent numbers of proliferating myoblasts (MB), quiescent myoblasts (G0), reactivated myoblasts (R24), and differentiated myotubes (MT) showed marked quantitative differences in the secretion of sEVs of mean diameter of 140-180 nm. Interestingly, G0 cells produced significantly more (4 fold) sEVs compared to MB, despite a substantially lower global metabolic rate. As G0 cells re-entered the cell cycle, the sEV release returned to lower rates characteristic of MB. Using western blotting, sEVs from all states were found positive for markers such as Alix, TSG101 which were further enriched in G0 derived sEVs. Interestingly, the expression of Kibra, a known positive regulator of EV biogenesis, is higher in G0 cells than in other cellular

states. Over-expression or knock-down of Kibra in MB, led to significantly enhanced or reduced sEV secretion, respectively. In experiments designed to measure uptake and function, we found that sEVs from different cellular states showed different uptake potential, and that G0 derived sEVs could facilitate better differentiation than those derived from other states.
Summary/Conclusion: Taken together, we provide evidence that quiescence in myogenic cells is associated with quantitative alterations in the amount of sEVs secreted, and that these sEVs have distinct signaling functions. Our studies suggest that cross-talk between quiescent and differentiated cells in tissue homeostasis may involve sEV-associated functions.
Funding: This work was funded by MUSTER-Indo Danish grant from the Govt of India Dept of Biotechnology to JD.

PT01.15 | Therapeutic testing of optic nerve head-derived exosomes for the survival of retinal ganglion cells after optic nerve crush

Esmahan Durmaz[1]; Zara Mehrabyan[2]; Steven Bernstein[2]; Marcela Votruba[1]; Ben Mead[1]
[1]Cardiff University, Cardiff, United Kingdom; [2]University of Maryland, Baltimore, USA

Introduction: Retinal ganglion cells (RGCs) form the innermost layer of the retina, which is responsible for transmitting visual information received from photoreceptors to the brain through the optic nerve. Therefore, the impairment or loss of RGCs can cause retinal diseases and even irreversible blindness. According to World Health Organization, 1 billion people suffer from blindness, of which 7.7 million of those are due to glaucoma. Progressive RGC loss due to elevation in intraocular pressure is a characteristic of glaucoma. Despite the decrease in IOP by current lowering agents, they are inefficient for neuroprotection in glaucoma. Therefore, there is need for neuroprotective treatment in clinical usage.
Small extracellular vesicles (sEV) are nanosized (50-150 nm) vesicles secreted from all cells and found in all body fluids, providing cell to cell communication through their composition such as mRNA, miRNA and proteins. Recent evidence shows that bone marrow mesenchymal stem cells (MSCs)-derived exosomes, human embryonic stem cells-derived exosomes, and umbilical cord MSCs-derived exosomes promoted the survival of RGCs. Since different stem cell-derived sEVs offer varying levels of efficacy, we hypothesised that stem cells isolated from the eye and surrounding tissues would produce the most therapeutically efficacious exosomes. This study aims to test optic nerve head (ONH) derived-sEV in a rat optic nerve crush model.
Methods: In this project, we used ONH-derived sEVs as a novel source that might be neuroprotective for RGC. To test therapeutic efficiency, we dissected rodent retinae, cultured and treated them with sEV. Lastly, RGC numbers and neurite regeneration were analysed through immunohistochemistry and microscopy.
Results: ONH-derived sEV promoted survival of RGC and neuritogenesis of RGC in primary culture.
Summary/Conclusion: According to the findings, optic nerve head derived sEV have therapeutic potential to the RGC protection as a cell-free therapy for degenerative eye diseases including glaucoma.

PT02: Technology and Methods: EV Separation from biological sources (not blood)

Chair: Rossella Crescitelli – Sahlgrenska Center for Cancer Research and Wallenberg Centre for Molecular and Translational Medicine, Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Chair: Maja Kosanović

PT01.16 | Clonal Immortalized Mesenchymal Stromal Cells appear as an ideal cell source for the production of therapeutic extracellular vesicles

Yanis Mouloud[1]; Nicole Labusek[2]; Chen Wang[3]; Helmut Hanenberg[4]; Dirk Hermann[3]; Josephine Herz[2]; Bernd Giebel[5]
[1]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, essen, Germany; [2]Department of Pediatrics I, Neonatology & Experimental Perinatal Neurosciences, University Hospital Essen, University Duisburg-Essen, Essen, Germany, Essen, Germany; [3]Department of Neurology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany., Essen, Germany; [4]Department of Pediatrics III, University Children's Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany; [5]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany

Introduction: Connected to their immunomodulatory properties mesenchymal stromal cells (MSCs) are considered as therapeutic agent for a variety of different diseases. Mechanistically, MSC exert a large proportion of their therapeutic effects via extracellular vesicles (EVs), which they also secrete in vitro. Indeed, we demonstrated therapeutic potentials of EVs prepared

from conditioned media of cultured bone marrow-derived MSCs in a treatment-resistant GvHD patient and in several animal models, including mouse models for ischemic stroke and hypoxia induced neonatal encephalopathy (HIE). Aiming to set up scaled production strategies and to standardize the production as much as possible, we have compared various strategies for immortalizing primary MSCs and evaluated the immunomodulatory potential of their EVs in vitro and in vivo.
Methods: Upon applying an hTERT-based lentiviral transduction strategy, we were able to efficiently immortalize MSCs and to expand them at the single cell level to obtain clonal immortalized MSC lines (ciMSCs). ciMSCs still fulfil bona fide MSC characteristics and secrete EVs with immunomodulatory properties.
Results: ciMSC-EVs retain the capability to supress T cell activation in a multi-donor mixed lymphocyte reaction assay. Furthermore, comparably to EVs from primary MSCs they suppress disease symptoms in ischemic stroke and HIE models.
Summary/Conclusion: Thus, we conclude, EVs from ciMSCs contain therapeutic potentials and ciMSCs appear as an ideal cell source for the scaled manufacturing of MSC-EV-based therapeutics.
Funding: We were able to efficiently immortalized MSCs that retains their bona fide characterisitics. The secreted EVs retains their immunomodulatory capabilities in vitro in a multi donor mixed lymphocyte reaction assay and in vivo in ischemic stroke and HIE models.

PT01.17 | Human liver stem cell-derived extracellular vesicles improve fibrosis in an in vivo model of non-alcoholic steatohepatitis and modulate lncRNA expression profile

Elena Ceccotti[1]; Stefania Bruno[2]; Giulia Chiabotto[2]; Chiara Pasquino[3]; Massimo Cedrino[4]; Giovanni Camussi[5]
[1]Department of medical science, University of Turin, Torino, Italy; [2]Department of medical science, University of Turin, Turin, Italy; [3]Molecular Biotechnology Center, Turin, Turin, Italy; [4]Unicyte, Torino, Italy; [5]Department of Medical Sciences, Torino, Italy

Introduction: We recently demonstrated that fibrosis and inflammation associated with non-alcoholic steatohepatitis (NASH) are improved by treatment with human liver stem cells (HLSCs) and their EVs. We are now investigating the molecular mechanisms involved in the regulation of hepatic fibrosis by EVs, focusing our attention on lncRNAs modulated by EVs
Methods: EVs were purified by ultracentrifugation and characterized in accordance with ISEV guidelines, by transmission electron microscopy, flow cytometry and Western Blot.
NASH has been induced in SCID mice through a methionine-choline-deficient diet. EVs (2.5 x 109) were i.v. administered twice a week, starting at week 2 and ending at week 4. To evaluate liver fibrosis and inflammation, histological and molecular analyses were performed using specific staining and real time PCR analysis. PCR array was used to screen 84 inflammation-related lncRNAs on liver RNA and 14 lncRNAs were validated through real time PCR analysis. The expression of lncRNA-Meg3 was further evaluated in activated human hepatic stellate cells (LX-2) treated with EVs
Results: EV-treatment significantly reduced liver fibrosis and inflammation, at both morphological and molecular levels. Liver RNA screening with lncRNA array indicated that both NASH and EV-treatment influenced the lncRNA expression profile.
Real time PCR validation of 14 selected lncRNAs showed that the expression of 10 lncRNAs was increased by NASH and partially restored to baseline levels by EV-treatment. Moreover, the expression of 4 lncRNAs was not reduced by NASH but by EV-treatment.
EV regulation of lncRNA-Meg3 expression in activated LX-2 showed a similar trend to that observed in NASH
Summary/Conclusion: HLSC-derived EVs exert anti-fibrotic and anti-inflammatory effects in NASH. EV-treatment may restore baseline levels of several lncRNAs deregulated in NASH and in activated LX-2, indicating their possible contribution to the anti-inflammatory and anti-fibrotic effect of EVs

PT02.01 | Proof of concept of using a membrane sensing peptide for sEVs affinity-based isolation

Beatriz Benayas[1]; Joaquín Morales[1]; Alessandro Gori[2]; Alessandro Strada[2]; Pilar Armisen[3]; Carolina Egea[3]; Marina Cretich[2]; María Yáñez-Mó[1]
[1]Universidad Autónoma de Madrid (UAM), Madrid, Spain, Madrid, Spain; [2]Consiglio Nazionale delle Ricerche, Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC), Milan, Italy, Milano, Italy; [3]Agarose Bead Technologies (ABT), Spain, Madrid, Spain

Introduction: Differential ultracentrifugation and size exclusion chromatography are the mostly used EVs isolating methods, but none of them can efficiently purify EVs from all contaminants present in a complex biofluid sample. Until now, affinity-based methods may not be a good option as there is no a known universal marker of EVs, leading to the selection of a given subpopulation depending on the capture antibody and membrane protein composition of EVs. Recently, a membrane-sensing peptide, derived from bradykinin and that selectively binds to highly curved membranes was reported and applied in a microchip-based method to analyse small EVs (sEVs) (Gori et al., 2020).

Our objective is to study the capacity of this peptide to work in selectively isolating sEVs from conditioned media through an affinity chromatographic method.
Methods: To address it, the peptide was modified with a poli-His tag to allow binding to divalent cations-carrying agaroses. After peptide-agarose binding under rotation, concentrated conditioned medium from melanoma SKMEL-147 cell line was incubated ON at 4°C under rotation. We used different antibodies to estimate peptide (anti poli-His) and sEVs (anti-CD81) binding. We also used anti-ApoB antibody to analyse lipoprotein contamination.
Results: Firstly, we observed that cobalt agarose was the most efficient cation in peptide binding. We have optimized the amount of peptide and incubation time necessary for a proper EV attachment, and we did not observe unspecific binding of lipoproteins. A modified agarose with a longer spacer arm resulted in better peptide and EVs binding. A branched peptide was also tested. Finally, we did some EV recovery test, and binding and elution conditions were set up.
Summary/Conclusion: In conclusion, bradykinin-derived peptide is a promising method for sEVs isolation, which allows proper purification without any bias for a given protein marker.

PT02.02 | Assessment of extracellular vesicle isolation methods from human stool supernatant

Emmalee J. Northrop-Albrecht; William R. Taylor; Bing Huang; John Kisiel; Fabrice Lucien
Mayo Clinic, Rochester, USA

Introduction: Colorectal cancer (CRC) is the 2nd most fatal cancer in the U.S. but is preventable with screening, including non-invasive stool testing. The contents of host and/or microbiome-derived extracellular vesicles (EVs) may provide complimentary biomarkers that augment the sensitivity for detection of CRC precursors. In this study, we assessed the quality and efficiency of different EV separation methods in stool.
Methods: Stool specimens from healthy individuals were obtained after IRB approval. EV separation methods (ultracentrifugation [UC], precipitation [EQ], size exclusion chromatography [SEC], and ultrafiltration [UF]) were applied to stool supernatant (n=5). Transmission electron microscopy (TEM) was used to confirm the presence of EVs and contaminants. Particle recovery was evaluated using nanoscale flow cytometry. RNA composition was assessed both pre/post- enzymatic treatment with an Agilent Bioanalyzer. RT-qPCR assays were designed for 16s and 18s ribosomal transcripts, and western blot was used to characterize human, bacterial and contaminating proteins.
Results: TEM confirmed the presence of EVs in stool specimens. Ultrafiltration had the highest particle recovery (mean± SEM; %; UF: 56.0 ± 7.9, SEC: 47.5 ± 5.6, UC: 30.0 ± 6.1, EQ: 28.6 ± 6.1), RNA (mean± SEM; ng; UF: 84.1 ± 20.2, UC: 169.5 ± 55.8, EQ: 15.4 ±1.8, SEC: 5.7 ± 2.0), and protein yield (mean± SEM; ug; UF: 199.1 ± 36.1, EQ: 44.8±15.4, UC: 29.1 ± 10.3, SEC: 13.6 ± 3.7). SEC was the most pure method based on TEM, protein and RNA evaluation. Bacterial rRNA was 1097x more abundant than human rRNA, and enzyme treatment prior to extraction revealed most RNA is non-vesicular. TSG101, CD63, and ompA proteins were present in EV fractions from all methods except UC.
Summary/Conclusion: SEC is the ideal separation method for stool based on multiple EV characterization techniques. It can be applied in future studies that use high throughput omics technologies to aid in the identification of novel biomarkers for early CRC detection.
Funding: This work was supported by the National Cancer Institute under CA214679 to JBK.

PT02.03 | Development of an easy, cost-effective methodology for isolation of small extracellular vesicles for obtainment of better yield and mass spectrometric compatibility

SIMRAN RASTOGI[1]; Komal Rani[2]; Saroj Kumar[3]
[1]DEPARTMENT OF BIOPHYSICS, ALL INDIA INSTITUTE OF MEDICAL SCIENCES, NEW DELHI, INDIA, NEW DELHI, India; [2]ALL INDIA INSTITUTE OF MEDICAL SCIENCES, NEW DELHI, India; [3]All India Institute of Medical Sciences, New Delhi, New Delhi, India

Introduction: Numerous techniques are present for the isolation of small-sized extracellular vesicles (sEVs) like ultracentrifugation (UC), chemical-based precipitation methods. Although they have their set of disadvantages and sometimes prove to not match the suitability criteria with patient samples. The present invention focuses on the development of a protocol that ensures efficient isolation from lower sample volumes has reduced processing time, requires minimal instrumentation, and possesses mass spectrometric compatibility.
Methods: The sEVs were isolated from the biofluid (saliva, blood plasma) using many combinations of crowding reagents. Nanoparticle tracking analysis aided in the primary selection of combinations based on the mean size of sEVs and the size distribution of sEVs sub-populations. The fluorescence NTA using CMDR lipid-binding dye and CD63-antibody AlexaFluor

488 were used for quantification. Isolated sEVs from biofluids were validated against anti-CD63 and anti-TSG101 antibodies via western blotting. The comparative profiling with existing methodologies like UC, SEC, and PEG-based precipitation was done to prove the utility of the current invention.

Results: In the present invention, we formulated two new chemical precipitant combinations COM X1 and COM X2 (patent under filing) that could efficiently isolate sEVs (30-150nm) with 98% purity, utilizing only 200-300μl of sample volume. The obtained combinations are compatible with mass spectrometry, unlike the PEG-based precipitation that led to ion suppression and interfered with target ions.

Summary/Conclusion: In an aspect, the present invention provides a method for sEVs isolation especially from a biological sample (saliva, blood plasma) using different chemical combinations, that are easy to make, available in every laboratory, and does not require any additional equipment. The present invention is cost-effective and could pave way for better sEVs based-biomarker discovery.

Funding: The funding for the work was provided by ICMR, India.

PT02.04 | Efficient freeze drier-based protocol to deplete extracellular vesicles from fetal bovine serum

Mohammad Ali Gholampour

Department of Medicine, Lung Biology Center, Cardiovascular Research Institute, University of California, San Francisco, CA, USA, San Francisco, USA

Introduction: One of the major concerns is to deplete contaminating EVs present in FBS. EVs present in FBS are co-isolated with cell-derived EVs and therefore act as contaminants that, affect any quantitative and/or qualitative assessments of EVs derived from the cultured cells.

Methods: For preparation of Freeze drier EV-depleted FBS (FD-dFBS), the directions below was followed to deplete EVs from FBS:

1- 4 mL FBS was loaded onto 50 mL falcon tube and stored at −80°C overnight.

2- The falcon tube containing frozen FBS was put in a freeze-drier machine and processed for 6 hours. In this step, the water was evaporated and precipitation of FBS was sedimented in falcon tube.

• Critical step: Do not close the door of falcon tube, just seal the door with sterile aluminum foil and make some pore by piercing the aluminum foil to exchange the temperature and vacuum.

3- The precipitation collected and dropped in a clean porcelain mortar. Then, it was rubbed at least for 2 minutes to completely become powder. 500 μL of PBS or media was added to soft powder of FBS and rubbed again at least 2 minutes. Finally, additional 3.5 mL of PBS or media added. Pipette it or use vortex until it dissolves completely.

4- The dissolved FBS was centrifuged for 30 minutes at 10,000 g in 4°C to remove debris and aggregate proteins.

• Hint: A portion of the FBS proteins may not dissolve very fast, therefore before centrifugation, it is better to incubate it 4-6 hours in 4°C.

Ultrafiltration EV-depleted FBS (UF-dFBS) was done. Briefly, FBS was loaded onto Amicon Ultra-15 Centrifugal Filter Units, and spun for 55 min at 3,000 g. Ultracentrifugation EV-depleted FBS (UC-dFBS) was subjected to centrifugation for 19 hours at 100,000× g at 4 ∘C.

Results: Analysis of the different EV-depleted FBS

Based on DLS analysis, while untreated FBS contained a heterogeneous population of EVs, with sizes ranging from < 50 nm to >500 nm, no vesicles could be detected in FD-dFBS. After an 18-h UC of the FBS, a small population of vesicles structures and protein aggregates persists in UC-dFBS, which may indicate low efficiency of ultracentrifugation. The UF-dFBS also displayed scarce vesicle-like structures. Characterization of the EV samples by TEM mostly supported the DLS analysis. While FD-dFBS had no EVs, UF-dFBS still contained some EVs or other EV-like particles that were mainly small. The diameter of most membrane vesicles was between 30 and 120 nm, with the spherical shape typical of EVs. The morphological analysis of UC-dFBS also displayed EVs population and protein aggregates. As expected, regular FBS contained both large and small EVs.

Summary/Conclusion: In this study, we have developed a novel, cost- and time-effective, standardized and simple protocol based on freeze drying to deplete EVs from FBS, and addressed the purity of this freeze drying EV-depleted FBS in comparison with ultrafiltration, ultracentrifugation, and regular FBS.

PT02.06 | Optimization of the isolation of extracellular vesicles from human-induced pluripotent stem cells

Hala Saneh[1]; Christine Finck[2]; Joanne Walker[3]; Heather Wanczyk[3]

[1]University of Connecticut / Connecticut Children's, Farmington, USA; [2]Connecticut Children's Medical Center, Hartford, USA; [3]UConn Health, Department of Pediatrics, Farmington, USA

Introduction: Extracellular vesicles (EVs) derived from stem cells have the potential to serve as vehicles for the treatment of various diseases such as neonatal hyperoxia-induced lung injury. Currently, their isolation and characterization are challenging, as no standardized protocols are available. This study focuses on determining the most efficient and effective technique for the isolation of EVs from human-induced pluripotent stem cells (hiPSCs) in terms of sample purity and yield, as well as preserved in-vitro functional characteristics. Herein we describe a standardized protocol that can be used to maximize the therapeutic potential of hiPSCs-derived EVs in future clinical applications.
Methods: Different methods were investigated for the isolation of EVs from hiPSCs-conditioned media. These included ultracentrifugation (classic differential and density-gradient), precipitation, immuno-magnetic capture, size-exclusion chromatography (SEC), and precipitation followed by SEC. The yield and purity of the isolated EVs were assessed by transmission electron microscopy, imaging flow cytometry, and nanoparticle tracking analysis. Additionally, in-vitro functionality was assessed by culturing A549 cells with isolated EVs for 48 hours following exposure to 50 μM of Hydrogen Peroxide. Cell viability was quantified using an Adenosine Tri-Phosphate detection assay.
Results: Vesicles isolated using SEC and precipitation techniques showed the best results in terms of yield and purity compared to the other methods. On imaging flow cytometry, SEC consistently yielded the highest rate of tetraspanins CD63 and CD81 positivity (49% and 67% respectively), followed by the combined precipitation/SEC technique (32% and 43% respectively). Lastly, the viability of A549 cells exposed to Hydrogen Peroxide was improved by 44% following treatment with EVs isolated by SEC. EVs recovered by the other techniques were less effective in improving cell viability.
Summary/Conclusion: Our data demonstrate that SEC is a promising and reproducible method that results in high yield, purity, and functionality of EVs isolated from hiPSCs. This project may pave the way for stem-cell free translational therapeutic applications in premature lung diseases.

PT02.07 | Identification of storage conditions stabilizing EV preparations

André Görgens[1]; Giulia Corso[1]; Daniel W Hagey[1]; Rim Jawad Wiklander[1]; Ulrika Felldin[1]; Yi Lee[1]; Beklem Bostancioglu[1]; Helena Sork[1]; Xiuming Liang[2]; Wenyi Zheng[2]; Dara Mohammad[1]; Simonides I. van de Wakker[3]; Pieter Vader[4]; Antje Zickler[1]; Oscar Wiklander[1]; Samir El Andaloussi[2]
[1]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, Stockholm, Sweden; [2]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, Huddinge, Sweden; [3]Department of Experimental Cardiology, University Medical Center Utrecht, Utrecht University, The Netherlands, Utrecht, Netherlands; [4]CDL research, University Medical Center Utrecht, Utrecht, The Netherlands, Utrecht, Netherlands

Introduction: Extracellular vesicles (EVs) play a key role in many physiological and pathophysiological processes and hold great potential for therapeutic and diagnostic use. Despite significant advances within the last decade, the key issue of EV storage stability remains unresolved and under investigated. Here, we aimed to identify storage conditions stabilizing EVs and comprehensively compared the impact of various storage buffer formulations at different temperatures on EVs derived from different cellular sources for up to two years.
Methods: EV features including concentration, diameter, surface protein profile and nucleic acid contents were assessed by complementary methods, and engineered EVs containing fluorophores or functionalized surface proteins were utilized to compare cellular uptake and ligand binding.
Results: We show that storing EVs in PBS over time leads to drastically reduced recovery particularly for pure EV samples at all temperatures tested, starting already within days. We further report that using PBS as diluent was found to result in severely reduced EV recovery rates already within minutes. Several of the tested new buffer conditions largely prevented the observed effects, the lead candidate being PBS supplemented with human albumin and trehalose (PBS-HAT).
Summary/Conclusion: We report that PBS-HAT buffer facilitates clearly improved short-term and long-term EV preservation for samples stored at -80°C, stability throughout several freeze-thaw cycles, and drastically improved EV recovery when using a diluent for EV samples for downstream applications.

PT02.08 | Defining an optimal methodology for the efficient isolation of EVs from skeletal muscle myoblasts

Maria F. Fernandez Rhodes; Soraya Williams; Bahman Adlou; Mark P. P. Lewis; Owen G. Davies
Loughborough University, Loughborough, United Kingdom

Introduction: Optimising effective skeletal muscle extracellular vesicle (SM-EV) isolation methods offering high levels of purity will be important to appreciate their composition and functionality in physiological and pathophysiological systems. Size-exclusion chromatography (SEC) is a ubiquitously applied method for the isolation of EV-enriched fractions that can be combined with ultrafiltration (UF) to increase sample throughput and allow for scalable and selective EV isolation. However, SEC has not previously been optimised from SM-EV isolation and the impact of UF column choice on recovery remains undefined.
Methods: Murine myoblast cultures were collected after 48 hours differentiation, pre-concentrated using Amicon® Ultra 15, 100KDa or Vivaspin®20, 100KDa UF columns and processed by SEC (IZON). Thirty individual fractions were collected and individually analysed using nanoparticle tracking analysis (NTA) and bicinchoninic acid (BCA) assay to calculate particle number and particle to protein (PTP) ratio. Transmission Electron Microscopy (TEM), western blotting (WB) and NanoFCM were applied to validate the presence of EVs.
Results: Differential patterns of EV markers were identified following pre-concentration with Amicon and Vivaspin UF columns. Alix and TSG101 could be detected up to fraction 13, while CD9 and Annexin A2 until fraction 6. The presence of high-density lipoproteins (ApoA+) was detected from fraction 6 onwards for both protocols by WB. Pooling identical fractions (2-10) to maximise EV recovery led to qualitative and quantitative variations in EV marker profiles and PTP ratios of 129 (Amicon) and 60 (Vivaspin). CD63+ and CD81+ particles were quantitatively (NanoFCM) increased following Amicon pre-concentration (CD63: 7.21x108 vs 4.35x108 and CD81: 6.5x108 vs 4.14x108 for Amicon and Vivaspin respectively; $p < 0.05$). Eliminating lipoprotein co-isolation by reducing the fraction window (1-5) resulted in a net loss of particles (32%). However, an increase in PTP ratio (8%) and significantly increased CD81+ particle recovery was observed (5.64x108 vs 4.14x108 for Amicon and Vivaspin respectively; $p < 0.05$).
Summary/Conclusion: Caution should be taken when pre-concentrating samples for SEC due to observed variations in EV outputs. The isolation of fractions 2-10 was tested to maximise EV recovery at the expense of sample purity but resulted in no significant increase in quantitative measures of EV tetraspanins. Reducing the fraction window (fractions 1-5) eliminated lipoprotein contamination while having no significant impact on EV recovery.
Funding: OGD acknowledges funding from the Academy of Medical Sciences, Wellcome Trust, Government Department of Business, Energy and Industrial Strategy, British Heart Foundation, Diabetes UK (SBF004\1090) and the EPSRC/MRC Doctoral Training Centre in Regenerative Medicine

PT02.09 | Comparison of extracellular vesicle isolation processes for therapeutic applications

Soraya Williams[1]; Maria F. Fernandez Rhodes[1]; Alice Law[2]; Ben Peacock[3]; Mark P. P. Lewis[1]; Owen G. Davies[1]
[1]Loughborough University, Loughborough, United Kingdom; [2]NanoFCM, Nottingham, United Kingdom; [3]NanoFCM Co., Ltd., Nottingham, United Kingdom

Introduction: Extracellular vesicles (EVs) continue to gain interest for therapeutic applications. However, progression to clinical translation is limited by the diversity of EV isolation methods and their compatibility with downstream analysis.
Methods: We compared EV recovery from C2C12 mouse myoblast conditioned media, isolating by ultracentrifugation (UC), polyethylene glycol precipitation (PEG), Total Exosome Isolation Reagent (TEIR), an aqueous two-phase system with and without repeat washes (ATPS and ATPS/R) and size exclusion chromatography (SEC). Additionally, to understand parameters governing method implementation (e.g. cost and scalability), we distributed an international survey using Qualtrics.
Results: Data collected by nanoparticle tracking analysis and a BCA protein assay indicated the highest particle and protein concentration was obtained with TEIR (1.15E+09) and SEC (4066μg/ml) respectively. Purity was measured by particle to protein ratio, with ATPS/R and UC displaying the highest purity and SEC the lowest. EV tetraspanin markers (CD63, CD9 and CD81) were analysed by western blot, ExoELISA and nano flow cytometry, indicating EV-enriched fractions isolated by all methods but with variable profiles across both isolation and analysis methods. However, evidence showed the potential interference of reagents with methods such as ATPS. To evaluate our collective findings, data was normalised to the highest output to plot radar charts. Outputs highlighted that although SEC displayed low purity, it provided the highest marker presence overall, followed by UC. Data also indicated a significant increase in CD81 when isolating by UC. When observing parameters governing implementation, SEC and UC were found to be favoured by respondents due to their overall efficiency. However, limitations in the scalability of SEC were reported.
Summary/Conclusion: Overall, all methods isolated EV-enriched fractions but with variable outputs that could impact downstream biological functions and therapeutic utility.
Funding: OGD acknowledges funding from the Academy of Medical Sciences, Wellcome Trust, Government Department of Business, Energy and Industrial Strategy, British Heart Foundation, Diabetes UK (SBF004\1090) and the EPSRC/MRC Doctoral Training Centre in Regenerative Medicine

PT02.10 | Development of a protocol to isolate extracellular vesicles from 3D cultures

Giuseppina Poppa[1]; Letizia Esposito[1]; Sandra D'Ascenzo[1]; Vincenza Dolo[2]; Ilaria Giusti[2]

[1]Department of Life, Health and Environmental Sciences- University of L'Aquila- 67100, L'Aquila- Italy, L'Aquila, Italy; [2]Department of Life, Health and Environmental Sciences- University of L'Aquila, L'Aquila- Italy, L'Aquila, Italy

Introduction: Even if the 3D cultures reflect the tumor's architecture more realistically, there are currently no standardized protocols to isolate extracellular vesicles (EVs) from tumor cell lines cultured in 3D models. Thus, the goal of our study was to develop a simple, practical, reproducible, and economical protocol to isolate EVs from 3D cell cultures.

Methods: Human ovarian cancer CABA I cells were cultured in RPMI+5% FBS (40 nm-filtered) by hanging drop to form tumor spheroids (30000 cells/spheroid). Spheroids were moved to and cultured (1 ml medium/10 spheroids) in Petri dishes coated with an anti-adhesion solution, commercially available. The supernatant was collected twice daily for 5 days, centrifuged at 4°C at 600xg (15 min), 1,500xg (30 min), and 100,000xg (Rotor 70Ti, Quick-Seal Ultra-Clear tubes, kadj 221, brake 9, Optima XPN-110 Beckman Ultracentrifuge) for 90 min. Isolated EVs were resuspended in PBS, quantified by measuring the EV-associated proteins level (by Bradford method), and analyzed by Transmission Electron Microscopy (TEM), Nanosight (NTA), Western Blots. Scanning Electron Microscopy (SEM) imaged EVs' release from spheroids. Cell viability in spheroids was evaluated by XTT.

Results: CABA I cultured by hanging drop form compact and proliferating spheroids (334% viability at 48h vs 100% attributed to 24h); SEM revealed the release of many EVs from their surface. Indeed, the supernatant of cultured spheroids contained EVs; the TEM morphological analysis showed spherical and intact particles, which for the most part have a size attributable to sEVs (size range 55-120 nm), as confirmed by NTA. The identity of EVs was further confirmed by positivity for specific EV markers (CD63, CD9, CD81). The amount of released EVs was 0,17-0,53 μg/spheroid.

Summary/Conclusion: Taking advantage of an anti-adhesion solution, compatible with EVs release and collection, we have set a simple method to isolate EVs from 3D spheroids in an effective and reproducible way.

PT02.11 | An optimized workflow for magnetic bead-based separation of extracellular vesicles enables studies on EV subpopulation functionality

Bella Monica[1]; Casper S. Triesscheijn[1]; Rowan Frunt[1]; Ioanna Paspali[1]; Mark Tielemans[1]; Olivier G de Jong[2]; Raymond M. Schiffelers[1]; Pieter Vader[3]; Sander A. Kooijmans[4]

[1]University Medical Center Utrecht, Utrecht, The Netherlands, Utrecht, Netherlands; [2]Utrecht University, Utrecht, The Netherlands, Utrecht, Netherlands; [3]CDL research, University Medical Center Utrecht, Utrecht, The Netherlands, Utrecht, Netherlands; [4]CDL Research, University Medical Center Utrecht, Utrecht, The Netherlands, Utrecht, Netherlands

Introduction: The molecular content of extracellular vesicles (EVs) is highly heterogeneous. Given that EV surface molecules determine their interactions with their environment, it is conceivable that EV functionality varies between EV subpopulations with different surface profiles. However, it is as of yet challenging to test this hypothesis as methods to isolate intact EV subpopulations based on surface markers are lacking. Here, we demonstrate recovery of intact and functional EV subpopulations from antibody-coated magnetic beads using an optimized release protocol.

Methods: EVs from HEK293T, MDA-MB-231 and cardiac progenitor cells (CPCs) were isolated using tangential flow filtration and size exclusion chromatography (SEC). EV subpopulations were captured on antibody-coated magnetic beads targeting tetraspanins or phosphatidylserine (PS). Design-of-experiments (DoE)-based statistical modeling was applied to optimize an elution buffer to release EVs from the beads. EV release and integrity were monitored using flow cytometry and proteinase protection assays, respectively. Released EV subpopulations were further characterized by transmission electron microscopy (TEM), Nanoparticle Tracking Analysis (NTA) and western blotting. EV functionality was assessed in cellular uptake and scratch wound migration assays.

Results: A DoE-based library of elution conditions was applied to magnetic beads which had captured MDA-MB-231 EVs via CD9 or CD81 antibodies. EV elution and integrity analyses revealed that pH of the elution buffer was the most critical factor affecting both EV elution efficiency for both antibodies and EV integrity. A second round of optimization was performed to identify optimal elution conditions to release EV subpopulations enriched for CD9, CD63, CD81 or PS from various cell sources. Released EVs appeared intact by TEM and NTA and lacked typical contaminants observed in the original SEC-derived EV isolates. Uptake experiments in various recipient cell types revealed differences in cell-type specificity and uptake efficiency between EV subpopulations. Furthermore, the regenerative capacity of CPC-derived EVs in scratch wound assays differed among EV subpopulations.

Summary/Conclusion: Using DoE methodology, we readily optimized a protocol to recover intact EV subpopulations from magnetic beads based on their surface marker expression, and revealed functional differences among them. This universal

capture-and-release platform can be used to study EV surface-functionality relationships, and holds the potential to enrich EVs with desirable characteristics for therapeutic purposes.
Funding: This work was supported by a VENI grant (no. 17296) of the Dutch Research Council (NWO).

PT02.12 | Collection and Characterization of extracellular vesicles from mouse brain interstitial fluid

Nicholas Fitz; Mary Ann Ostach; Yi Lu; Radosveta koldamova; Iliya Lefterov
University of Pittsburgh, Pittsburgh, USA

Introduction: The collection and characterization of extracellular vesicles (EVs) in the brain interstitial fluid is an important advancement in better understanding the communication between neurons and glia. Furthermore, changes in the cargo of these EVs could modulate pathological progression, impact preclinical treatments or serve as biomarkers for neurodegenerative diseases.
Methods: Cerebral open flow microperfusion (cOFM) was refined in awake freely moving mouse models for collection of brain interstitial fluid. EVs were isolated and concentrated utilizing the Norgen Bioteck Exosome Purification kits. Isolated brain interstitial fluid EVs were then further characterized based on size and concentration with nanoparticle tracking analysis and electron microscopy and protein composition of EV markers by Western blot. Finally, following RNA isolation we utilized RNA-seq to profile different classes of noncoding RNAs.
Results: We report in both ex vivo and in vivo that cOFM can be a vital tool in the collection of brain interstitial fluid EVs. We were able to collect EVs with a size of ~120 nm at a concentration 2.3x107 particles/ml. These EVs were enriched in CD63, CD81 and flotillin and lacked cellular markers. Following RNA isolation, we observed enrichment in brain specific noncoding RNAs: SNORD115 and SNORD116 utilizing PCR. Using RNA-seq, we profiled four significant classes of noncoding RNAs: miRNAs, snoRNAs, tRNAs, and piRNAs from the isolated brain interstitial fluid EVs which had unique profiles.
Summary/Conclusion: The results from our study outline a new in vivo technique for the recovery and characterization of EVs from the brain interstitial fluid of mice. The cOFM allows for continuous sampling of interstitial fluid in brain tissue and could be used to monitor changes in EV cargo associated with development and progression of neurodegenerative diseases or the impact of therapeutics. With the ability to simultaneously collect large molecules with cOFM, there is the potential to correlate changes in EV profiles with changes in pathological extracellular proteins associated with different diseases or neuroinflammation states.
Funding: This work was funded by NIH: AG057565, AG056371, AG066198 and AG075069.

PT02.13 | Evaluation of four size-exclusion chromatographies to separate extracellular vesicles from human intracranial cerebrospinal fluid

Vedrana Krušić Alić[1]; Mladenka Malenica[1]; Siniša Zrna[2]; Maša Biberić[2]; Lara Valenčić[3]; Lada Kalagac Fabris[2]; Aleksandar Šuput[2]; Karmen Wechtersbach[4]; Nika Kojc[4]; Mario Kurtjak[5]; Natalia Kučić[1]; Kristina Grabušić[1]
[1]University of Rijeka, Faculty of Medicine, Rijeka, Croatia, Rijeka, Croatia; [2]Pula General Hospital, Pula, Croatia, Pula, Croatia; [3]Clinical Hospital Centre Rijeka, Rijeka, Croatia, Rijeka, Croatia; [4]Institute of Pathology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, Ljubljana, Slovenia; [5]Advanced Materials Department, Jožef Stefan Institute, Ljubljana, Slovenia, Ljubljana, Slovenia

Introduction: Size-exclusion chromatography (SEC) is emerging as a possibly preferential method for isolation of extracellular vesicles (EVs). This is because SEC separates EVs from soluble proteins, and preserves the EV structure. The method is based on sieving the sample through a porous stationary phase enabling faster migration and thus quicker elution of larger particles like EVs. Various materials can be used as stationary phase, whereby Sepharose-based SEC was already shown to be successful in EV separation. However, efficiency of different stationary phases in SEC separation of EVs has not been evaluated. This study compared Superose 6 PG, Sephacryl S-400, Sepharose CL-6B and commercial SEC column in separating EVs from human cerebrospinal fluid (CSF).
Methods: Three severe traumatic brain injury patients were included into the study after obtaining informed consent from family. CSF samples were collected during external drainage for monitoring intracranial pressure at days 1-3 after injury and analysed by immunoblot for albumin, EV protein markers CD9 and CD81, and lipoprotein markers ApoE and ApoAI. Pool of 9 CSF samples in volume of 2.8 ml per experiment was separated by four SECs: columns packed with Superose 6 PG, Sephacryl S-400 or Sepharose CL-6B (S6B), and a commercial column for EV separation. 46 fractions were collected and analysed by slot-blot followed by Ponceau staining and immunodetection for albumin, CD9, CD81, ApoE and ApoAI. Size and concentration of

nanoparticles in fractions were determined by tunable resistive pulse sensing. Selected fractions were analysed by transmission electron microscope and western blot.
Results: All CSFs contained EVs, lipoproteins and high level of albumin. All SECs provided separation of nanoparticles from soluble proteins and lipoproteins, but S6B with its mean 1.08E+09 of total isolated nanoparticles provided the highest enrichment of nanoparticles which also included CD9+ and CD81+ EVs.
Summary/Conclusion: S6B is suitable for separation of EVs from CSF.
Funding: This work was supported by the Croatian Science Foundation IP-2019-04-1511 to KG, University of Rijeka grant uniri-biomed-18-5-1131 to KG and University of Rijeka grant uniri-biomed-18-279 to MM. Slovenian Research Agency Program No P3-0054 (Pathology and Molecular Genetics) funded the research.

PT02.14 | PDMS – free microfluidic device fabrication and testing for high throughput extracellular vesicle separation

Roberts Rimsa[1]; Miks Priedols[2]; Janis Cipa[1]; Antons Miscenko[2]; Beate Bekere[2]; Gatis Mozolevskis[1]; Arturs Abols[2]
[1]Institute of Solid-State Physics University of Latvia, Riga, Latvia; [2]Latvian Biomedical Research and Study Centre, Riga, Latvia

Introduction: Currently standard EV isolation methods have issues with purity, efficiency, and EV functionality. More advanced strategies include alteration of streamlines within microfluidic channels; thus, utilizating differential lateral displacement of differently sized particles. Most available microfluidic systems use small volumes of sample and typically have low flow rates. Such low volumes and flow rates are not feasible if the goal is to extract EVs from bioreactor media and urine. Additionally, these devices predominantly are fabricated from PDMS (Polydimethylsiloxane), while it is well known to absorb and interact with lipophilic molecules. Therefore, new methods and devices for EV isolation from high volume samples are necessary.
Methods: As the first step we evaluated two PDMS alternatives – OSTE (Off-stoichiometry thiol-ene polymer) and SEBS (styrene-ethylene-butylene-styrene) materials with respect to their optical, fat-soluble molecule, EV absorption properties and RT-qPCR inhibition. Next, we microfabricated EV separation devices from OSTE polymer based on asymmetric flow field flow fractionation (A4F) principle and optimised them with standard EV sample produced within bioreactor, isolated with SEC and characterised by NTA, WB and TEM by applying in-house developed pressure system. Finally, the devices currently are tested for EV isolation directly from bioreactor media and compared with EV isolated from media by SEC and UC.
Results: Our results showed that OSTE have superior characteristics for EV isolation in comparison to PDMS and SEBS. Next, optimisation experiments showed that volume flow rate within the channel system should be below 100 μl/min since higher volume flow rates result in EV losses. Meanwhile, EV isolation directly from bioreactor media is ongoing and will be presented at the conference.
Summary/Conclusion: A4F devices fabricated from OSTE for EV isolation are more suitable than PDMS devices.
Funding: Nr. lzp-2019/1-0142

PT02.16 | A novel method for isolation of spontaneously-released small extracellular vesicles from mouse and human brain

Ioannis Sotiropoulos[1]; Patrícia P. Gomes[2]; Cristian Bodo[3]; Carlos J. Nogueras-Ortiz[4]; Martina Samiotaki[5]; Minghao Chen[6]; Carina Soares-Cunha[7]; Joana Silva[7]; Bárbara Coimbra[7]; George Stamatakis[8]; Liliana Santos[7]; George Panayotou[5]; Clarissa Waites[9]; Christos Gatsogiannis[6]; Nuno Sousa[7]; Dimitrios Kapogiannis[10]; Bruno Costa-Silva[11]
[1]ICVS Institute, Medical School, University of Minho, Braga, Portugal; [2]ICVS - School of Medicine, University of Minho, Braga, Portugal; [3]Champalimaud Center for the Unkown, Lisbon, Portugal; [4]Laboratory of Clinical Investigation, National Institute on Aging, Baltimore, USA; [5]Institute for Bioinnovation, Biomedical Sciences Research Center "Alexander Fleming", Athens, Greece; [6]Department of Structural Biochemistry, Max Planck Institute of Molecular Physiology, Dortmund, Germany; [7]Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal; [8]Institute for Bioinnovation, Biomedical Sciences Research Center "Alexander Fleming", Vari, Greece; [9]Department of Pathology and Cell Biology, Taub Institute for Research on Alzheimer's Disease and the Aging Brain, Columbia University Medical Center, New York, USA; [10]National Institutes of Health, Baltimore, USA; [11]Champalimaud Center for the Unknown, Lisbon, Portugal

Introduction: Extracellular vesicles (EVs), particularly exosomes, exhibit great potential for the diagnosis, prognosis and treatment of brain disorders such as Alzheimer's disease, representing an advantageous tool for Precision medicine; the latter demands high-quality biomarkers, especially for complex brain disorders, where pathological heterogeneity and diverse clinical presentations complicate the development of precise patient-tailored treatments. Thus, the collection and characterization of physiologically relevant exosomes are of the utmost importance. However, it is still challenging to identify, isolate and quantify exosomes

efficiently, accurately, and selectively. For instance, current approaches for exosome isolation from brain rely on tissue dissociation, which may contaminate the exosome fraction by cellular disruption/damage leading to purer/contaminated EVs yield and thus, conflicting, incongruent or inaccurate characterization of exosome profile.
Methods: Overcoming this drawback, we developed a novel method to isolate exosome-enriched EVs from mouse and human brain, relying on their spontaneous release.
Results: To confirm the efficacy of the release method and its advantages over the existing, digestion-based approaches, we have utilized different state-of-the-art and innovative analytical platforms and approaches (e.g., Cryogenic electron microscopy, High-sensitivity flow cytometry, proteomic analysis, ExoView analysis) that help us to structurally, biochemically and functionally characterize the captured EVs in in vitro and in vivo studies. Based on the above multiscale analytical platforms, we hereby present an efficient purification method that captures a more physiologically relevant, sEVs-enriched population spontaneously released by mouse and human brain tissue.
Summary/Conclusion: This spontaneous release method may contribute to the characterization and biomarker profile of physiologically relevant brain-derived exosomes in brain function and pathology.

PT02.17 | A simple microfluidics approach for the isolation of brain-derived extracellular vesicles

Mehdi Kabani[1]; Luc Bousset[1]; Han Wei Hou[2]; Hong Boon Ong[3]; Marc Dhenain[1]
[1]CNRS, Fontenay aux Roses, France; [2]School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore 639798, Singapore, Singapore, Singapore; [3]School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore, Singapore

Introduction: The development of methods to isolate intact and biologically relevant extracellular vesicles (EVs) from brain tissues is of great interest given the wide range of physiological and pathological roles they play in the central nervous system. Dissociating the brain tissue without damaging the cells and releasing intracellular content, minimizing contamination by non-EV and lipoprotein particles, avoiding protein degradation and EVs aggregation are some of the many technical challenges that need to be overcome. The aim of this study is to assess, both quantitatively and qualitatively, the advantages of isolating brain-derived EVs by inertial microfluidics as compared to standard ultracentrifugation-based approaches.
Methods: EVs were isolated from enzymatically dissociated adult mouse brains either by ultracentrifugation at 100,000 x g and iodixanol density gradients (Hurwitz et al., J Neurosci Methods 2018) or by using an inertial-based microfluidic device (Tay et al., Lab Chip 2021). EVs characterization by Western blot, nanoparticle tracking analysis, transmission electron microscopy (TEM) and cryo-electron microscopy is currently underway.
Results: While brain-derived EVs were successfully isolated using both ultracentrifugation and microfluidic approaches, the latter allowed for an improved yield, simpler and less laborious operation, and a much shorter processing time. TEM analysis showed that the size distributions and morphologies of EVs obtained by both methods were similar. We will present the results from ongoing quantitative and qualitative assays.
Summary/Conclusion: Microfluidic approaches are a useful alternative to isolate EVs from brain (or other) tissues while avoiding some issues such as the ultracentrifugation-induced aggregation of EVs. We will test this methodology to isolate EVs from human brain tissues and devise functional assays to assess their quality.

PT02.18 | A new improved method for the isolation of bacterial extracellular vesicles by the depletion of "extracellular vesicle like particles" in bacteriological culture medium

Lena Hoang My Le[1]; Joel R. Steele[2]; Ralf B. Schittenhelm[2]; Richard L. Ferrero[3]
[1]Hudson Institute of Medical Research, Monash University, Springvale, Australia; [2]Monash Proteomics and Metabolomics Facility, Department of Biochemistry and Molecular Biology, Monash University, Australia, Clayton, Australia; [3]Centre for Innate Immunity and Infectious Diseases, Hudson Institute of Medical Research, Australia, Clayton, Australia

Introduction: Bacterial extracellular vesicles (BEVs) are isolated from microorganisms that have been grown in culture media containing animal-derived products. Consequently, these media are likely to contain animal EVs that may have an effect on downstream analyses. Preliminary data showed that Brain Heart Infusion (BHI) broth, a bacteriological culture media, contained particles that were similar in size to bacterial and eukaryotic EVs, thereby suggesting that BHI broth may contain animal-derived EVs. Here, we developed an optimised protocol for BEV purification that would eliminate animal-derived EVs from the bacteriological culture medium. Furthermore, we compared the properties of BEVs isolated using this new protocol with those isolated from bacteria grown in standard medium.

Methods: To deplete BHI broth of animal-derived "extracellular vesicle-like particles" (EVLPs), we used tangential flow filtration with a Vivaflow membrane (50,000 MWCO). The filtrate was used to cultivate Helicobacter pylori. BEVs isolated from this new protocol with those isolated from bacteria grown in standard medium was then subjected to Nanoparticle tracking analysis (NTA), proteomics and biochemical analysis for comparison.
Results: NTA results showed that BEVs prepared from the non-depleted BHI medium contained greater particle numbers. EVLPs within BHI did not react with any eukaryotic EV marker antibodies (Epcam, Alix, CD9 and CD69), suggesting that these are not true eukaryotic EVs. Despite BEVs isolated from the two methods inducing similar interleukin-8 responses in mammalian cells, proteomic data suggested differences in their protein composition. While 399 H. pylori proteins were common to BEVs purified by both methods, 57 were unique to BEVs isolated from H. pylori grown in depleted BHI. Five were uncharacterised H. pylori proteins. Furthermore, there were 10x less bovine proteins and 3.4x more H. pylori proteins in BEVs prepared from non-depleted BHI. The decrease in bovine protein improved the quality of the sample for Mass spectrometry analysis.
Summary/Conclusion: Collectively, we showed that EVLPs present within BHI broth are not true EVs but may still interfere with downstream analyses, such as with proteomic studies where these hinder the full characterisation of BEV proteins. Furthermore, we have successfully described an optimised method for BEV isolation that increases the purity of isolated BEVs and reduces potential confounding effects of EVLPs.

PT02.19 | A GMP compliant chemically-defined culture medium sustains increased production of small extracellular vesicles while conserving their main properties and functions

Aliosha I. Figueroa-Valdés[1]; Catalina de la Fuente[2]; Ana María Vega-Letter[3]; Yessia Hidalgo[4]; Maroun Khoury[4]; Francisca Alcayaga-Miranda[5]
[1]Laboratory of Nano-Regenerative Medicine, Centro de Investigación e Innovación Biomédica (CiiB), Universidad de los Andes, Santiago, Chile, Santiago, Chile; [2]Consorcio Regenero, Chilean Consortium for Regenerative Medicine, Santiago, Chile, Santiago, Chile; [3]Laboratory of Molecular and Cellular Immunology, Faculty of Medicine, Universidad de los Andes, Santiago, Chile, Santiago, Chile; [4]IMPACT, Center of Interventional Medicine for Precision and Advanced Cellular Therapy, Santiago, Chile, Santiago, Chile; [5]Universidad de los Andes, Santiago, Chile

Introduction: small Extracellular Vesicles (sEV) are acquiring a major interest in the cell therapy field. However, their successful translation will rely ultimately on their large-scale production necessary to achieve the doses-required quantities.
Methods: We aimed to compare the sEV production from various types of cells including mesenchymal stromal cells (MSCs) in several harvest time points (2, 4 and 6 days) using Oxium®EXO, standard DMEM and a commercially available medium developed specifically for sEV production. Phenotype and cell viability analyses of parental cells were performed after the sEV production cycle. sEV secretion rate to the supernatant was evaluated by nanoparticle tracking analysis (NTA). sEV isolation by ultracentrifugation allowed characterization by NTA, flow cytometry and in vitro cell uptake assays, a in mice in vivo biodistribution study and a comparison of sEV's miRNA cargo profile.
Results: MSC's phenotype and differentiation capacity was maintained in the three mediums after sEV production cycle. Oxium®EXO allowed a better cell viability after 6 days of production, as well as a 3-fold increase in total particle secretion to the supernatant and a 4-fold increase in those particles sized between 50 to 200nm, compared to DMEM or the commercial medium. The isolated-sEV characterization showed the presence of CD63, CD9 and CD81 on the three isolated types of sEV. No differences were observed as well in the in vitro cell uptake assay nor the in mice in vivo biodistribution pattern. The miRNA cargo profile will be discussed.
Summary/Conclusion: Oxium®EXO allowed an increased particle secretion rate while conserving the classic sEV functional properties of internalization into acceptor target cells and biodistribution in vivo, supplying the amount and quality of sEV for the development of potentially cell-free therapies.
Funding: In part by Chilean National Agency for Research and Development (ANID) FONDECYT Regular project #1190411, by Consorcio Regenero S. A., Chile and by Cells for Cells S. A., Chile.

PT02.20 | Characterization of EVs isolated by a novel magnetic bead-based method utilizing automated western blotting

Martin Schlumpberger[1]; Rachel Talis[2]; Francisco Ramirez[2]; Karolin Spitzer[1]; Caroline Metzen[1]; Nike Bahlmann[1]; Chris Heger[2]; Markus Sprenger-Haussels[1]
[1]QIAGEN GmbH, Hilden, Germany; [2]Bio-Techne, Analytical Solutions Division, San Jose, USA

Introduction: The study of extracellular vesicles (EVs) not only keeps producing new insights into fundamental biologic processes, but also continues to provide new opportunities in the diagnosis and treatment of a wide range of diseases. Despite that, the field is still lacking methods for isolation of EVs that provide not only the required efficiency and specificity, but also standardization, scalability, short processing time and potential for full automation, required for routine use. Here we present a novel, magnetic bead-based workflow, utilizing biochemical affinity, followed by characterization of the isolated EVs according to MISEV criteria using an automated analytical platform.
Methods: Total EVs were isolated by magnetic beads from different volumes of pre-filtered plasma or cell culture supernatant. Intact vesicles were eluted from the beads and the EV-depleted biofluid fraction retained for parallel analysis. Each fraction (3 μL) was analyzed for the presence or absence of MISEV-recommended protein markers by Simple Western, an automated capillary electrophoresis-based western platform. Isolated EVs were further characterized using Nanoparticle Tracking Analysis (NTA), and by their RNA content.
Results: In comparison to EVs isolated by a commercially available biochemical affinity column-based workflow, eluates obtained by the new magnetic bead-based process showed highly similar enrichment of EV marker proteins, and depletion of contaminating non-EV proteins, as well as similar size profiles and RNA content.
Summary/Conclusion: The novel method presented provides new options for EV isolation, including future automation, and is expected to be particularly useful for routine applications.

PT02.21 | Easy and consistent purification of CD9, CD63, and CD81 positive functional extracellular vesicles

Tom Krol; Curtis Yingling; Joy Hayden; Tess Swope
Clara Biotech, Lawrence, USA

Introduction: The lack of a standardized tool that supports an efficient method for the isolation of extracellular vesicles (EVs) by subtype, with consistency between users, has limited potential research outcomes in the EV field. One solution to this barrier is the use of novel magnetic "Nano Poms" beads which combine immunoaffinity and photo-release technology. The magnetic beads contain graphene "Nano Poms" which increase surface area, which allow for simple isolation of EVs from a variety of samples such as plasma, cell culture medium, urine, and milk. The technology is also capable of using a variety of antibodies to isolate different subtypes of EVs.
Methods: The novel magnetic "Nano Poms" beads use antibodies to three tetraspanins (CD9, CD63, and CD81), allowing capture of EVs expressing those markers. For isolating EVs from plasma, 1 mL of plasma is centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant is mixed with the "Nano Poms" beads and incubated at 4°C overnight on a rotator. The next morning the magnetic "Nano Poms" beads are washed with PBS and moved to a glass vial. EVs are released under UV light during a 20-minute incubation while shaking at 100 rpm at 4°C. Magnetic "Nano Poms" beads are separated, and supernatant is transferred to 0.65 mL microtube for downstream analysis.
Results: This method consistently provided 1 x 109 CD9, CD63, and CD81 positive EVs with a narrow peak around 100 nm in size, as shown by nanoparticle tracking analysis. The results were consistent regardless of the user.
Summary/Conclusion: The novel magnetic "Nano Poms" beads are capable of isolating pure EVs by avoiding non-specific binding through the use of a photo-release peptide. The platform is capable of aiding in research and development, diagnosis, and therapeutics by isolating specific intact EVs subtypes, which opens the door to isolate more EVs subtypes. Ease and consistency between users are the strengths of this isolation method demonstrated by this study.
Funding: This work was sponsored by Clara Biotech.

PT02.22 | Müller cells are a potential source of EVs within the retina

Melanie Schwämmle[1]; Xu Cheng[2]; Günther Schlunck[1]; Hansjürgen Agostini[1]; Irina Nazarenko[3]; Felicitas Bucher[1]
[1]Eye Center, Medical Center, Faculty of Medicine, University Medical Center Freiburg, Freiburg, Germany, Freiburg, Germany; [2]Institute for Infection Prevention and Hospital Epidemiology, Faculty of Medicine, University Medical Center Freiburg, Freiburg, Germany; Xiangya Hospital Affiliated To Central South University, Changsha, Hunan Province, PR China, Changsha, China (People's Republic); [3]Institute for Infection Prevention and Hospital Epidemiology, Faculty of Medicine, University Medical Center Freiburg, Freiburg, Germany, Freiburg, Germany

Introduction: Müller cells (MCs) are the major macroglia cell type in the retina. By spanning all retinal layers, MCs can interact with almost every retinal cell. However, the communication of MCs with surrounding neuronal and vascular cells is insufficiently understood. Two intercellular communication mechanisms have been described for MCs: direct interaction via gap junctions

and indirect paracrine communication. Nevertheless, increasing evidence points to a communication via extracellular vesicles (EVs). This project aims to assess the possible existence of murine MC-derived EVs by establishing an EV-isolation protocol from a primary MC culture and by characterizing the isolated EVs.

Methods: Culturing of murine MCs was started at P11/P12 by retina isolation, tissue homogenization and cell incubation in growth medium. At passage 2, medium was changed to differentiation medium for 7 days. To optimize MC differentiation and EV isolation, MCs were cultured under different media conditions including EV-depleted media gained by ultracentrifugation. MC identity under different cell culture conditions was confirmed using ICC. EV-isolation from MC-conditioned media, produced for 72h, was performed using tangential flow filtration and size exclusion chromatography. Characterization was conducted by NTA, TEM, ELISA, WB and MicroBCA.

Results: Characterization of MCs cultured in EV-depleted medium for different time periods were consistent with MCs in standard MC culture expressing GS and Kir4.1, markers used to label MCs in vivo. We were able to isolate MC-derived EVs if EV-depleted media were being used at early stages of MC cultivation. The isolated particles were positive for the EV-specific markers CD9 and CD81 as well as for the MC-specific marker AQP4. Moreover, nanosized structures enclosed by a lipid-bilayer could be visualized by TEM.

Summary/Conclusion: We successfully established a method for the isolation of MC-derived EVs. Using our protocol further analyses can now be performed.

Funding: MS, FB: Else-Kröner-Fresenius Stiftung; FB: DFG BU3135/3-1, Berta-Ottenstein-Fellowship

PT02.23 | SubXTM Technology for Isolation of Virus-Free Exosomes

Andrei Malykh[1]; Andrey Pavlov[1]; Vladimir Evtushenko[2]
[1]CAPITAL BIOSCIENCES, INC., Gaithersburg, USA; [2]A.M. Granov Russian State Center of Radiology and Surgery Technologies, Saint Petersburg, Russian Federation, St. Petersburg, Russia

Introduction: Overlapping size between viruses and exosomes (extracellular vesicle; EV) is the major obstacle for the isolation of pure EVs by most popular commercial kits based on EV precipitation by ultracentrifugation, PEG salting-out, hydrophobic binding with silicon carbide, or size exclusion chromatography. Historically, these methods have been employed for viral isolation, so one can expect co-isolation of viruses with EVs, especially from virus-containing liquids. Earlier we demonstrated that SubX technology is appropriate for isolation of both cfDNA and EVs from the same bioliquid sample. The different affinity of the SubX molecules to EV membrane phospholipids and phosphate residues of nucleic acids allows for the efficient separation of EVs from cfDNA. Due to the high affinity to nucleic acids, SubX squeezes out proteins from the nucleoprotein complexes and forms SubX-DNA/RNA pellet insoluble in the exosome reconstitution buffer (ERB). Based on this observation we suggested that EVs can be purified of the viruses since viral proteins are substituted by SubX and viral DNA/RNA will remain in pellets.

Methods: Cell line B95-8 shedding Epstein-Barr Virus (EBV) was used as to develop the protocol for exosomes and viral DNA isolation from culture medium utilizing SubX Exo-DNA isolation kit. RNA-carrying recombinant lentiviruses were used as spikes in different bioliquids. EBV DNA quantity was determined by qPCR with primers for EBNA 1 gene. Extracellular genomic DNA released by B95-8 cells was detected using primers for 36B4 gene. RNA quantity was determined by RT-qPCR using primers specific for lentivirus used. Detection of SubX-DNA and exosomes binding was done by measurement of particle sizes using Zetasizer Nano ZS and Nanosight NS300.

Results: SubX molecules bind exosomes and form micron-size aggregates that are easily pelleted in a brief 14K x g centrifugation step. SubX-DNA/RNA complexes also precipitate at the same centrifugation force. Exosomes are easily solubilized in ERB from the pellet, while tightly bound DNA/RNA remains insoluble. Less than 0.5% of viral DNA was associated with exosome fraction, while post-exosome pellet contained >99% DNA. This is over 200-fold purification of exosomes from viruses. Thus, we separate two distinct types of extracellular material – intact exosomes and mixture of viral plus cfDNA in a single protocol from the same sample. Also, less than 0.25% of B95-8 genomic cfDNA is detected in exosome fraction. Current results suggest that the EV-associated DNA measured after application of the SubX technology does not contain cf- or viral DNA.

Summary/Conclusion: SubX technology based on specific capture of phosphate-clusters permits isolation of exosomes free of non- phospholipid vesicles, protein aggregates, DNP/RNP, as well as viral contamination. It also appears efficient for isolation of viral DNA or RNA from bioliquids (plasma, urine, cell culture medium).

PT03: Physiology and pathology: Central and peripheral nervous systems

Chair: Tiana F. Koukoulis – The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, Australia

Chair: Hargita Hegyesi, Semmelweis University Department of Genetics, Cell and Immunobiology

PT03.01 | Cerebrospinal fluid extracellular vesicles and miRNA cargo as disease specific biomarkers for Alzheimer's disease

Julie A. Saugstad[1]; Ursula S. Sandau[1]; Trevor J. McFarland[2]; Erika Duggan[3]; John P. Nolan[3]; Joseph F. Quinn[2]
[1]Oregon Health & Science University, PORTLAND, USA; [2]Oregon Health & Science University, Portland, USA; [3]Scintillion Institute, San Diego, USA

Introduction: Alzheimer's disease (AD) is the most common cause of dementia, and the sixth leading cause of death in the US. Given that brain changes in AD begin 10-20 years before symptoms appear, it is essential to identify brain changes that occur early in AD and to develop effective therapies to stop or slow AD progression. We previously identified miRNA biomarkers for AD in human cerebrospinal fluid (CSF). Here our study assessed the specificity of extracellular vesicles (EVs) and their miRNAs as biomarkers for AD vs. Parkinson's disease (PD), a related neurodegenerative disorder.
Methods: EVs from human AD, PD, and controls (CTL) (n=10/group) were isolated from lumbar CSF using ultrafiltration combined with size exclusion chromatography. EV concentrations and sizes were determined by vesicle flow cytometry. MiRNAs were quantied using a custom TaqMan array with probes for 36 previously established AD miRNAs.
Results: There was no effect on the total number of EVs in CSF in the AD and PD groups vs. CTL. However, we did observe an increase in EV size in AD and PD vs. CTL. The arrays detected 30 miRNAs in total CSF, and 25 miRNAs in the CSF EVs. Four of the 25 CSF EV miRNAs were detected in both AD and PD with a 1.5 fold decrease in expression, and an area under the curve (AUC) > 0.7 for classifying either AD or PD from CTL. Five of the CSF EV miRNAs had a 1.5 fold change in expression only in AD, relative to CTL. These five miRNAs performed well individually and in combination to classify AD, but not PD, from CTL. Furthermore, the expression levels of CSF EV miRNAs generally performed better at classifying AD than the total CSF miRNAs.
Summary/Conclusion: Human CSF EV miRNAs can classify AD from PD, and show better performance than total CSF miRNAs.

PT03.02 | Characterization of extracellular vesicles produced by astrocytes from the spinal cord of SOD1G93A late symptomatic mice

Roberta arianna Zerbo[1]; Carola Torazza[1]; Fabrizio Fabbiano[2]; Marco Milanese[1]; Vito Giuseppe D'Agostino[3]; Giambattista Bonanno[1]
[1]Department of Pharmacy, Pharmacology and Toxicology Unit, University of Genoa, Genoa, Italy, Genoa, Italy; [2]Department of Cellular, Computational and Integrative Biology, University of Trento, Trento, Italy, Trento, Italy; [3]Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy

Introduction: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that affects upper and lower motor neurons (MNs), leading to muscle atrophy and paralysis. ALS is a non-cell autonomous disease, astrocytes playing a central role in clinical progression.
Since extracellular vesicles (EVs) own a key role in cell-to-cell communication, we compared EVs derived from SOD1G93A (an ALS animal model) and WT mouse astrocytes. We also studied the effect of EVs from SOD1G93A astrocytes on WT MN viability.
Methods: Astrocytes were prepared from 120-day-old spinal cord SOD1G93A and WT mice. The tissue was mechanically dissociated in DMEM medium + N2 factor and astrocytes were cultured at 37°C and 5% CO2 for 20 DIV before experiments.
EVs were isolated by nickel-based isolation (NBI) from the supernatant of astrocytes by using positively charged agarose beads and eluted by means of EDTA-NaCl elution buffer.
MNs were isolated by gradient from the spinal cord of E13,5 SOD1WT embryos and cultured. MNs were counted for viability from day 8 to day 14 after seeding.
EV-RNA was extracted from NBI-isolated EVs using the Single Cell RNA extraction kit (Norgen Biotek) and analyzed by RNA sequencing.
Results: The amount of EV recovered from SOD1G93A and WT astrocyte was 6,73x105 and 1,78x106 respectively (cell number: SOD1G93A 6.37x105; WT 1.61x105). We did not detect EV size or Z potential differences.

We completed the RNA sequencing to define the microRNA repertoire of SOD1G93A and WT astrocyte-derived EVs. Data are underwenting bioinformatic analysis.
MN viability was significantly reduced when MNs were exposed to EV from SOD1G93A astrocytes compared to untreated MNs
Summary/Conclusion: Our results indicate that pathological astrocytes influence MN viability in ALS via EV secretion and suggest that EVs from SOD1G93A astrocytes may retain a unique signature defining their neurotoxic activity. Data from RNA sequencing should straightly shape EV characteristics.
Funding: MUR-PRIN, grant n. 2017F2A2C5

PT03.03 | Conventional dendritic cells reprogrammed by extracellular vesicles from amniotic fluid stem cells rescue autoimmune neuroinflammation

Giorgia Manni[1]; Marco Gargaro[1]; Eleonora Padiglioni[1]; Simona Fontana[2]; Alessandra Di Veroli[3]; Gabriele Cruciani[3]; Marco Cipolloni[4]; Alessandro Di Michele[5]; Vincenzo Nicola Talesa[1]; Rita Romani[1]; Francesca Fallarino[1]
[1]Department of Medicine and Surgery, University of Perugia, Perugia, Italy; [2]University of Palermo, Dep. of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), Palermo, Italy; [3]Department of Chemistry, Biology and Biotechnology, University of Perugia, Perugia, Italy; [4]TESPharma S.R.L., Corciano, Perugia, Italy; [5]Department of Physic and Geology, University of Perugia, Perugia, Italy

Introduction: Dendritic cells (DCs) are potent antigen-presenting cells that control adaptive immunity and balance effector and regulatory components of the immune response. Extracellular vesicles (EVs), released from human amniotic fluid stem cells (HAFSCs), are characterized by important immunoregulatory properties. In this study, we investigated the potential of HAFSC-EVs to promote tolerogenic effects on specific subsets of murine DCs.
Methods: We first produced EVs from HAFSCs cultured in serum-free medium. Ultracentrifugation of HAFSC-conditioned cell medium allowed isolation of EVs that were characterized by scanning electron microscopy (SEM). Nanoparticle tracking analysis (NTA) distribution plots showed vesicle size.Western blot analysis confirmed that the isolated fraction contained EVs and exosomes markers. Lipidomic, proteomic and miRNA analysis were used to completely characterize EVs. Murine DCs and T cells were isolated from bone marrow and spleen respectively. Confocal microscopy and cytofluorimetric analysis were used to evaluate EV uptake to cells. Experimental autoimmune encephalomyelitis (EAE) model was induced in C57BL/6 female mice immunized with MOG35–55 peptide.
Results: We demonstrated that HAFSC-EVs are preferentially internalized by conventional dendritic cell type 2 (cDC2), but not by other cDCs, both in vitro and in vivo. Protein and miRNA cargo analysis revealed the enrichment of several immunoregulatory pathways in HAFSC-EVs. Indeed, immunogenic cDC2 conditioned with HAFSC-EVs acquired strong tolerogenic functions. Transfer of cDC2 conditioned with HAFSC-EVs in vivo resulted in suppression of autoimmune responses and significant improvement in the clinical score of EAE.
Summary/Conclusion: These results demonstrate that HAFSC-EVs, which are naturally loaded with immunoregulatory mediators, contribute to reprogram inflammatory cDC2 to tolerogenic functions, leading to the control of autoimmune responses.

PT03.04 | EVs derived from CSC-exposed myeloid lineage cells increase IL-1β levels in CNS cells

Sunitha Kodidela[1]; Asit Kumar[1]; Namita Sinha[2]; Santosh kumar[1]
[1]UTHSC, Memphis, USA; [2]UTHSC, Mrmphis, USA

Introduction: Cigarette smoke condensate (CSC), a component of cigarette/tobacco smoking, increases pro-inflammatory molecules in myeloid lineage cells. However, the cellular mechanism by which CSC contributes to CNS inflammation is unclear. We have recently demonstrated that long-term exposure of CSC to HIV -uninfected(U937) and infected (U1) macrophages are known to induce packaging of pro-inflammatory molecules, particularly IL-1β, in extracellular vesicles(EVs). Therefore, we hypothesize that exposure of EVs derived from CSC-treated monocyte/macrophages to CNS cells can increase their IL-1β levels, contributing to neuroinflammation.
Methods: The U937 and U1 differentiated macrophages were treated once-daily with CSC (10μg/ml) for 7-days. After 7 days of treatment, we collected the media, isolated EVs, and characterized those according to the ISEV guidelines. The EVs isolated from 1ml of media from above U937 and U1 cells were treated to 1ml of media containing human astrocytic and neuronal cells (0.1million cells/well; 12 well plate) once daily for three days and two days respectively in the presence of CSC. At the end of the treatment, the cells were harvested and examined for protein levels superoxide dismutase-1 (SOD1), catalase (CAT), IL-1β, and Glial fibrillary acidic protein (GFAP) using western blot. The differences in the relative expression of protein levels between groups were compared using ANOVA and $p < 0.05$ is considered significant.

Results: We observed that the U937 cells have lower expression of IL-1β compared to their respective EVS, confirming that most of the produced IL-1β get packaged into EVs. Our results suggest that the astrocytic and neuronal cell lines which received EVs derived from CSC exposed macrophages (U937 and U1 cell lines) showed increased expression of IL-1β levels (~500 folds) compared to the control and CSC groups alone. In addition, the expression of IL-1β was furthermore high in the group that received the exposure of both EVs and the CSC.
Summary/Conclusion: These results suggest that CSC might contribute to neuroinflammation by EV-mediated transfer of increasing IL-1β in astrocytic and neuronal cells
Funding: This study is supported by funding from the NIH grant DA047178 (Santosh Kumar)

PT03.05 | Neuronal primary cilia in morphine tolerance

Rong Ma[1]; Naseer Kutchy[2]; Guoku Hu[1]
[1]University of Nebraska Medical Center, Omaha, USA; [2]St. George's University, True Blue, Grenada

Introduction: Tolerance leads to not only limited use of opioids, including morphine, as analgesics but also increased risk of overdose and death. Critical features of morphine tolerance involve both neurons and astrocyte functions. There is little understanding of how astrocyte-neuron communication that can normally provide the essential networks underlying brain function is modified by morphine and contribute to morphine tolerance. Recent studies by others and us have demonstrated that pharmacological inhibition of extracellular vesicle (EV) release prevents morphine tolerance.
Methods: C57/B6 mice were administered morphine for five days to develop tolerance, determined using the tail-flick. Mouse primary astrocyte-derived EVs (ADEVs) were isolated using both size exclusion chromatography (SEC) and ultracentrifugation approaches followed by characterization of EVs using zetaview for EV size distribution and number, and Western blotting for EV markers as well as electron microscopy for EV morphology. Mouse primary neurons were treated with either control-ADEVs or morphine-ADEVs for 48h followed by assessment of primary cilia. Pharmacological and genetic approaches were used to determine the role of EVs in primary ciliogenesis. Primary cilia were assessed by fluorescent immunostaining for primary cilia markers.
Results: We found that morphine-stimulated astrocyte-derived EVs (morphine-ADEVs) can be taken up by neurons and promote neuronal primary ciliogenesis. Moreover, we also found that SHH was increased on morphine-ADEVs that can activate SHH signaling in neurons. Our in vivo results demonstrate that inhibiting primary cilia in neurons prevents morphine tolerance in mice.
Summary/Conclusion: ADEV-mediated neuronal primary ciliogenesis and SHH activation contribute to the development of morphine tolerance.
Funding: NIH- R21DA046831, R21DA042704 and MH112848

PT03.06 | Neuron-to-neuron of polyGR transmission through extracellular vesicles causes degeneration in receiving cells

Maria Elena Cicardi[1]; Davide Trotti[2]
[1]thomas jefferson university, Philadelphia, USA; [2]thomas Jefferson Univesrity, Philadelphia, USA

Introduction: Amyotrophic lateral sclerosis (ALS) and fronto-temporal dementia (FTD) are two neurodegenerative disease which belong to the same disease spectrum. The most common genetic cause of ALS-FTD is a hexanucleotide repetition (G4C2) present in the intron 1 of the C9orf72 gene. This mutation results in three main toxic events: C9orf72 protein reduction, RNA foci formation and aberrant repeat associated non-AUG translation (RAN-T). The products of RAN-T are five dipeptide repeat proteins (DPRs: polyGA, polyGP, polyGR, polyPA and polyPR).
Methods: Extracellular vesicles extraction from mouse blood/cell culture media by UC and size exclusion chromatography (IZON columns)
Analysis of EVs by NTA, Cytoflex, proteomic,
Bioactivity of extracellular vesicles through live imaging of neurons
Bioactivity of extracellular vesicles through in vivo injections is mice
Primary cortical nuerons, hiPSCS
Results: Employing a mouse model which conditionally expresses polyGR we found that a fraction of the extracellular vesicles (EVs) present in the serum encloses polyGR. We thus analyzed whether neurons produce EVs loaded with RAN-T products. Indeed, DPRs were detected in neuronal EVs and glutamate stimulation was able to increase the number of polyDPR+ EVs. Interestingly, we found higher percentage of polyGR+ EVs compared to other DPRs. We then investigate if the internalization

of polyGR+ EVs elicited toxicity in recipient neurons. We treated cortical neurons with polyGR+ EVs and by live imaging we found a significant reduction in cell viability compared to control EVs treated neurons paralleled by TDP-43 nuclear depletion which is hallmark of ALS-FTD pathology. When we performed in vivo experiments injecting polyGR+ EVs in mouse spinal cord we observed loss of NeuN positive cells, paralleled by astrocytes and microglial recruitment at the injection site. Treating human derived motor neurons (MNs) with polyGR+ EVs and we found that only C9-ALS derived MNs show increased neurodegeneration possibly owing to polyGR+ EVs induced RAN-T which we were able to measure thanks to a luciferase RAN-T reporter.

Summary/Conclusion: In conclusion we demonstrated that polyGR reception into neurons was able to cause neuronal toxicity, TDP-43 nuclear depletion and increase in RAN-T substantiating the hypothesis that EVs mediated neuron-to-neuron spreading of polyDPRs is one of the possible pathways in which neurodegeneration spreads across the central nervous system during ALS or FTD.

Funding: NIH - R01 to DT
DoD post doctoral fellowship to MEC

PT03.07 | Osteopontin mRNA transferred via extracellular vesicles: a repair mechanism after stroke?

Paul Kügler[1]; Santra Brenna[2]; Clemens Falker-Gieske[3]; Yuqi Gui[1]; Hermann Altmeppen[4]; Tim Magnus[4]; Berta Puig[5]
[1]UKE, HAMBURG, Germany; [2]Department of Neurology, Experimental Research in Stroke and Inflammation (ERSI), University Medical Center Hamburg-Eppendorf, Hamburg, Germany., HAMBURG, Germany; [3]Georg-August-Universität, Göttingen, Germany; [4]UKE, Hamburg, Germany; [5]Department of Neurology, Experimental Research in Stroke and Inflammation (ERSI), University Medical Center Hamburg-Eppendorf, Hamburg, Germany., Hamburg, Germany

Introduction: After ischemic stroke, where blockage of a brain artery impedes the supply of glucose and oxygen, the directly affected neurons die by necrosis, whereas brain cells surrounding the ischemic area (penumbra) remain metabolically active and can potentially be rescued. Several extracellular signals, including those received via extracellular vesicles (EVs), will influence the fate of brain cells at the penumbra in the hours/days following the ischemic insult. To decipher the nature of these critical extracellular stimuli, is fundamental to understand the pathophysiology of stroke.

Methods: The aim of the present study was to analyse the mRNA cargo of brain-derived EVs (BDEVs) at two time-points, 24h (acute phase) and 7 days (recovery phase), after transient Middle Cerebral Artery Occlusion (tMCAO, a widely used mouse model of stroke) to gain insight into their potential participation in brain recovery processes. In a previous study, we proved that a commercially available mRNA expression panel allowing for multiplexed assessment of 770 genes is suitable to analyze BDEV mRNAs without the need for previous RNA isolation, thus simplifying the protocol and limiting sample/information loss. We here applied the same targeted approach together with an improved BDEVs isolation protocol using a iodixanol gradient.

Results: Nanoparticle Tracking Analysis (NTA) showed that the amount of BDEVs is significantly increased at 7 days after reperfusion, together with the presence of more mRNAs being significantly upregulated (160 mRNAs) compared to 24h after stroke (with 78 mRNAs significantly increased). The analysis of gene ontology (GO) pathways for biological processes indicated a decrease in the "inflammatory response" (GO:0006954) and an increase in "immune system process" (GO:0002376) and "immune response" (GO:0006955) at 7 days compared to 24h. Fittingly, many highly overexpressed mRNAs at 7 days can be ascribed to microglia, the resident immune cells of the brain. Lastly, we detected that the most upregulated mRNA in BDEVs at 7 days after stroke is Spp1, which encodes for osteopontin (OPN), a phospho-glycoprotein secreted by macrophages and microglia and acting protective after stroke. By immunofluorescence analysis, we observed that OPN is upregulated at the cortical penumbral area.

Summary/Conclusion: The results presented here point to a possible contribution of BDEVs loaded with Spp1 mRNA to the mechanisms of survival/recovery of neurons after stroke.

PT03.08 | Study of deregulated microRNA and their targets in small extracellular vesicles of Fronto-temporal Dementia patients

maria Garofalo; Daisy Sproviero; Francesca Dragoni; Eleonora Corridori; Alfredo Costa; Giulia Perini; Matteo Cotta Ramusino; Orietta Pansarasa; Stella Gagliardi
IRCCS Mondino Foundation, Pavia, Italy

Introduction: Fronto-Temporal Dementia (FTD) is characterized by aggregation of proteins (TDP-43 and Tau) in the frontal and temporal lobes with microvacuolation and relevant deregulation of RNA-binding proteins (RBPs).

We investigated miRNA cargo of small Extracellular Vesicles (SEVs) derived from plasma of FTD patients and healthy controls. The purpose was to evaluate deregulated microRNAs in patients to identify new peripheral biomarkers. Moreover, we aimed to identify mRNA targets involved in FTD pathogenesis.

Methods: SEVs were isolated from plasma of 9 FTD patients and 9 healthy volunteers by differential centrifugation and characterized by Nanosight. MicroRNA libraries were generated using Small RNA-Seq Library Prep Kit (Lexogen) and sequenced on a NextSeq 500 (Illumina). Interaction prediction was carried out on TarBase v.8 database.

Results: We found a total of 197 Differentially Expressed microRNAs, 99 up-regulated and 98 down-regulated. Then, we looked for directly validated mRNA targets of the most deregulated microRNAs in our analysis. Interestingly, hsa-miR-522-5p, down-regulated in our profiling, targets RTN3 that in turn interacts with and modulates BACE1, and the up-regulation of hsa-miR-203a-3p may impact on TNF and IL-12 levels. Moreover, hsa-miR-181c-5p was up-regulated and its role was already linked to a negative feedback network of TDP43. We also found a down-regulated microRNA in common with Alzheimer's disease, hsa-miR-1260b, involved in Wnt pathway.

Summary/Conclusion: In conclusion, our data highlight the importance of microRNAs cargo examination in EVs of FTD patients. In fact, their potential is exploitable both for biomarkers discovery and for study of gene expression alteration in FTD pathogenesis.

Funding: This research was funded by Italian Ministry of Health (Grant N°5*1000 anno 2016, Ricerca Corrente 2018–2020, Young research project GR-2016-02361552, AIFA-co-ALS); EuroNanoMed III JTC 2018; Fondazione Regionale per la Ricerca Biomedica for TRANS–ALS (FRRB 2015-0023); Fondazione Cariplo 2017 (Extracellular vesicles in the pathogenesis of Frontotemporal Dementia 2017-0747; Association between frailty trajectories and biological markers of aging 2017-0557).

PT03.09 | The effect of serum on human astrocyte reactivity and their extracellular vesicles in vitro

Katherine E. White[1]; Robert Layfield[1]; Daniel Scott[1]; Robert Markus[1]; Raquel Mesquita-Ribeiro[2]; Zubair Ahmed Nizamudeen[3]; Denise McLean[1]; Kenton P. Arkill[1]; Sebastien Serres[4]

[1]University of Nottingham, Nottingham, United Kingdom; [2]School of Life Sciences, University of Nottingham, Nottingham, United Kingdom; [3]University of Nottingham, Oxford, United Kingdom; [4]Life Sciences Nottingham university, Nottingham, United Kingdom

Introduction: Astrocytes are abundant glial cells in the central nervous system (CNS) that undergo phenotypic and functional changes in response to injury and disease. Whilst not fully understood, these changes can be used to classify astrocytes as quiescent or reactive to study astrocyte-derived extracellular vesicles (ADEVs). Reactive astrocytes are more common in neurodegenerative disease so ADEVs could act as biomarkers for CNS disease and may be involved in disease transmission. Current in vitro models typically use serum such as fetal bovine serum (FBS) to culture healthy astrocytes; however serum may induce astrocyte reactivity. This project therefore aims to investigate the effect of FBS on the morphology and gene expression of human astrocytes and ADEVs by developing a serum-free model.

Methods: Primary human astrocytes were cultured in serum-free or serum media and characterised using ICC, qPCR and RNA-SEQ. ADEVs were collected in 40ml of serum-free media either from a serum-free model over 2 weeks, or for 72 hours in the serum model. Ultrafiltration was completed to concentrate the media, followed by size exclusion chromatography using qEVorignal 70nm columns to isolate ADEVs. ADEVs were then characterised using western blotting, TEM and dSTORM. Finally, proteomic analysis was completed on EVs and cell lysates from each model using mass spectrometry (LC-MS/MS).

Results: Serum-cultured astrocytes showed morphological changes and were associated with upregulated markers of astrocyte reactivity compared to serum-free culture (n=3). RNA-SEQ found 1474 unique protein-coding RNA exclusive to serum-free cultures (n=4). Gene ontology analysis found 577 significantly upregulated pathways in serum-free astrocytes including cell-adhesion pathways, axon and synapse development and ion channel activity. Proteomic analysis found distinct differences in EV protein cargo and cell lysates in both models with ~200 unique proteins identified in EV samples (n=3).

Summary/Conclusion: These findings suggest that there are clear differences in astrocyte phenotype when using serum-containing media and therefore serum should not be used to study quiescent astrocytes and their ADEVs. Instead, we recommend serum-free cultures whilst serum-based cultures could still be used as a model of reactive astrocytes.

Funding: Funded by the BBSRC doctoral training program.

PT03.10 | The Protective Effects of miR-210 Modified Endothelial Progenitor Cells Released Exosomes in Hypoxia/Reoxygenation Injured Neurons

Ji Bihl[1]; Sri Yerrapragada[2]; Harshal Sawant[1]

[1]Marshall University, Huntington, USA; [2]Wright State University, Dayton, USA

Introduction: We have previously demonstrated that endothelial progenitor cells (EPCs) provide beneficial effects on ischemic stroke by reducing oxidative stress, which could be through EPCs-released exosomes (EPC-EXs). EXs are emerging as a bioagent for mediating cell-cell communications via their carried microRNAs (miR). miR-210 is shown to provide a neuroprotection effect against ischemic stroke. Here, we aimed to determine whether the combination of EPC-EXs and miR-210 would provide an enhanced protective effect on neurons.
Methods: The hypoxia and reoxygenation (H/R) model were applied to neurons to mimic the ischemic injury of neurons. EPCs were transfected with miR-210 mimic to elevate the level of miR-210 in cells and EPC-EXs (miR210-EPC-EXs). For functional studies, EPC-EXs were co-incubated with H/R-injured neurons, then the cell viability and reactive oxygen species (ROS) production were determined.
Results: The results showed 1) H/R induced apoptosis and ROS overproduction in neurons; 2) miR-210 mimic increased the level of miR-210 in both EPCs and EPC-EXs; 3) EPCs cultured in serum-free medium released more exosomes in comparison with cells grown in complete growth media, suggesting serum starving induce the release of EXs; 4) After transfection, EPCs grown in complete media had almost 50 times higher miR-210 level than EPCs had in serum-free media, while the EPCs-EXs isolated from the complete media has lower miR-210 expression than from the serum-free media in a time-dependent manner, suggesting the transfer of miR-210 through EXs; 5) After co-incubation, EPC-EXs and miR210-EPC-EXs could be uptaken by neurons, miR210-EPC-EXs could elevate the miR-210 level in neurons; 6) miR210-EPC-EXs were more effective in promoting cell viability and decreasing apoptosis and ROS production.
Summary/Conclusion: The present study demonstrated that EPCs-carried miR-210 could be released and transferred to neurons in a time-dependent manner and that miR-210 loading can enhance the protective effects of EPC-EXs on H/R-induced neuron apoptosis, oxidative stress, and decreased viability.
Funding: This work was supported by the National Institute of Neurological Disorders and Stroke (1R01NS102720).

PT03.11 | Understanding the role of extracellular vesicles in the pathophysiology of Alzheimer's disease

Mohsin Shafiq[1]; Ladan Amin[2]; Andreu Matamoros-Angles[1]; Alexander Hartmann[3]; Stefano Da Vela[4]; Ilka Egger[1]; Emina Karadjuzovic[1]; Ayesha Zafar[1]; Behnam Mohammadi[1]; Carolin Seuring[5]; Bente Siebels[6]; Hannah Voß[6]; Minna Holopainen[7]; Reijo T Käkelä[7]; Hartmut Schlüter[6]; Hermann Altmeppen[8]; David A Harris[2]; Dmitri Svergun[4]; Markus Glatzel[1]
[1]Institute of Neuropathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, Hamburg, Germany; [2]Department of Biochemistry, Boston University School of Medicine, Boston, MA, USA., Boston, USA; [3]Institute of Neuropathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, hamburg, Germany; [4]EMBL European Molecular Biology Laboratory Hamburg, Germany, Hamburg, Germany; [5]Multi-User CryoEM facility, Centre for Structural Systems Biology (CSSB) Hamburg, Germany, Hamburg, Germany; [6]Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, Hamburg, Germany; [7]Helsinki University Lipidomics Unit (HiLIPID), Helsinki Institute for Life Science (HiLIFE) and Biocenter Finland, Helsinki, Finland, Helsinki, Finland; [8]UKE, Hamburg, Germany

Introduction: Cellular prion protein (PrPC) is a GPI-anchored receptor glycoprotein with highest expression in the nervous and the immune system. It is also abundantly expressed on surface of the extracellular vesicles (EVs). In Alzheimer's disease (AD), although a decline in brain PrPC levels is reported but EV-expressed PrPC, along with shed extracellular PrPC forms, is suggested to sequester Aβ oligomers (Aβo) and to accelerate Aβ fibrillation, yet formal evidence is still lacking. Effects of PrPC decline on the EV compositions also lack comprehensive studies. Here, we aim to study the role of PrPC expressing EVs in Aβ fibrillization and to highlight physiological alterations associated with EV levels of PrPC.
Methods: PrPC-containing (WT) and PrPC-deficient (KO) EVs were obtained from WT and PrPC-KO Neuro-2a (N2a) cells, respectively. After achieving ∼70% confluency in DMEM (+ 10% FBS), cells were incubated (∼16h) with serum-free DMEM. EVs pellets were prepared from conditioned DMEM by differential centrifugation steps (1500xg, 15min; 10,000xg, 30min; and 100,000xg, 1h10min). EVs were characterized using NTA, immunoblotting, and cryo-EM. Small angle X-ray scattering (SAXS), super-resolution microscopy (SRM), Cryo-EM, proteomic and lipidomic profiling, and associative biochemical and biophysical methods were employed to further the study objectives.
Results: SAXS studies helped us to identify potent Aβo-sequestering properties of WT-EVs, which were further confirmed by SRM and aggregation assays. Lipidomic and proteomic profiling of WT- and KO-EVs pointed towards marked differences in the lipid and protein make-up (i.e., higher abundance of certain kinases, RNA- and DNA-binding proteins) of the WT- and KO-EVs.
Summary/Conclusion: Our findings provide new evidence for crucial roles carried out by PrPC expressing EVs in the pathophysiology of AD, i.e., their involvement in Aβ aggregation, and their potential involvement in AD-specific intercellular communication in the brain.

Funding: The experimental work has been funded by Joachim Herz foundation, University of Hamburg [PIER Hamburg / Boston seed grant (project ID: PHM-2019-03), PIER seed project (project ID: PIF-2020-10)] and the Forschungsförderungsfonds der Medizinischen Fakultät, University medical center Hamburg-Eppendorf.

PT04: Therapeutics: Pre-clinical in vivo studies and clinical trials

Chair: Eva Rohde – SCI-TReCS GMP Unit at Paracelsus Medical University, Salzburg, AUSTRIA

Chair: Sébastien Banzet – Institut de Recherche Biomédicale des Armées / INSERM UMR-MD 1197

PT04.01 | Antirheumatic therapy is associated with changes in extracellular vesicles proteomic profiles in patients with rheumatoid arthritis

Reidun Øvstebø[1]; Thao Nguyen[2]; Tuula Nyman[3]; Maria Stensland[3]; Trude Aspelin[1]; Anne-Marie Siebke Trøseid[4]; Mark W. Feinberg[5]; Morten W. Fagerland[6]; Ivana Hollan[7]; Stefan Agewall[8]
[1]The Blood Cell Research Group, Department of Medical Biochemistry, Oslo University Hospital, Ullevaal, Norway, Oslo, Norway; [2]Lillehammer Hospital for Rheumatic Diseases, Lillehammer, Norway and University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway, Lillehammer, Norway; [3]Department of Immunology, University of Oslo and Oslo University Hospital, Norway, Oslo, Norway; [4]The Blood Cell Research Group, Department of Biochemistry, Oslo University Hospital Ullevaal, Oslo, Norway, Oslo, Norway; [5]Harvard Medical School, Boston, USA and Division of Cardiology, Brigham and Women´s Hospital, Boston, USA, Boston, USA; [6]Oslo Centre for Biostatistics and Epidemiology, Research Support Services, Oslo University Hospital, Norway, Oslo, Norway; [7]Beitostølen Health and Sport Centre, Beitostølen, Norway and Norwegian University of Science and Technology, Gjøvik, Norway, Lillehammer, Norway; [8]University of Oslo, Faculty of Medicine, Institute of Clinical Medicine, Oslo, Norway and Department of Cardiology, Oslo University Hospital, Ullevål, Norway, Oslo, Norway

Introduction: Extracellular vesicles (EVs) are associated with inflammation and cardiovascular diseases (CVD). However, the role of EVs in rheumatoid arthritis (RA) and their impact on CVD in RA, as well as EVs response to treatment is far less studied. Therefore, we investigated whether qualitative and quantitative characteristics of circulating EVs differ between RA patients and healthy controls (HC), and whether they are influenced by antirheumatic therapy.
Methods: We assessed 20 RA patients before and after using methotrexate alone (MTX, n=10) or Adalimumab with MTX co-medication (ADA+MTX, n=10) for six weeks, in addition to 8 age- and sex-matched HC. All patients starting with ADA had been previously unsuccessfully treated with MTX. Plasma EVs were isolated by size exclusion chromatography (0.5 mL fractions, fractions 7-9), and characterized by nanoparticle tracking analysis, NanoDrop One, western blot, transmission electron microscopy, and label-free quantitative proteomics combined with Ingenuity Pathway Analysis of the proteomics data.
Results: Plasma EV numbers, sizes and total protein concentrations did not differ between patients and HC, and were unaffected by antirheumatic therapy. Proteomic analysis identified 1513 proteins, wherein 169 proteins had significantly different baseline levels between patients and HC (FDR $p < 0.05$). These proteins were mostly related to cardiometabolic disease and upstream regulation of inflammatory pathways linked to RA, such as integrins, interleukin-8, actin cytoskeleton signaling. From patient samples, 317 proteins were significantly affected after 6 weeks on antirheumatic treatment. 115 proteins changed in response to MTX, whereas ADA+MTX had no significant impact on these proteins. Among them there were several upstream regulator proteins involved in pathogenesis of RA, including downregulation of vascular endothelial growth factor ($p=0.012$) and MYC ($p=0.0003$), and upregulation of CD437 ($p < 0.0001$).
Summary/Conclusion: EV proteins differed between RA patients and HC, and were affected by antirheumatic treatment within six weeks. Surprisingly ADA+MTX had no significant impact on EV proteins compared to MTX monotherapy. EVs proteomic content may indicate a contributing role in pathogenesis of RA and CVD development.
Funding: The study was funded by The Norwegian Women´s Public Health Association. Financial support to the EV analyses was provided by Lillehammer Hospital for Rheumatic Diseases

PT04.02 | EVs isolation from Trypanosoma cruzi generates intense tissue parasitism and inflammation in the experimental model in Chagas disease

Nicholy Lozano[1]; SOFIA OLIVEIRA MIGUEL[2]; Ana Claudia Torrecilhas[3]
[1]UNIFESP, Sao Paulo, Brazil; [2]UNIFESP, São Paulo, Brazil; [3]Federal University of Sao Paulo, Sao Paulo, Brazil

Introduction: Trypomastigotes of T. cruzi release EVs enriched in gp85/Trans-sialidase superfamily and alpha-galactosyl-containing glycoconjugates. EVs induce potent inflammatory immune response and host cell invasion of macrophages in vitro The aim of our research project is analysis the effect of EVs in animal infection with Chagas Disease
Methods: BALB/c female mice (8-10 week-old) were obtained from the animal facility at the UNIFESP. BALB/c mice pretreated with EVs (108 particles/ animal) followed by trypomastigote (500 parasites/ animal via intra peritoneal). The heart, spleen, bladder, lung, skeletal muscle and intestine were obtained from the animal pretreatment with EVs and infection with T. cruzi and controls (T.cruzi infection). One half was fixed in neutral buffered formalin, embedded by routine technique in paraffin wax and sectioned at 5 um for hemmatoxylin and eosin (H&E) staining
Results: All organs of infected mice submitted to previous treatment with PBS or EVs were removed 15 days after infection, fixed, embedded in paraffin, and sectioned for hemmatoxylin and eosin (H&E) staining. The heart, spleen, bladder, lung, skeletal muscle and intestine of the animals pre-inoculated with EVs showed an intense parasitism with an extensive inflammatory reaction, when compared to the control. Approximately 10 or more amastigote nests were found in the infected animal pre-inoculated with EVs.
Summary/Conclusion: The results indicate that vesicles released by trypomastigotes of T. cruzi can modulate the infection in vivo by inducing cytokines, and might play a role during the acute phase of the disease. These findings suggest that pre-inoculation of EVs produces an exacerbated inflammatory reaction in the animal all organs maybe by modulation cytokine production
Funding: FAPESP, CAPES and CNPq

PT04.03 | Exercise-Derived Exosomes Prevent Diabetes-Associated Osteoporosis

Neetu Tyagi; Jyotirmaya Behera; Yu-Ting Zheng; Rubens P. Homme
UNIVERSITY OF LOUISVILLE, Louisville, USA

Introduction: Recent evidence suggests that physical exercise released exosomes have been identified as novel players to mediate cell-to-cell communication in promoting skeletal development. However, the impact of EX on the progression of bone loss and deterioration of mechanical strength/quality in diabetic mice, induced by a high-fat diet and streptozotocin (HFD/STZ), remains unexplained. Hypothesis: In the present study, we investigate the effect of physical exercise released exosomes (Ex-EVs) on bone mass and mechanical quality using a diabetes mice model. Micro-CT scans and mechanical testing revealed that trabecular bone microarchitecture and bone mechanical properties were improved upon EX in diabetic mice.
Methods: Circulating exosomes were isolated from the plasma of with or without exercise training 10-12 weeks old mice subjected to 15-weeks treadmill exercise. Using the standard differential ultracentrifugation method and characterized by transmission electron microscopy, NanoSight & western blot analyses. Micro-CT scans and mechanical testing were used to test trabecular bone microarchitecture and bone mechanical properties.
Results: Treatment of EX-EVs restored the bone mechanical quality in diabetic mice by preventing inflammasome-associated pyroptosis response and promoting osteogenesis. Using miScript miRNA array-based screening, we discovered a particular miRNA, miR-218, as a novel target of EX-EVs induced Fndc5/irisin expression. Mechanistically, the data found that diabetes upregulated miR-218 via oxidative stress-dependent action and suppressed the Fndc5/irisin expression by binding to its 3'-UTR. Indeed, the decreased level of irisin expression further triggers the expression of pyroptosis-associated proteins-NLRP3, Caspase-1, and GSDMD in diabetic bone tissue.
Summary/Conclusion: This study demonstrates an understanding of the previously undefined role of exercise-induced skeletal irisin in ameliorating diabetes-associated bone loss via inhibiting the miR-129-FNDC5/pyroptosis axis. It possibly provides a mechanism for a therapeutic effect on metabolic osteoporosis.
Funding: National Institute of Health (NIH) gran: t AR-067667

PT04.04 | Impact of mesenchymal stromal/stem cell isolation strategies on functional activities of their extracellular vesicles

Oumaima Stambouli[1]; Robin Dittrich[1]; Fabiola Nardi Bauer[1]; Tobias Tertel[1]; Peter A A. Horn[2]; Bernd Giebel[1]
[1]*Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany;* [2]*University Hospital Essen, Essen, Germany*

Introduction: Mesenchymal stromal cell (MSC)-derived extracellular vesicles (EVs) are increasingly considered as therapeutic agents. Remarkably, as monitored in a multi-donor mixed lymphocyte reaction (mdMLR) assay, and in different animal models, including ischemic stroke and acute Graft-versus-Host Disease models, only a proportion of our MSC-EV products, all of which

have similar EV and protein concentrations, reveal immunomodulatory activities. Coupled to our experience that some MSC stocks reproducibly allow manufacturing of EV products with and others without detectable immunomodulatory capabilities, we hypothesize the differences derive from heterogeneities in parental MSC populations. Aiming to understand the heterogeneity of MSC-EV products, we explored whether initial human bone marrow (BM) seeding procedures have any impact on obtained MSCs or their EV products, respectively.

Methods: MSCs were raised from unprocessed BM aspirates and from mononuclear cells (MNCs) harvested thereof, originally either seeded into human platelet lysate (hPL) supplemented DMEM low media or in cytokine and hPL supplemented EBM media. After 24h, non-adherent cells were removed and remaining cells continuously cultured in hPL supplemented DMEM low media in all four settings. Growth rates of obtained MSCs including their senescence status, their cell surface phenotypes and osteogenic and adipogenic differentiation capabilities were analyzed. Furthermore, their EVs were prepared, phenotypically characterized and investigated for their immunomodulatory and proangiogenic activities.

Results: Overall, aspirate-derived MSCs, especially those from the DMEM seedings, appear more mature then MNC-derived MSCs. They grew slower and reached senescence quicker than MNC-derived MSCs. Furthermore, MSCs obtained from EBM seedings revealed higher expansion capacities than those obtained from DMEM seedings. Despite these differences, all EV products obtained from early MSC passages revealed comparable immunomodulatory activities in the mdMLR assay. In contrast, EV preparations obtained from the aspirate-EBM seeding promoted tube formation of endothelial cells in Matrigel more efficiently than those obtained from the other MSCs.

Summary/Conclusion: Our data imply, initial seeding strategies impact the characteristics of obtained MSCs as well as that of their EVs.

PT04.05 | Placenta EV-associated regulation of the Toll-like Receptor 4 (TLR4) pathway, a key mechanism in delaying aging

Jisook Moon[1]; Yuri choi[2]; Chul Kim[2]; Jin Woo Hwang[2]; Chang Hun Huh[3]; Moonbeom Choi[4]; Jin Young Lee[2]; Ji-Min Park[5]
[1]College of Life Science, Department of Biotechnology, CHA University, Seongnam, Republic of Korea; [2]CHA University, seoul, Republic of Korea; [3]Seoul National University Bundnag Hospital, seoul, Republic of Korea; [4]CHA University, Seoul, Republic of Korea; [5]TS Cell Bio, seoul, Republic of Korea

Introduction: Extracellular vesicles (EVs) can modulate a myriad of biological phenomenon as well as disease development through cell-cell communication, and both the naïve and the modified EVs have showed prospects as therapeutic interventions. However, the EV mediated modulation of normal aging has not been studies detailed yet.

Methods: In this study, we tested the regulation of aging process by human placenta derived mesenchymal stem cells (hpMSCs), which already showed the recovery potentials of hpMSCs in the degenerative disease models in our studies, and the human placenta derived EVs. We first intravenously infused hpMSCs into the aged mice from18-19 month old and repeated three times at every six weeks followed by assessment of diverse behavioral and molecular analyses.

Results: Compared to the age matched controls, we observed in the multiply treated mice significant improvement of the cognitive and locomotive activities, enhancement of genes related neuronal activities, and suppression of aging associated genes including p16INK4a in their hippocampi. In addition, the hpMSCs delayed the cellular senescence of the cocultured human cells, such as primary fetal neural progenitor cells (hfNPCs) and lung fibroblast cells in a transwell system, indicating that the hpMSC mediated effects are systemic and consequences of secreted materials including EVs. Through the comprehensive analysis with the total RNAs from hippocampi of the treated mice and the placenta EV containing microRNA, we identified the TLR4 signlaing targeting several microRNAs, such as miR 92a, miR-150, miR -22 and miR-122, that are able to modulate normal aging and aging related decline of cognitive function. Both the EVs and the target miRNA mimics partially delayed the cellular senescence and protected the hfNPCs against Aβ42 toxicity.

Summary/Conclusion: Here we first report that the hpMSCs are able to modulate aging process systemically in aged mice and the outcomes are at least consequences of the placenta EV associated modulation of Toll-like Receptor 4 (TLR4) pathway.

Funding: This research was financially supported by the Ministry of Trade, Industry, and Energy (MOTIE), Korea, under the "Regional Innovation Cluster Development Program (OpenLab, P0004793)" supervised by the Korea Institute for Advancement of Technology (KIAT) and the Bio & Medical Technology Development Program of the National Research Foundation (NRF) 2019M3A9H1103765.

PT04.06 | Pre-clinical efficacy of intra-articular administration of mesenchymal stromal cells-derived small extracellular vesicles for the treatment of knee osteoarthrosis

Aliosha I. Figueroa-Valdés[1]; Ana María Vega-Letter[2]; Cynthia García[2]; Catalina de la Fuente[3]; Yessia Hidalgo[4]; Nicolas Georges[5]; Patricia Luz-Crawford[6]; Maroun Khoury[4]; Francisca Alcayaga-Miranda[5]
[1]Laboratory of Nano-Regenerative Medicine, Centro de Investigación e Innovación Biomédica (CiiB), Universidad de los Andes, Santiago, Chile, Santiago, Chile; [2]Laboratory of Molecular and Cellular Immunology, Faculty of Medicine, Universidad de los Andes, Santiago, Chile, Santiago, Chile; [3]Consorcio Regenero, Chilean Consortium for Regenerative Medicine, Santiago, Chile, Santiago, Chile; [4]IMPACT, Center of Interventional Medicine for Precision and Advanced Cellular Therapy, Santiago, Chile, Santiago, Chile; [5]Universidad de los Andes, Santiago, Chile; [6]Laboratorio de Inmunología Celular y Molecular, Centro de Investigación Biomédica, Facultad de Medicina, Universidad de Los Andes, Santiago, Chile; IMPACT, Center of Interventional Medicine for Precision and Advanced Cellular Therapy, Santiago, Chile, Santiago, Chile

Introduction: Knee osteoarthrosis (OA) is a leading cause of pain and disability. Our group developed a stem cell-based therapy with umbilical cord-derived mesenchymal stromal cells (UC-MSC) showing significant pain and function improvements in treated patients. The clinical need for a "ready-to-use" approach and in consideration of the advantages of the use of small extracellular vesicles (sEV), a preclinical study was performed to assess the potential of UC-MSC-derived sEV in a mouse model of OA.
Methods: sEV were produced and isolated by differential centrifugation and characterized following MISEV 2018 guidelines by nanoparticle tracking analysis, flow cytometry, western blot & transmission electron microscopy. An HTG/EdgeSeq profiling array to characterize sEV's-miRNAs and bioinformatic analyses to look for OA-related targets were performed. Finally, a collagenase-induced OA murine model was established and analyzed by microcomputed tomography, histopathology and histomorphometry techniques.
Results: sEV featured a size of ~150nm, classical cup-shape morphology, presence of CD63, CD9, CD81, Flotillin-1 and Syntenin-1 and absence of Calnexin and TOMM20. The sEV-miRNA cargo profiles were nearly identical among three UC-MSC donors; notably, three of the detected miRNAs constituted almost ~70% of the total miRNA's reads, with putative targets associated to immune system, angiogenesis and extracellular matrix-related genes. In the murine OA model, tissue analysis showed a decrease in OA progression in sEV-treated mice as evidenced by a lower bone mineral density index and specific bone surface ratio compared to sham control. Histological analyses showed partial joint regeneration.
Summary/Conclusion: sEV treatment inhibits cartilage matrix degradation and promotes regeneration with no need for invasive surgical procedures. These findings support a cell-free sEV-based treatment for OA.
Funding: Cells for Cells S.A., Chile & Consorcio Regenero S.A., Chile.

PT04.08 | Scalability of Production and Activity of Amniotic Fluid Stem Cell Extracellular Vesicles from 3D Hollow Fiber Bioreactor and 2D culture

Charmi Dedhia[1]; Paolo Neviani[1]; John js. Cadwell[2]; Sargis Sedrakyan[3]; Laura Perin[4]
[1]Children's Hospital Los Angeles, Los Angeles, USA; [2]Fibercellsystem, New Market, USA; [3]Children's Hospital Los Angeles - University of Southern California, Los Angeles, USA; [4]Children's Hospital Los Angeles-University of Southern California, Los Angeles, USA

Introduction: EV clinical translation is inhibited by limitations in the scale-up of EVs production. Hollow fiber bioreactors (HFBR) support culture of large numbers of cells, at high densities, with concentrated EVs. Culture conditions may affect EV composition and potency. Here we compare the production, potency, identity, and therapeutic potential of EVs collected from cells grown in culture dish (2D) vs a small and medium-sized HFBR (3D).
Methods: 1 × 10e6 human clonal stem cells from amniotic fluid (hAFSC from consented donors) were seeded in 2D (145cm2), 1.6 × 10e7 hAFSC were seeded on a small cartridge (FiberCell C2025D; 450cm2), and 1.8 × 10e8 hAFSC on medium cartridge (FiberCell C2011; 4,000cm2) with fibronectin coating. All cultures used Chang medium with 20% of ES-FBS, starved for 24hr and then EVs collected. The effect of harvest frequency was tested (8hr, 24hr, 72hr). 2D-EVs and 3D-EVs were compared by Nanosight, potency assay (by WB), identity (by Exoview), and therapeutic effect (in vivo injections in an animal model of chronic kidney disease, Alport Syndrome).
Results: 2D production was ~5.5 × 10e9EV/ml/24hrs while 3D was ~2.8 × 10e10EV/ml (first four 24hrs) and ~4.4 × 10e10EV/ml (two days of hourly harvests). The medium cartridge produced similar concentrations of EVs but at 10X harvest volume indicating linear scalability.
Very little difference in EV concentration was observed during harvest intervals, with a very similar size distribution. This could indicate either significant EV re-uptake or inhibition of EV secretion dependent upon free EV in the supernatant. Both 3D-EVs trapped VEGF in vitro (an in vitro established potency assay) and expressed CD9, CD81, CD63, CD80, CD86, and VEGFR1

as 2D-EVs. 3D-EVs ameliorated proteinuria and histology when injected into Alport mice and also trapped VEGF in vivo as 2D-EVs.

Summary/Conclusion: 3D-EVs had comparable properties and bio-activity to 2D-EVs, but the HFBR produced 10x to 100X more EVs. Cell culture conditions for hAFSC still need optimization in the HFBR, however, a currently available 1.2m2 cartridge provides a 50X scale-up potential. The HFBR is a closed system that can be cGMP compliant. In conclusion, the HFBR can produce a sufficient number of EVs to support pre-clinical and clinical applications of EVs with at least similar properties to EVs produced in 2D methods.

PT04.09 | Small Extracellular Vesicle are Responsible for Bystander Immunity in Targeted Radiotherapy

Julie Constanzo[1]; Soumaya Turpault[2]; Isabelle Vila[3]; Jihad Karam[2]; Stanislas Du Manoir[2]; Nathalie Bonnefoy[2]; Nadine Laguette[3]; Marie Morille[4]; Jean-Pierre Pouget[1]

[1]IRCM, Inserm U1194, Université de Montpellier, Institut Régional du Cancer de Montpellier (ICM), Montpellier, F-34298 France, Montpellier, France; [2]Inserm U1194, Montpellier, France; [3]IGH, UMR 9002, Montpellier, France; [4]ICGM, Montpellier University, CNRS, ENSCM, Montpellier, France, Montpellier, France

Introduction: Beside conventional radiotherapy using X-rays dedicated to localized tumors or oligometastasis, targeted radionuclide therapy (TRT) allows to specifically irradiate diffuse and metastatic tumors. TRT consists of the administration of radiopharmaceuticals made of monoclonal antibodies or peptides coupled to a radionuclide emitting alpha, beta and Auger particles. We already demonstrated that TRT induces anti-tumor immunity at low activity. Here, we investigate the role of small extracellular vesicles (sEVs) released by irradiated cells in triggering anti-tumor immunity.

Methods: B16F10 melanoma cells were subcutaneously injected in C57BL/6J and athymic mice, or in cGAS-/- and in STING-/- mice. Mice received intraperitoneal injections of TA99 mAb targeting TYRP-1/gp75 tumor antigen radiolabeled with 5MBq 177Lu-TA99 (beta TRT), or intratumor injection of sEVs purified from cells exposed to 2MBq/ml 177Lu-TA99 (TRT-sEVs) or from non-treated cells (NT-sEVs). Macrophages and DC were exposed in vitro to sEVs purified from B16F10 CRISPR ctrl, B16F10 cGAS-/- and B16F10 STING-/- exposed to beta TRT.

Results: In vivo, Beta TRT efficacy was shown to require T-cells for adaptive immunity, with a factor 3 in tumor growth delay (**$p = 0.001$) compared to non-treated (NT), and no difference between TRT and NT was observed in athymic nude mice. Then, we focused on sEVs as a second messenger released by cancer cells that may activate an antitumor immune response through the STING pathway. EVs purified from beta-TRT exposed cells demonstrated a strong tumor growth delay and survival (***$p = 0.0007$). The dsDNA content between NT-sEVs and TRT-sEVs was comparable, which suggest that dsDNA was not responsible for the observed in vivo effect. Also, we demonstrated using TCGA database analysis in vivo that cGAS gene expression is required in cancer cells to obtain a significant therapeutic efficacy of radiotherapy, while STING is not. In addition, knocked-out cGAS genes in host immune cells did not affect TRT response, while knocked-out STING genes did, suggesting that cGAMP is an extracellular messenger involved in activation of immune cells. Finally, compared to TRT purified sEVs-CTRL, sEVs-cGAS-/- do not activate macrophages nor DC in vitro, demonstrating that sEVS may carry cGAMP.

Summary/Conclusion: sEVs contribute to TRT efficacy by mediating an antitumor immune response in vivo.

PT04.10 | Towards continuous production of extracellular vesicles: productivity of batch-refeed cell cultures

Carolina Paganini[1]; Hannah Boyce[1]; Mauro Manno[2]; Antonella Bongiovanni[3]; Paolo Arosio[1]

[1]Institute for Chemical and Bioengineering, ETH Zurich, Zurich, Switzerland, Zurich, Switzerland; [2]Cell-Tech HUB at Institute of Biophysics (IBF), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy; [3]Cell-Tech HUB at Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy

Introduction: The low yields of production of extracellular vesicles (EVs) seriously challenge research and clinical application of successful products (Paganini et al, Biotech. J., 1800528, 2019). Until now, few efforts have been done to increase upstream yields of EVs. Examples include switching the cell cultivation from flasks to hollow fiber bioreactors and stirred tank bioreactors operated in batch mode. Moreover, little is known about how these changes in the upstream operations impact the rate of EV production and the properties of the final EV mixtures. Here, we simulate perfusion cultures with batch-refeed cultures, evaluate their productivity and compare them with batch cultures.

Methods: 293F cells are cultured in shake flasks and maintained at two different cell densities for 14 days. The medium is collected and replaced daily. EVs are separated by immuno-magnetic isolation, quantified and the productivities of the two batch-refeed cultures and of a batch culture are measured.

Results: With our experimental setup we can determine the rate at which the EVs are produced in batch and batch-re-feed systems. In addition, proteomic analysis of EVs isolated by SEC reveals the biochemical characteristics of the EVs produced by the same culture at different days.
Summary/Conclusion: We present important information on the productivity of EVs in different cultivation modes. These results indicate a promising strategy to increase the upstream yield of EVs and hence obtain more material for research and clinical trials.
Funding: H2020-EU.1.2.1-FET Open programme via the VES4US Grant agreement 801338
H2020-EU.1.2.2-FET Proactive programme via the BOW Grant agreement 952183

PT04.13 | Serum Derived Extracellular Vesicles Promote Recovery of Diabetic Nephropathy Damaged Kidneys in NSG mice

Cristina Grange[1]; Massimo Cedrino[2]; Giovanni Camussi[3]; Maria Felice MF Brizzi[4]
[1]Department of Medical Sciences, University of Turin, Turin, Italy; [2]Unicyte, Torino, Italy; [3]Department of Medical Sciences, Torino, Italy; [4]Deaprtment of Medical Sciences, Torino, Italy

Introduction: Extracellular Vesicles (EVs) released by stem cells carry several bioactive molecules such as proteins and RNA (mRNAs and miRNAs) that could be transferred to target cells inducing phenotypic changes. EVs can reprogram injured cells by activating regenerative processes in acute tissue injury. The aim of this study was to evaluate whether serum derived EVs inhibit the progression of chronic kidney injury in a mouse model of diabetic nephropathy (DN).
Methods: To develop DN, NSG mice were injected with 35 mg/Kg of streptozotocin for 4 consecutive days. All treated mice developed diabetes (glycaemia > 250 mg/ml) within 10 days, and DN within 1 month. Mice were intravenously treated with serum derived EVs once a week for 4 weeks, from the onset of diabetes. Kidney function were evaluated by analysing blood and urine for physiological parameters and tissues for morphological studies.
Results: EV treatments resulted in significant amelioration of physiological parameters such as reduction of albumin/creatinine excretion ratio and plasma creatinine compared to control DN mice. Histological analyses revealed a significant reduction of glomerular and interstitial fibrosis, Bowman's space enlargement and tubular damage. At variance, when serum derived EVs were administered after the development of diabetic nephropathy, 1 month after the onset of diabetes, they resulted ineffective in the reversion of renal damage and fibrosis.
Summary/Conclusion: This study demonstrate that serum derived-EVs inhibit the progression of DN, when EVs are administered as soon as the onset of diabetes. This effect likely relies on EV slowdown of renal damage and signs of diabetic nephropathy.
Funding: Unicyte AG and 2i3T, Incubatore d'Imprese of the University of Turin, Turin, Italy.

PT04.14 | The Combination Treatment of Umbilical Cord Mesenchymal Stem Cells (UC-MSCs) and Secretome for Diabetic Foot Ulcer

Arif T. Prasetyo[1]; Reiva F. Dwiyana[2]; Cynthia R. Sartika[3]; Rima Haifa[4]; Nadya Karina[3]; Nabilla F. Naura[3]; Ditta K. Devi[3]; Adina N.P. Putri[3]
[1]Tivaza Clinic, 40171 Bandung, West Java, Indonesia, Bandung, Indonesia; [2]Division of Plastic Reconstructive and Aesthetic Surgery, Department of Surgery, Faculty of Medicine Universitas Padjajaran, 45363 Bandung, West Java, Indonesia, Department of Dermatology and Venerology, Faculty of Medicine Universitas Padjadjaran, 45363 Bandung, West Java, Indonesia, Bandung, Indonesia; [3]PT. Prodia StemCell Indonesia, Kramat VII No. 11, 10430 Senen, Central Jakarta, Indonesia, Jakarta Pusat, Indonesia; [4]Prodia StemCell Indonesia, Jakarta, Indonesia

Introduction: Diabetic foot ulcer (DFU) is a chronic disease that can lead to subsequent limb amputation. The standard treatment for diabetic foot ulcer includes wound debridement, wound dressing, management of infection, control of ischemia, revascularization, and off-loading pressure to promote healing. Even though DFU requires a long-term treatment for successful healing, most of its sufferers fail to recover resulting in limb amputation.
An alternative and effective treatment is required to accelerate the healing process of diabetic foot ulcer (DFU). Stem cell therapy is one of the regenerative medicines that has emerged as a promising treatment for DFU. It can repair and even replace damaged tissues or organ. In this study, mesenchymal stem cells (MSCs) are chosen over other stem cell populations due to their outstanding advantages such as ability to differentiate into various cell types (multipotent), cytokines and growth factor secretion, homing ability, and modulation of anti-inflammatory response.
Methods: A 65-years-old male patient presented with diabetic ulcer on the left foot since September 2020. Diagnosed with type 2 diabetes mellitus in the last 5 years, the patient has been controlling his blood sugar levels. The patient has undergone 3 times surgery, 2 times wound debridement, and defect closure through skin graft and flap. The surface areas of the back lower calf and

outer ankle ulcers were 7 cm x 1 cm and 2 cm x 2 cm, respectively. The depth of the outer ankle ulcers was 3 cm with exudation and pus around the area, swelling and pain of the tissue were also observed. Umbilical cord-derived mesenchymal stem cells (UC-MSCs) and their Secretome was given as a treatment. Ten million of UC-MSCs was injected directly into the ulcers followed by Secretome spraying all over the area once in 3 days when changing wound dressing.

Results: Based on the Picture 1 & 2, the ulcer size was reduced within a few weeks. The ulcer size on back lower calf (Picture 1) were gradually reduced from A) 7x1cm into B) 5x1cm, C) 5x1cm and D) 3x1cm. On the outer ankle, the size of ulcer is also gradually decreased during the observation (Picture 2). The size become smaller from the baseline were 2x2 cm into 0.5x0.5 cm at the last observation.

As shown on Table 1, the yellowish pigmentation or slough around the ulcer also improved. This is also supported by the moisture of the skin which became moist at the last observation. The presence of epithelialization and necrotic tissue was examined clinically. Necrotic tissue can be seen as blackening of the ulcer, whereas the epithelialization seen as red pigmentation. Another improvement shown from this therapy was the depth of ulcer is significantly reduced from 3 cm to 0.5 cm.

Summary/Conclusion: In conclusion, this current report is one of the available evidence that confirmed the use of UC-MSCs and Secretome as a promising treatment for diabetic foot ulcer patients.

PT04.15 | Therapeutic effect of MSC-NTF exosomes in experimental bleomycin-induced lung injury

Haggai Kaspi[1]; Chen Dekel[1]; Ralph Kern[2]; Stacy Lindborg[2]; Chaim Lebovits[2]; Revital Aricha[1]
[1]*Brainstorm cell therapeutics, Petach Tikva, Israel;* [2]*Brainstorm cell therapeutics, New York, USA*

Introduction: Mesenchymal stem cell (MSC)-derived small extracellular vesicles (sEVs) have been proposed as a potential therapy for pulmonary disorders associated with inflammation and fibrosis, due to their significant immunomodulatory and regenerative properties. We have previously demonstrated that in LPS-induced mice ALI/ARDS model, sEVs from MSCs induced to secrete increased levels of regenerative and immunomodulatory factors (Exo MSC-NTF), had a superior effect over treatment with naïve MSC-derived sEVs (Exo MSC). In this study we aimed to examine the advantage of Exo MSC-NTF over Exo MSC focusing on different lung injury.

Methods: Using the bleomycin model, we compared the effect of the two types of sEVs: Exo MSC and Exo MSC-NTF. Treatment was administered intratracheally 1 and 5 days after bleomycin instillation, and experimental observations were made through day 14. Analysis of mRNA in the lungs was performed using NanoString technology. EVs cargo analysis was performed by mass spectrometry and validated by ELISA.

Results: Exo MSC-NTF showed greater improvements compared to Exo MSC in multiple parameters, including increased blood oxygen saturation, reduction in lung pathology including fibrosis and inflammatory cell infiltrates, regulation of proinflammatory cytokines in bronchoalveolar lavage fluid, as well as regulation of gene expression related to multiple pathways in the lung. Cargo analysis of proteins in Exo MSC and Exo MSC-NTF demonstrated higher expression of regenerative factors, including amphiregulin (AREG) and leukemia inhibitory factor (LIF) in Exo MSC-NTF.

Summary/Conclusion: We observed positive preclinical results suggesting that intratracheal administration of Exo MSC-NTF may have potential as a clinical therapy for pulmonary pathologies associated with inflammation and fibrosis and may be more effective at modifying physiological, pathological, and biochemical outcomes than sEVs isolated from naïve MSCs.

PT05: Stem cells EVs

Chair: Marta Monguió-Tortajada – 1. IVECAT, REMAR-IGTP Group & Nephrology Service, "Germans Trias i Pujol" Health Science Research Institute & University Hospital, Can Ruti Campus, Badalona, Spain 2. Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain 4. ICREC Research Program, Health Science Research Institute Germans Trias i Pujol, Can Ruti Campus, Badalona, Spain 5. CIBERCV, Instituto de Salud Carlos III, Madrid, Spain

Chair: Wolfgang Holnthoner – Ludwig Boltzmann Institute for Traumatology

PT05.01 | A look into the neuro-regenerative potential of miRNAs in Wharton's jelly-derived small extracellular vesicles (sEV)

Vera Tscherrig[1]; Valérie Haesler[2]; Patricia Renz[1]; Sophie Cottagnoud[2]; Daniel Surbek[2]; Andreina Schoeberlein[2]; Marianne Jörger-Messerli[2]

[1]1) Department of Obstetrics and Feto-maternal Medicine, University Women's Hospital, Inselspital, Bern University Hospital, Bern, Switzerland and Department for BioMedical Research (DBMR), University of Bern, Switzerland; 2) Graduate School for Cellular and Biomedical Sciences (GCB), University of Bern, Bern, Switzerland, Bern, Switzerland; [2]Department of Obstetrics and Feto-maternal Medicine, University Women's Hospital, Inselspital, Bern University Hospital, Bern, Switzerland and Department for BioMedical Research (DBMR), University of Bern, Switzerland, Bern, Switzerland

Introduction: In the past, our research group and others have shown promising results on the use of mesenchymal stromal cell-derived small extracellular vesicles (MSC-sEV) as a therapeutic approach for neuronal injuries. MSC-sEV carry small non-coding RNAs such as microRNAs (miRNAs), which are predicted to target mRNAs encoding for proteins that are involved in premature birth-related white matter injury (WMI). We hypothesize that miRNAs, released by sEV upon uptake in their target cells have a key function in the observed beneficial effects of MSC-sEV.
Methods: We isolated MSC from the connective tissue of human umbilical cords, the Wharton's jelly. The cells were stained for typical MSC-markers by immunohistochemistry. sEV were purified from the conditioned cell medium by serial ultracentrifugation followed by size exclusion chromatography (SEC). The protein and RNA contents of each SEC fraction were measured with a NanoVue Plus™. The fractions with the highest protein content were characterised by proteomics, western blot, ImageStream and ZetaView analyses. The miRNA content of the sEV was measured by quantitative PCR. Pathway enrichment of the miRNAs was analysed following Next Generation Sequencing.
Results: The SEC fractions were positive for the sEV markers CD81, CD63 and syntenin-1, and the MSC markers CD73, CD90 and CD105. Further, they contained high amounts of miRNAs, such as miR-21-5p, miR-22-5p, miR-27b-3p, and members of the let-7 family. The targets of the highly abundant miRNAs in the sEV fractions are involved in apoptotic and inflammatory processes and drive oligodendrocyte differentiation.
Summary/Conclusion: The miRNAs released by MSC-SEV might influence WMI outcomes. The regulatory potential of miRNAs in gene expression are currently analysed using agomir / antagomir assays in an in vitro model of WMI, as well as dual luciferase assays. To analyse the integrity of the sEV we will check for the CD73 enzyme activity.

PT05.02 | "Wharton's Jelly MSCs derived exosomes as cell-free therapeutic strategy for intervertebral disc regeneration"

Veronica Tilotta[1]; Cicione Claudia[2]; Giuseppina Di Giacomo[2]; Luca Ambrosio[2]; Fabrizio Russo[2]; Rocco Papalia[2]; Gianluca Vadalà[2]; Vincenzo Denaro[2]
[1]Campus Bio-Medico University(UCBM), Rome, Italy; [2]Campus Bio-Medico University of Rome, Rome, Italy

Introduction: Intervertebral disc degeneration (IDD) affects more than 80% of the population. Current approaches to treat IDD are based on conservative or surgical procedures relieving the pain. Studies reported as paracrine factors as extracellular vesicles released by mesenchymal stem cells (MSCs) may regenerate IVD. The aim of this study is to investigate the therapeutic effects of Wharton's Jelly MSCs derived exosomes on human nucleus pulposus cells (hNPCs) in an in vitro.
Methods: Exosomes were isolated by tangent filtration of Wharton's Jelly derived MSCs conditioned media. The exosomes were quantified by bicinchoninic acid assay, exosomal morphology was characterized by transmission electron microscope, western blot analysis was performed for markers expression and nanoparticles tracking analysis for vesicular size and quantification.
hNPCs were treated with growth medium (control) and MSCs-exosomes at 10 ug/ml, 50 ug/ml and 100 ug/ml. At different time points each group was analyzed for: cell proliferation [flow cytometry]; nitrate [Griess] and glycosaminoglycan (GAG) production [DMBB]; histological staining for extracellular matrix (ECM) analysis; gene expression levels of catabolic and anabolic genes [real time-polymerase chain reaction, qPCR]. Furthermore, exosomes were labeled with PKH26 and the uptake was detected by confocal microscopy.
Results: An increase of hNPCs proliferation was reported in exos 10 μg/ml sample group. Nitrate production was significantly reduced at 100 μg/ml. GAG content was enhanced in a dose dependent-manner by all exos concentrations under study. Histological analysis suggested that there is not a significant difference between treated and untreated hNPCs' ECM synthesis. Gene expression levels were modulated by exosomes compared to the control.
Summary/Conclusion: Our preliminary results supported the potential use of exosomes as cell-free treatment of IDD. MSC-exosomes ameliorate hNPCs growth, attenuate ECM degradation and oxidative stress-related IDD progression. These findings offer new opportunities for the potential use of exosomes as an attractive alternative strategy to the effects of cell-therapy. compromised by the harsh microenvironment of the disc.
Funding: Financial support was received from the Horizon 2020 research and innovation program "iPSpine" # 825925 and "RESPINE"ID:NCT03737461.ClinicaTrials.gov

PT05.04 | Development of senomorphics based on intercellular communication through sEV to rejuvenate senescent mesenchymal stem cells

Rocío Mato-Basalo[1]; Sergio Lucio-Gallego[1]; Marta Sacristán-Santos[1]; María Del Pilar Miranda-Quintana[1]; Miriam Morente-López[1]; María C. Arufe[2]; Juan Antonio Fafián Labora[1]
[1]Grupo de investigación en Terapia Celular y Medicina Regenerativa, Departamento de Fisioterapia, Medicina y Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidade da Coruña, INIBIC- Complejo Hospitalario Universitario A Coruña (CHUAC), CICA, A Coruña, Spain, A Coruña, Spain; [2]University of La Coruña, A Coruña, Spain

Introduction: Higher life expectancy of population brings health problems associated with age. Enhance our knowledge on the aging process mechanisms is essential to develop new therapies to palliate this health problems and improve quality life in people. Mesenchymal stem cells (MSCs) have a high therapeutic potential facing age-related diseases. Senescent MSCs enter in a stage characterized by loss of proliferation and pluripotency capacities, related with the deterious of the tissues and organs. These senescent MSCs produce a secretoma called senescence-associated secretory phenotype (SASP) formed by chemokines, interleukins, lipid mediator and extracellular vesicles. Our group, it was discovery that the intercellular communication through small extracellular vesicles (sEV) influence on the propagation of senescence and a proinflammatory message, main characteristics of inflamm-aging by activation of non-classical SASP through sEV. The senomorphics are drugs with interesting tool to modulate functionality senescent sEV to paracrine senescence transmission. Because of that, we wanted to know how three pharmacological inhibitors of p65 pathway (JSH-23, MG132 and curcumin) could modulate the functionality of non-classical SASP mediated by sEV.
Methods: For that, we induced senescence into mesenchymal stem cells. After that, the cells were treated with the pharmacological inhibitor of p65 (JSH-23, MG-132 and Curcumin). The sEV were isolated by ultracentrifugation. The size and production were evaluated using NTA. With respect to the functionality, the isolated sEV from senescent MSC-treated with pharmacological inhibitors of p65, were used to treat non-senescent and senescent MSCs using the same concentration of particles for 6 days. Later, it was evaluated the paracrine transmission and the rejuvenation capacities by evaluation of cell proliferation and senescence phenotype using crystal violet staining and b-galactosidase assay.
Results: Our data shown that inhibition of p65 pathway decreased the production of sEV in the MSC-treated with the three compounds. Besides, the sEV from MSC-treated with JSH-23, MG-132 and Curcumin loss the capacity paracrine senescence transmission on non-senescent MSCs. Otherwise, they can rejuvenate senescent MSCs.
Summary/Conclusion: The pharmacological inhibitor of p65 have a high potential to change the functionality of non-classical SASP mediated by sEV. Because of that, we proposed JSH-23, MG-132 and Curcumin as senomorphics drugs to rejuvenated senescent MSCs. These three senomorphics could use to treat age-related diseases and help to improve elderly people's quality of life.
Funding: JFL was funded Xunta de Galicia (ED481D-2021-020). MCA was granted by the Spanish National Health ISCIII(PI20/00497)

PT05.05 | Evaluation of local vs. systemic biodistribution of mesenchymal stromal cell-derived extracellular vesicles for the treatment of pulmonary diseases

Anna Maria Tolomeo[1]; Gaia Zuccolotto[2]; Alessandro Penna[2]; Ricardo Malvicini[3]; Giada De lazzari[4]; Chiara Franco[5]; Michele Grassi[6]; FABIO MAGAROTTO[7]; Michela Pozzobon[8]; Antonio Rosato[2]; Maurizio Muraca[6]; Federica Collino[9]
[1]Department of Cardiac, Thoracic and Vascular Science and Public Health, University of Padova, Padua, Italy, Padova, Italy; [2]Istituto Oncologico Veneto IOV-IRCCS, Padova, Italy, Padova, Italy; [3]Instituto de medicina traslacional, trasplante y bioingeniería (IMeTTyB-CONICET), buenos Aires, Argentina; [4]Department of Women's and Children's Health, University of Padova, Padua, Italy., padova, Italy; [5]Department of Medicine (DIMED), Rheumatology Unit, University of Padova, Padova, Italy; [6]Department of Women's and Children's Health, University of Padova, Padua, Italy., Padova, Italy; [7]Department of Women and Children Health, Università di Padova, Italy, PADUA, Italy; [8]Department of Women's and Children's Health, University of Padova, Padua, Italy. Fondazione Istituto di Ricerca Pediatrica Città della Speranza, Padua, Italy, Padova, Italy; [9]Laboratory of Translational Research in Paediatric Nephro-urology, Department of Clinical Sciences and Community Health, University of Milano, Milan, Italy., Milan, Italy

Introduction: Mesenchymal stromal cell-derived extracellular vesicles (MSC-EV) are extensively studied as therapeutic tools in pulmonary diseases, including COVID-19. Local MSC-EV administration, intratracheal (IT) or by aerosol, is being used in ongoing clinical trials. Even if EV biodistribution is a critical determinant of therapeutic activity, it has never been evaluated following local vs. systemic administration for the treatment of lung diseases.

Methods: We set up a method in which MSC-EV were labelled with a common fluorescent lipophilic dye, DiR (1,1-dioctadecyl-3,3,3,3-tetramethy-lindotricarbocyanine iodide), and then were extensively washed with PBS using 100kDa Amicon filters. Two different administration routes were tested in vivo, intravenous (IV) and intratracheal (IT). DiR-EV were administered in Balb/C mice (2.0x1010 EV/mice) and monitored at 1, 3 and 24h. A negative control group receiving the dye alone was included to correct for non-EV specific signal.
Results: Whole body analysis, after 3h from IV injection, showed accumulation of EV in the spleen and liver, compared to IT EV injection, where EVs localized in lungs and trachea. After 24h mice treated with IV EV injection showed a stronger positivity in the abdominal region of the body and a low signal in the low posterior region. The analyses of isolated organs confirmed the accumulation of EV in spleen, liver, and lungs. A positive signal was also detected in lymph nodes, heart, intestine and kidney. After 24h from IT EV injection a stronger positivity was detected selectively in the isolated lungs. Heart, intestine and kidney were characterized by low positive signals
Summary/Conclusion: Analysis of the in vivo imaging data, highlights a selective delivery and permanence of EV cargo in specific organs based on their route of administration. In particular, these results show that IT administration can increase the concentration of MSC-EV in the target organ, limiting their systemic biodistribution and possibly extra-pulmonary effects. This data can help both selecting the most appropriate way of administration of MSC-EV and understanding their mechanism of action for the treatment of lung diseases

PT05.06 | Evaluation of secretome enriched in human MSC-EV: characteristic's and immunomodulatory effects

Guillaume Valade[1]; Bileyle Lorenzini[2]; Marion Grosbot[3]; Bastien Rival[3]; Sylvie Goulinet[4]; Philippe Mauduit[4]; Marina Trouillas[3]; Sébastien Banzet[3]; Juliette Peltzer[3]
[1]Institut de Recherche Biomédicale des Armées - INSERM UMR-MD 1197, Clamart - (92140), France; [2]INSERM UMR-MD 1197, Villejuif, France; [3]Institut de Recherche Biomédicale des Armées / INSERM UMR-MD 1197, Clamart, France; [4]Inserm UMR-MD 1197, Villejuif, France

Introduction: Hemorrhagic shock can lead to immediate death by bleeding or later by organ failure. The early administration of mesenchymal stromal cells (MSC) moderates the immune response and reduces organ dysfunction. Here, we explore the therapeutic potential of biological products based on MSC-derived extracellular vesicles (MSC-EVs). Such products, easily stored and available immediately, would be very convenient for emergency use. Our aim is to explore in vitro, the anti-inflammatory and immunomodulatory potential of MSC secretome, more or less enriched in EV, and to evaluate the effects of the culture conditions on secretome characteristic's.
Methods: The conditioned medium (CM) enriched in MSC-EVs was obtained after 72h, from a pool of 9 donors of human bone marrow MSCs, either in depleted medium (α-MEM only) or in association with EV-free platelet lysate. The secretomes were purified by Tangential Flow Filtration (TFF) +/- size-exclusion chromatography (SEC). Three types of enrichments were evaluated, depending on filtration conditions: i) purified EVs fraction, ii) soluble proteins fraction and iii) the association of EV and soluble proteins. Their immunomodulatory properties were evaluated in vitro by mixed leucocyte reaction (MLR) and anti-inflammatory assay.
Results: In EV free platelet lysate condition, we found a better cell survival and MSC-EVs secretion than in α-MEM only. Preliminary results showed a dose-dependent immunosuppressive effect in MLR and an anti-inflammatory activity of EVs fraction. Functional activity of the other different fractions is in progress.
Summary/Conclusion: These preliminary results show an improvement in the production of secretomes enriched in MSC-EVs, by reducing production times and by increasing the amount of EVs produced and ultimately, by reducing costs. We are now trying to find out which fraction would be the most efficient in our applications.

PT05.07 | EVs from Trichinella spiralis muscle larvae exert immunomodulatory potential in chronic inflammatory Th2 disorders

Sofija Glamočlija[1]; Anna Schmid[2]; Nataša Ilić[1]; Alisa Gruden-Movsesijan[1]; Saša Vasilev[1]; Irma Schabussova[2]; Maja Kosanović[1]
[1]Institute for the Application of Nuclear Energy, INEP, University of Belgrade, Belgrade, Serbia; [2]Medical University of Vienna, Vienna, Austria

Introduction: Excretory-secretory products of Trichinella spiralis muscle larvae (ES L1) have immunomodulatory properties i.e. reduce hypersensitivity to both allergens and autoantigens. Recently, we have shown that extracellular vesicles isolated from ES

L1 (TsEVs) exert immunomodulatory properties on human monocyte derived dendritic cells. Now, we aim to investigate whether TsEVs can also exert beneficial immunomodulatory effects in murine model of ovalbumin (OVA)-induced allergy.
Methods: TsEVs were enriched from ES L1 by differential centrifugation and ultrafiltration. Experimental allergic airway inflammation was induced in BALB/c mice by intraperitoneal injection of OVA in alum on days 1 and 14. On days 21-24 mice were challenged with intranasal application of OVA, 30 min after intranasal administration of TsEVs or PBS and sacrificed two days later. Blood samples were taken for serum IgE determination. Lungs and spleens were extracted for the isolation of immune cells. Phenotype of immune cells was determined by flow cytometry and their cytokine production by ELISA assays.
Results: TsEVs treatment of allergic mice lead to diminished numbers of alveolar macrophage and CD103+ dendritic cells (DC) in lungs compared to allergic control while numbers of CD11b+ DCs and their Ly6C+ subset was increased, along with CD8+ and CD19+ T cells. Upon restimulation with OVA, splenocytes and lung immune cells of TsEVs-treated mice produced lower levels of Th2 cytokines, while the production of IFN-g was elevated only in lung immune cells. Lower IgE levels were found in TsEVs-treated mice compared to sham-treated controls.
Summary/Conclusion: TsEVs exert immunomodulatory properties in murine model of allergic airway inflammation by diminishing inflammation and thus they may represent the basis for novel allergen-independent therapeutics in treatment of respiratory allergies.

PT05.08 | Exploring the potential of mesenchymal stem cell extracellular vesicles as novel therapeutics for inflammatory bowel disease

Mona Belaid[1]; Giorgia Pastorin[2]; Driton Vllasaliu[3]
[1]King's College London (KCL) and National University of Singapore (NUS), London, United Kingdom; [2]National University of Singapore, Singapore, Singapore; [3]King's College London, London, United Kingdom

Introduction: Mesenchymal stem cell (MSC) extracellular vesicles (EVs) show great promise for repair and regeneration of injured tissues and their immunomodulatory effect makes them strong potential therapeutics for inflammatory bowel disease (IBD), which was the focus of this work.
Methods: Conditioned medium (CM) was harvested from cultured human bone marrow MSCs to isolate EVs. CM was precleared of dead cells and cellular debris by differential centrifugation at 4°C at 500g for 5 min twice then 2000g for 15 min. The recovered supernatant was filtered through 0.22 μm filters and subjected to ultracentrifugation onto a 25% (w/w) sucrose cushion prepared in deuterium oxide. After centrifugation at 100,000g at 4°C for 1.5 h, the sucrose layer was resuspended in phosphate-buffered saline (PBS) and washed by ultracentrifugation at 100,000g at 4°C for 1.5 h to pellet the EVs. The EVs were then resuspended in PBS and stored at − 80°C for further use. Isolated MSC EVs were characterised for yield, size and protein marker expression using bicinchoninic acid assay, nanoparticle tracking analysis and the Exo-Check antibody array kit, respectively. A wound healing (scratch) assay evaluated the effect of MSC EVs on the proliferation of intestinal epithelial Caco-2 cells, while the anti-inflammatory activity of MSC EVs was determined in LPS-activated macrophages via nitrite quantitation.
Results: MSC EVs were < 200 nm in diameter and EV protein marker expression was confirmed by the presence of tetraspanins CD63 and CD81 and cytosolic proteins TSG101 and ALIX. The scratch assay showed that Caco-2 cells treated with MSC EVs demonstrated accelerated wound closure over time compared to control. Furthermore, an effect of MSC EVs on nitrite concentrations in activated macrophages was apparent.
Summary/Conclusion: In conclusion, MSC EVs exhibit wound repair and anti-inflammatory activity which could have potential therapeutic applications in IBD.

PT05.09 | Extracellular vesicles from senescent mesenchymal stromal cells preserve their senoprotective effect in osteoarthritis

Jérémy Boulestreau[1]; Marie Maumus[2]; Christian Jorgensen[3]; Daniele Noël[1]
[1]Inserm, Montpellier, France; [2]Bauerfeind, Montpellier, France; [3]University of Montpellier, Montpellier, France

Introduction: Osteoarthritis (OA) is the most prevalent rheumatic disease characterized by progressive loss of cartilage and alterations in all compartments within joints. The highest risk factor in this degenerative disease is age and an accumulation of senescent cells in cartilage and synovium has been shown to contribute to the functional decline of joints. We previously demonstrated that extracellular vesicles (EVs) from mesenchymal stromal cells (MSCs) largely mediate the therapeutic effect of parental cells in OA. Here, we investigated whether EVs from adipose tissue-derived MSCs (ASC-EVs) possess senoprotective effects in a new model of induced senescence in OA chondrocytes.
Methods: Human chondrocytes isolated from OA patients and ASCs from healthy donors were induced to senescence using 25μM etoposide for 24 hours. Senescence was assessed by quantifying proliferation rate, SA-βGal activity, nuclear γH2AX foci

number, phalloidin staining and expression of cyclin dependent kinase inhibitors (CDKI) by RT-qPCR. ASC-EVs were isolated by differential ultracentrifugation and characterized by size, concentration, total protein content, structure (cryo-TEM) and immunophenotype at day 7 and 12. OA chondrocytes were cultured with ASCs or ASC-EVs for 7 days after senescence induction. Expression of senescent, chondrocytic and inflammatory markers was quantified by RT-qPCR and SASP factors were quantified by ELISA in supernatants.

Results: Etoposide-induced senescence in chondrocytes was characterized by growth arrest, increase of SA-βGal+ cell number, increase of p15, p21, p27 expression, of nuclear γH2AX foci, of stress fibers and cell surface confirming the induction of main senescence features even though secretion of SASP factors was not significantly up-regulated. The addition of ASCs or different doses of ASC-EVs at the time of senescence induction prevented the increase of SA-βGal+ cell number and significantly reduced the number of γH2AX+ chondrocytes as well as their nucleus surface. No effect of ASC-EVs was found on CDKI expression whereas the secretion of several SASP factors (IL6, IL8, MMP3, MMP13, HGF, VEGF) was decreased. Interestingly, ASC-EVs slightly up-regulated the anabolic markers of chondrocytes (AGG and type II COLLAGEN) and decreased the expression of catabolic markers (MMP13, AP). Of note, a similar protective effect was observed using senescent ASC-EVs.

Summary/Conclusion: Our results demonstrate that ASC-EVs, produced by either healthy or senescent ASCs, exert a senoprotective and chondroprotective effect in OA chondrocytes. Further studies are warranted to investigate their effect on other cell compartments, notably synoviocytes, in the joints.

Funding: Study was supported by a research grant from FOREUM, Foundation for Research in Rheumatology.

PT05.11 | Neuroprotective of nasally administered human adipose tissue mesenchymal stem cell (hAT-MSC)-derived extracellular vesicles (EVs) 24h after stroke in normotensive and differences to hypertensive rats

Francieli Rohden[1]; Luciele Varaschini Teixeira[1]; Luis Pedro Bernardi[2]; João Victos Matos e moreira[3]; Fernanda Dos Santos De Oliveira[4]; Elizabeth Obino Cirne Lima[4]; Fátima Guma[3]; Diogo Onofre souza[3]

[1]Universidade Federal do Rio Grande do Sul and Instituto de Cardiologia de Porto Alegre, Porto Alegre, Brazil; [2]UFRGS and UFCSPA, Porto Alegre, Brazil; [3]Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [4]Hospital de Clínicas de Porto Alegre - HCPA, Porto Alegre, Brazil

Introduction: Ischemic stroke is a major cause of death and disability, intensely demanding innovative and accessible therapeutic strategies. To propose an approach, using extracellular vesicles (EV) secreted from human mesenchymal stem cells isolated from adipose tissue (hAT-MSC), with a prolonged period for therapeutic intervention and a non-invasive route for administering the treatment of stroke in normotensive and hipertensive rats.

Methods: Wistar rats (90-120 days) were subjected to focal permanent ischemic stroke (IS), 24 hours after, were treated intranasally with EV (200 μg/kg) secreted by hAT-MSC. We analyzed front paws symmetry (Cylinder Task), short- and long-term memory (Open Field and Novel Object Recognition Task) and angiogenesis. Evaluation of the interaction of EVs with cells in the peri-infarct region was performed 18h after intranasal administration of EVs labeled with fluorescence, cells were labeled with antibody NeuN, GFAP and to label Hoescht nucleus. Wistar Kyoto Spontaneously hypertensive rats (SHR) (90-120 days) were subjected to IS and we analyzed front paws symmetry (Cylinder Task).

Results: In Wistar rats, EV treatment recovered front paws symmetry and short- and long-term memory induced by ischemic stroke. Additionally, we observed stimulation of angiogenesis in the peri-infarction region in animals treated with EVs. In the peri-infarct region there is a greater number of EVs and in this region the EVs seem to have a preference for neurons (preliminary results). For SHR rats, we have preliminary results, where we can observe that the outcome of ischemia induction is very different and with a chronic effect and without spontaneous recovery in the symmetry of the forepaws. We are performing the EV dose and time curve for SHR rats.

Summary/Conclusion: In line with these findings, our work highlights hAT-MSC-derived EVs as a promising therapeutic strategy for stroke.

Funding: Instituto Nacional de Ciência e Tecnologia-INCT-EN (2014-465671/2014-4), Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Ministério da Saúde, Coordenação de Aperfeiçoamento de pessoal de Nível Superior-CAPES, Fundação de Amparo à pesquisa do Estado do Rio Grande do Sul-FAPERGS, Universidade Federal do Rio Grande do Sul-UFRGS.

PT05.12 | Proliferation-promoting effect of stachys geobombycis derived nanovesicles (SDNVs) in human keratinocytes

Tingting Chen[1]; Bingxiang Ma[2]; Shi Lu[1]; Yaokun Xia[1]; Lupeng Zeng[3]; Huaying Wang[4]; Wanhua Shi[5]; Xi Zhang[6]; Jing Zhang[7]; Jinghua Chen[1]
[1]Fujian Medical University, Fuzhou, China (People's Republic); [2]Fujian Medical University, Longyan, China (People's Republic); [3]Fujian Medical University, Zhangzhou, China (People's Republic); [4]Fujian Medical University, Sanming, China (People's Republic); [5]Fujian Medical University, Quanzhou, China (People's Republic); [6]Fujian Medical University, Ningde, China (People's Republic); [7]Fujian Agriculture and Forestry University, Fuzhou, China (People's Republic)

Introduction: In recent years, plant derived nanovesicles (PDNVs) have attracted much attention due to their simple preparation, high biosafety and biocompatibility. In this study, we isolated Stachys geobombycis derived nanovesicles (SDNVs), and found that they could significantly promote the proliferation of human keratinocytes (HaCaT), which has not been reported in previous researches.
Methods: SDNVs were obtained by juicing and centrifugation. Their morphology, diameters and zeta potentials were characterized by transmission electron microscope (TEM) and laser particle size analyzer (LPSA). In addition, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was used to determine the effect of SDNVs on HaCaT cell proliferation. Cells were incubated with different concentrations of SDNVs (0-1.56 μg/mL protein, determined by bicinchoninic acid assay) for 12 h, and cell viability was then calculated according to the absorbance of samples at wavelength of 490 nm.
Results: The obtained SDNVs showed a typical cup-shaped like structure with high monodispersity. The LPSA result showed that the average size of SDNVs was 133.8 nm, which was consistent with the TEM result. In addition, the average zeta potential of SDNVs was -6.9 mV, in accordance with negatively charged PDNVs. We also found that SDNVs could significantly promote HaCaT cell proliferation in a dose-dependent manner.
Summary/Conclusion: The above results showed the potential of SDNVs as a drug for wound healing. And, SDNVs can also serve as nanocarriers to load other agents for synergistic treatment for skin regeneration.
Funding: The work is supported by the innovation of science and Technology, Fujian province (2019Y9008)

PT05.13 | Protective effects of extracellular vesicles derived from mesenchymal stem cells against ischemia-reperfusion injury of hearts donated after circulatory death: study in a pig model

Anna Maria Tolomeo[1]; Assunta Fabozzo[2]; Domenico Ventrella[3]; Valentina Lombardi[2]; Fabio Zanella[2]; Alberto Elmi[3]; Marco Andreis[4]; Giulia Todeschini[5]; Ricardo Malvicini[6]; Camilla Aniballi[3]; Marco Ruggeri[7]; Giuseppe Astori[7]; Gianfranco Santovito[8]; Maurizio Muraca[9]; Maria Laura Bacci[3]; Gino Gerosa[4]
[1]Department of Cardiac, Thoracic and Vascular Science and Public Health, University of Padova, Padua, Italy, Padova, Italy; [2]Cardiac Surgery Unit, Hospital University of Padova, Padua, Italy, Padua, Italy; [3]Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy, Bologna, Italy; [4]Department of Cardiac, Thoracic and Vascular Science and Public Health, University of Padova, Padua, Italy, Padua, Italy; [5]L.i.f.e.L.a.b. Program, Consorzio per la Ricerca Sanitaria (CORIS), Veneto Region, Padua, Italy, Padua, Italy; [6]Instituto de medicina traslacional, trasplante y bioingeniería (IMeTTyB-CONICET), buenos Aires, Argentina; [7]Hematology Vicenza General Hospital, Vicenza, Italy; [8]Department of Biology, University of Padova, Padua, Italy, Padua, Italy; [9]Department of Women's and Children's Health, University of Padova, Padua, Italy, Padua, Italy

Introduction: Insufficient supply of cardiac grafts represents a severe obstacle in heart transplantation. Donation after Circulatory Death (DCD), in addition to conventional donation after brain death, is one promising option to overcome the organ shortage. However, DCD organs undergo an inevitable longer period of warm unprotected ischemia between circulatory arrest and graft procurement. Mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) have shown remarkable protective effects against ischemia-reperfusion injury. We thus aimed at improving the quality of grafts from DCD donors by treatment of MSC-EVs.
Methods: Pig were euthanized upon barbiturate overdose and after 20 min of a flat EKG (no touch period), the chest was opened, the heart harvested and subsequently connected to the extracorporeal machine perfusion. MSC- EVs, isolated by ion exchange chromatography, were added to the perfusion solution (1E+11 particles) and the heart was perfused for 2 hours. Then, heart tissue biopsies were taken to assess mitochondrial morphology, antioxidant enzyme activity and lipid peroxidation. Biochemical parameters of myocardial viability and function were assessed in the perfusate.
Results: The treatment with MSC-EVs significatively prevented mitochondria swelling, mitochondrial cristae loss and oxidative stress in myocardiocytes. The protective effect of MSC-EVs was confirmed by the delayed increase of the cardiac enzymes CK and TnC in the perfusate.

Summary/Conclusion: We showed that MSC-EVs can improve graft quality by protecting the myocardium against oxidative stress. Further studies are ongoing to identify the most effective dose and to assess the effects of these nanoparticles on heart function.

PT05.14 | The secretome of equine bone marrow mesenchymal stem cells enhanced regenerative phenotype of equine articular chondrocytes

Manon Jammes[1]; Romain Contentin[1]; Frédéric Cassé[1]; Bastien Bourdon[2]; Arnaud Bianchi[3]; Fabrice Audigié[4]; Thomas Branly[1]; Emilie Velot[5]; Philippe Galéra[1]

[1]Normandie Univ, UNICAEN, BIOTARGEN, F-14000 Caen, France, Caen, France; [2]Normandie Univ, UNICAEN, BIOTARGEN, F-14000 Caen, France, Rennes, France; [3]Université de Lorraine, CNRS, IMoPA, F-54000 Nancy, France, Nancy, France; [4]Center of Imaging and Research on Locomotor Affections on Equines (CIRALE), Unit Under Contract 957 Equine Biomechanics and Locomotor Disorders (USC 957 BPLC), French National Research Institute for Agriculture Food and Environment (INRAE), Ecole Nationale Vétérinaire d'Alfort, F-94700 Maisons-Alfort, France, Goustranville, France; [5]Assistant Professor, Université de Lorraine, CNRS, IMoPA, F-54000 Nancy, France, Nancy, France

Introduction: Equine osteoarthritis (OA) leads to cartilage degradation, impaired animal well-being, premature cessation of sportive activity and financial losses. Mesenchymal stem cells (MSC)-based therapies are promising for cartilage repair, but face limitations inherent to cell itself. The use of MSC secretome enables to overcome those limitations while keeping MSC properties.
Methods: The effect of equine bone marrow MSC secretome on equine articular chondrocytes (eAC) (monolayers or organoids) was evaluated with indirect co-culture or MSC-conditioned media (CM). The proliferation and migration of eAC, and the organoid quality were assessed through the expression healthy/OA markers of chondrocytes. To isolate exosomes from secretome, CM were filtered, concentrated and precipitated with polyethylene glycol and protamine sulfate. Exosome characterization was performed through western-blot and transmission electron microscopy. In addition, immunomodulatory effectors after MSC priming with interleukin (IL)1-β were evaluated to optimize immunomodulation properties of MSC secretome for future experiments.
Results: eAC co-cultured with MSC expressed higher proliferation associated molecules and collagen mRNA levels. MSC-CM did not affect eAC proliferation, while they induced an increase of collagen II and I protein amounts, particularly after IL-1β priming. We also showed the absence of MSC-CM cytotoxicity on eAC and an increase in eAC migratory capacities in in vitro repair. Further characterization of the MSC secretome confirmed the presence of exosomes and their involvement in the observed effects. Exosome functions on eAC regenerative phenotype remain under investigation.
Summary/Conclusion: These results demonstrate that MSC secretome influences eAC phenotype and could relieve OA symptoms or delay OA outcomes. Moreover, MSC secretome contains exosomes which need further investigation to better understand the mechanisms underlying MSC secretome beneficial effects on eAC.

PT05.15 | Treatment of hepatic fibrosis by using human stem cell-derived extracellular vesicles

Van Dat Bui[1]; Jae Hyung Park[2]; Hwa Seung Han[3]

[1]Sungkyunkwan University, Suwon-si, Republic of Korea; [2]Sungkyunkwan Universtiy, Suwon-si, Republic of Korea; [3]KIST, Suwon-si, Republic of Korea

Introduction: Liver fibrosis is one of the major causes of morbidity and mortality worldwide. Until now, there are no effective therapies or FDA-approved antifibrogenic agents, making liver transplantation the only curative treatment for decompensated cirrhotic liver disease. Mesenchymal stem cell (MSC) therapy has emerged as a potential way for treating chronic diseases due to its regenerative capability. Though, it has significant limitations such as cell moving, transforming into unexpected cell types, or growth of cancers. In order to overcome these concerns, a novel cell-free therapy was developed in this study to treat hepatic fibrosis, by using human adipose stem cell-derived extracellular vesicles (hADSC-EVs)
Methods: The hADSC-EVs were isolated from conditioned media of ADSCs using tangential flow filtration (TFF) method, then were dispersed in PBS and stored at -80°C. There are several in vitro experiments were conducted to confirm characteristics of the EVs and check whether these EVs could have effects on eliminating fibrotic signals in vitro. Next steps, we intravenously injected hADSC-EVs into the liver-fibrotic mice to confirm their in vivo therapeutic effects
Results: The hADSC-EVs had spherical shape with medium size around 100 nm and their Zeta potential was about -23.70 mV. There were some biomarkers of EVs such as CD9 and CD63 indicated that hADSC-EVs also expressed such biomarkers. The α-SMA was overexpressed in TGF-β1 treated group and significantly down-regulated in hADSC-EVs (1x108 particles) treated groups. Besides that, two different types of fibrosis-associated markers (COL-1), and MMP-2) in the activated LX-2 cells were

analyzed and their obtained results were similar with α-SMA results. We labeled hADSC-EVs with Cy5.5 dye to explore hADSC-EVs after administrating into mice. We found that hADSC-EVs mostly accumulated in liver in post 3h or 24h. The serum levels of AST, ALT, and ALP in the hADSC-EVs treated group were similar with control group, implying that the mice model of liver fibrosis was successfully established. The morphologies of livers were different between hADSC-EVs untreated groups and hADSC-EVs treated groups. Moreover, the hADSC-EVs treated groups showed similar liver surface to normal mouse groups. These results were further supported by the histo-pathological examination after H&E, MT, and α-SMA staining and western blot

Summary/Conclusion: After intravenous injected into the liver-fibrotic mice, hADSC-EVs preferred to maintain inside the livers, especially in damaged liver tissue, notably suppressed fibrogenic markers and finally restored hepatic functions. In conclusion, exosomes derived from hADSC have a dramatic potential to treat chronic hepatic fibrosis

Funding: Ministry of Science and ICT (2017R1D1A1B03034888 and 2015R1A6A3A04059033)

PT06: Cancer: I (Lessons learned from cell lines)

Chair: Marco Falasca – Curtin University

PT06.01 | CD20 expression, TrkB activation and functional activity of Diffuse Large B Cell Lymphoma-derived small extracellular vesicles

Hafidha Bentayeb[1]; Marine Aitamar[2]; AKIL Hussein[2]; Vignoles Chantal[2]; Branchaud Maud[3]; Julie Abraham[4]; Nathalie Guachard[2]; Jean Feuillard[4]; Marie-odil Jaubertaud[4]; Hamasseh Chirvani[5]; Danielle Troutaud[2]

[1]Centre George François Leclerc Dijon france, Dijon, France; [2]Limoges university, Limoges, France; [3]Limoges university, Rouen, France; [4]Chu de limoges, Limoges, France; [5]Institut ROCH, Paris, France

Introduction: Diffuse Large B-cell Lymphoma (DLBCL) is an aggressive non-Hodgkin lymphoma and a clinically and biologically heterogeneous disease with two main sub-groups (ABC and GCB). Among these,
ABC DLBCL is associated with substantially worse outcomes. Standard treatment is based on combination of chemotherapy (CHOP) with immunotherapy (anti-CD20, i.e: rituximab, R-CHOP). Despite therapeutic
advances, approximately 10-15% of patients exhibit primary refractory disease and 20-25% relapse after initial response to therapy. Small extracellular vesicles (sEVs) including exosomes, carrying the CD20, could
be involved in immunotherapy resistance in diffuse large B cell lymphoma (DLBCL). We have reported endogenous brain-derived neurotrophic factor/TrkB (tropomyosin-related kinase B) survival axis in DLBCL.
Here, we performed a comparative study of sEV production by germinal center B cell (GCB) and activated B cell (ABC)-DLBCL cell lines, and analysed TrkB activation on this process.

Methods: GCB (SUDHL4 and SUDHL6) and ABC (OCI-LY3, OCI-LY10 and U2932) cell lines were used. sEVs were characterised using nanoparticle tracking analysis technology and western blot. CD20 content was
also analysed by enzyme-linked immunoassay, and complement-dependent cytotoxicity of rituximab was investigated. 7,8-Dihydroxyflavone (7,8-DHF) was used as a TrkB agonist. In vivo role of sEVs was evaluated in a xenograft model.

Results: sEVs production varied significantly between DLBCL cells, independently of subtype. CD20 level was consistent with that of parental cells. Higher CD20 expression was found in sEVs after TrkB activation, with a trend in increasing their concentration. sEVs determined in vitro and in vivo protection from rituximab, which seemed CD20 level-dependent; the protection was enhanced when sEVs were produced by 7,8-DHF-treated cells.

Summary/Conclusion: DLBCL-derived sEVs have the differential capacity to interfere with immunotherapy, which could be enhanced by growth factors like neurotrophins. Evaluating the sEV CD20 level could be useful for disease monitoring.

PT06.02 | Development of EV-based cancer vaccination using transcription-induced chimeric RNA as a neoantigen

Xiao Xiong[1]; Xiurong Ke[1]; Lu Wang[1]; Yusheng Lin[2]; Shuhong Wang[1]; Jingfang Liu[1]; Kai Li[1]; Yichen Luo[1]; Fan Liu[1]; Hao Zhang[1]

[1]Institute of Precision Cancer Medicine and Pathology, Department of Pathology, School of Medicine, Jinan University, Guangzhou, China (People's Republic); [2]Department of Hematology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands., Groningen, Netherlands

Introduction: Esophageal squamous cell carcinoma (ESCC) is an extremely aggressive malignancy with a dismal prognosis, indicating urgent need for development of alternate and/or complementary immunotherapeutic treatment options in advanced

ESCC. Although somatic mutations are important sources of cancer-specific neoepitopes to generate cancer vaccine, recent studies revealed that somatic mutations commonly occur in many normal tissues, particularly in aging normal esophageal tissues. Transcription-induced chimeric RNAs (chiRNAs) are fused transcripts from unrelated genes generated through aberrant 'read-through/splicing' or 'trans-splicing'. These chiRNAs may be suitable to serve as targets in cancer vaccines for cancer types known to have low mutation burden and in which mutation-based immunogenic neoantigens are rare or even non-existent. Furthermore, a cell-free extracellular vesicle (EV)-based vaccine approach offers several practical advantages over a cell-based vaccine, including long shelf-life when frozen, scalability and genetic modifiability. Previously, we reported a group of chiRNAs in enriched in human ESCC. We wondered whether the ESCC-enriched chiRNAs could be exploited for the development of EV-based therapeutic vaccine.

Methods: We developed a novel cell-free anticancer vaccine based on EVs from the chiRNA-transduced dendritic cells (DC). EVs were purified using ultracentrifugation and characterized by transmission electron microscope, western blot and nanoparticle tracking analysis. Survival and tumor growth were monitored in mice bearing syngeneic chiRNA-expressing ESCC.

Results: We confirmed that chiRNA specifically expressed in cancer, suggesting it may be developed into an immunogenic neoantigen. Vaccination of immunocompetent mice bearing syngeneic chiRNA-expressing ESCC with EV-based vaccine potently inhibited tumor growth and prolonged survival.

Summary/Conclusion: Our data demonstrate that transcription-induced chiRNA can be used as a source of cancer-specific neoantigen for cancer types known to have low mutation burden and in which mutation-based immunogenic neoantigens are rare or even non-existent. Moreover, the result showed that EV-based vaccines have stable immunomodulatory capacity and can induce anticancer immunity.

Funding: National Natural Science Foundation of China (82072683, 81773087, 81071736, 81572876 and 30973508 to H.Z.);
Flagship specialty construction project General surgery (Funding no.: 711003)
Natural Science Foundation of Guangdong Province of China (2021A1515012522 and 9151018004000000 to H.Z.);
Science and Technology Planning Project of Guangdong Province of China (2019A030317024 to H.Z.);
Special Project on the Integration of Industry, Education and Research of Guangdong Province (2011A090100024 to H.Z.).

PT06.03 | Different expression of integrins in ganglioside-remodeling melanoma EVs

Kei Kaneko[1]; Yuhsuke Ohmi[2]; Mariko Kambe[2]; Farhana Yesmin[3]; Momoka Momoka[2]; Yoko Kitaura[2]; Takako Ito[4]; Orie Tajima[2]; Koichi Furukawa[2]; Keiko Furukawa[5]
[1]Chubu University, kasugai, Japan; [2]Chubu University, Kasugai, Japan; [3]Nagoya University, Kasugai, Japan; [4]Chubu University, KAsugai, Japan; [5]Chuvu University, Kasugai, Japan

Introduction: Gangliosides, sialic acid-containing glycosphingolipids, are known as a regulator of cell proliferation, and human malignant melanomas express high levels of ganglioside GD3. To analyze the roles of GD3, we established GD3-over expressing cells (GD3+) from a GD3-lacking mutant cell line (GD3-) by introducing GD3 synthase cDNA. Using these cell lines, we demonstrated that GD3 enhances tumor malignancy such as cell proliferation and invasion activities, and upregulates phosphorylation levels of focal adhesion kinase (FAK), p130Cas and paxillin. We also reported integrin b1 localizes in glycolipid-enriched microdomain (GEM)/rafts of GD3+ melanomas. The aim of this study is to clarify the behaviors and roles of GD3 and integrins in the extracellular vesicles (EVs) from ganglioside-remodeling melanomas.

Methods: EVs were isolated by ultracentrifugation from the culture medium of GD3+/GD3- melanoma cells. Isolated EVs were analyzed by using Western blotting. GD3- melanoma cells were incubated with GD3+ melanoma-derived EVs. After incubation, cells were lysed and analyzed by Western blotting.

Results: Among integrin family members, some integrins (alpha 4, alpha V, beta 1, beta 1) were highly expressed in GD3+ EVs. Addition of GD3+ melanoma-derived EVs to cultured cells resulted in the induction of activation of ERK1/2 and Akt, suggesting that GD3 on EVs might play important roles in the regulation of tumor environments. Function of integrins in ganglioside-remodeling cell-derived EVs are now under investigation.

Summary/Conclusion: We analyzed expression patterns of integrins in EVs, and verified that they were different between GD3+ and GD3- melanoma cell lines. Gangliosides may contribute to selective packaging of integrins into EVs.

PT06.04 | Differential "barcoding" of extracellular vesicles from diverse cellular origins and its implication for therapeutics in ovarian cancer

Johnny Nguyen
Honours Student, Brisbane, Australia

Introduction: Johnny Nguyen BSc, Carlos Salomon PhD, Andreas Moller PhD, Andrew Lai PhD, Priyakshi Kalita de Croft PhD UQ Centre for Clinical Research – Exosome Biology Laboratory, The University of Queensland, St Lucia, Australia, School of Biomedical Sciences

Ovarian cancer is an important health issue with a lasting impact on the whole population. It is the sixth most reported cancer and the fifth leading cause of cancer-related deaths in women. EV's have the greatest potential in cancer therapeutics as a natural carrier due to their organotrophic qualities.

A major gap in the field is understanding the predetermined pathway of EVs. We believe the protein composition ("barcode") on the surface of EV is the signal that allows the EVs to interact with different target cells affecting EV biodistribution and kinetic parameters. We aimed to profile surface proteins of ovarian cancer derived EVs to identify potential targets of EV and determine their kinetic distribution on varying ovarian cell lines.

Methods: Ovarian cancer EV's were isolated from SKOV-3, OVCAR-3 and Met-5a cell lines. CM was centrifuged at 500g and the supernatant was further processed for EV isolation via ultracentrifugation at 100,00g. EV surface proteins were marked and isolated using a biotinylating and isolation kit. Mass spectrometry was used to analyse the peptides which provided a list of isolated surface proteins. Using NTA we measured the uptake and kinetics of Ovarian Cancer EV's with differential membrane profiles on target cells.

Results: This investigation explored the characterisation of extracellular vesicles from diverse cellular origins.

Summary/Conclusion: We were able to identify membrane proteins in ovarian cancer-derived EVs and successfully perform uptake experiments to determine EV/Cell kinetics.

PT06.05 | Extracellular vesicles carrying miRNA-195-5p sensitizes melanoma cells to MAPK inhibitors

Nathalia L. Santos[1]; Silvina Bustos[2]; Patrícia Reis[3]; Tainara Francini Felix[4]; Roger Chammas[5]; Luciana Nogueira de Sousa Andrade[6]

[1]Cancer Institute of São Paulo State, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil; [2]ICESP, Sao Paulo, Brazil; [3]Department of Surgery and Orthopedics, Faculdade de Medicina, Universidade Estadual de São Paulo (UNESP), Botucatu - SP, Brazil., Botucatu, Brazil; [4]Department of Surgery and Orthopedics, Faculty of Medicine, São Paulo State University (UNESP), Botucatu, SP, Brazil, São Paulo, Brazil; [5]Center for Translational Research in Oncology (LIM24), Instituto do Cancer do Estado de Sao Paulo (ICESP), Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo (HCFMUSP), Sao Paulo CEP 01246-000, SP, Brazil, Sao Paulo, Brazil; [6]Center for Translational Research in Oncology (LIM24), Instituto do Câncer do Estado de São Paulo (ICESP), Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HCFMUSP), São Paulo, Brazil, São Paulo, Brazil

Introduction: Despite the significant improvements achieved by MAPK inhibitors treatment, the majority of melanoma patients with metastatic disease relapse within few months after treatment initiation. The presence of antitumoral molecules within Extracellular Vesicles (EVs) is commonly related to increased response rates and better prognosis. Hsa-miR-195-5p is down-regulated in melanoma, favoring cell proliferation and drug resistance, as recently shown by our group. We aimed to analyze whether restoring miR-195-5p expression could modulate EVs cargo and the role of these particles in naïve cells drug response.

Methods: The BRAF mutated A375, SKMel-5 and SKMel-28 cells were transfected with miR-195-5p or control mimic (10nM). After 72 hours EVs were isolated by differential ultracentrifugation, resuspended in PBS and characterized by NTA analysis, electron microscopy and by the presence of common EVs markers through western blot. Prior to functional experiments EVs were treated with RNAse A to eliminate non-vesicular RNAs.

Results: MiR-195-5p overexpression resulted in increased EVs release by melanoma cells, which presented 150-220 nm diameter range. Characterization of EVs miRNA profile was performed by digital barcode technology and showed that EVs derived from cells overexpressing miR-195-5p presented a higher content of this miRNA, along with increased levels of the tumor suppressors hsa-miR-152-3p and hsa-miR-202-3p. Uptake of miR-195 EVs by naïve tumor cells resulted in decreased proliferation and viability. Upon MAPK inhibitors treatment, miR-195 EVs resulted in increased cell death and decreased clonogenic potential. Moreover, EVs electroporated with miR-195 impaired in vivo tumor growth and reduced the repopulation capacity of cells previously exposed to MAPK inhibitors.

Summary/Conclusion: Restoring miR-195-5p expression represents a promising strategy to sensitize melanoma cells to targeted therapy.

Funding: This work was supported by FAPESP (process number 2019/07278-0).

PT06.06 | Extracellular vesicles derived from CAR-T Cells: A potential therapy for cancer

Anat Aharon; Galit Horn; Tali Hana Bar-Lev; Einav Zagagi Yohay; Tova Waks; Maya Levin; Naamit Deshet Unger; Irit Avivi; Anat Globerson Levin
Tel Aviv Sourasky Medical Center, Tel Aviv, Israel

Introduction: Chimeric antigen receptor (CAR)-T cells are genetically engineered T cells, directed against a tumor associated antigen (TAA). Extracellular-vesicles (EVs) derived from CAR-T cells (CAR-T EVs) may preserve CAR-T activity and overcome one of the major obstacles responsible for CAR-T cell failure in patients with solid tumors.
This study aimed to compare CAR-T EVs to their parental cells and explore their cell penetration and cytotoxic activity.
Methods: Anti-HER-2 CARs were stimulated with specific target cells. EVs were isolated from the cell media and characterized for their content and functions.
Results: We found that CAR-T EVs contained a mixture of small and large EVs. Stimulated Anti-HER-2+CAR-T EVs expressed lower cytokines levels compared to their parental CAR-T cells (such as IFN). Higher levels of Granzyme B were found in CAR-T EVs (≥20×) compared to EVs from unstimulated cells ($p < 0.001$). Anti-HER-2+ CAR-T EVs bound and penetrated specifically into HER-2 expressing target cells. Similar cytotoxic effects measured by Caspase-3/7 activity were found in CAR-T cells and their derived EVs. However, while the CAR-T cells induced massive apoptosis during the first 24 hours, CAR-T EVs required 60−90 hours.
Summary/Conclusion: In summary, HER-2-expressing CAR-T EVs provide a novel and potent immunotherapy approach that may be effective against solid tumors.
Funding: KAMIN grant from the Israel Innovation Authority.

PT06.07 | Ganglioside GD2 enhances malignant properties of melanoma by co-operating with integrin

Farhana Yesmin[1]; Robiul Hasan Bhuiyan[2]; Yuhsuke Ohmi[3]; Momoka Momoka[3]; Tetsuya Okajima[3]; Kei Kaneko[4]; Keiko Furukawa[5]; koichi Furukawa[3]
[1]Nagoya University, Kasugai, Japan; [2]Chubu Unoversity, Kasugai, Japan; [3]Chubu University, Kasugai, Japan; [4]Chubu University, kasugai, Japan; [5]Chuvu University, Kasugai, Japan

Introduction: Cancer-associated gangliosides have been used in cancer diagnosis and therapy. To clarify mechanisms by which gangliosides enhance tumor properties, we analyzed roles of GD2 and GD2-carrying exosomes by identifying GD2-associated molecules.
Methods: EMARS/MS using anti-GD2 antibody revealed that integrin β1 was one of GD2-associated molecules on the membrane. Their association was verified by IP/IB, immunocytochemistry as well as PLA. RT-CES revealed that GD2 expression strongly enhanced adhesion activity of melanoma cells. Addition of anti-GD2 antibody during RT-CES resulted in definite suppression of the cell adhesion.
Results: Immunoblotting with PY20 showed stronger bands at 180, 130, and 100 kDa in GD2+ cells than in GD2- cells, and they disappeared after knock-down of integrin β1. MS analysis using the bands detected in IP/IB with PY20 revealed that EGFR and FAK exist. Knock-down of integrin β1 resulted in decreased proliferation, invasion and cell adhesion of GD2+ cells. Integrins shifted to the raft fraction from non-raft fraction in GD2+ cells but not in GD2- cells. To clarify the roles of exosomes in GD2 functions, addition of GD2+ cell-derived exosomes to GD2- cells was tried, showing increase of cell adhesion.
Summary/Conclusion: Now, involvement of exosomes in the roles GD2 and integrins in the malignant properties of melanomas such as cell adhesion, proliferation and metastasis is under investigation.
Funding: This study was supported by the Grants-in-Aids from the Ministry of Education, Culture,
Sports and Technology of Japan (MEXT) (15H04696, 17K19616, 18H02628, 19K22518, 19K07393,
21K06828, 21H02699), and by JST-CREST (Grant Number: JPMJCR17H2).

PT06.08 | HPV-positive head and neck squamous cell carcinoma cells release small extracellular vesicles carrying immunoregulatory proteins and HPV peptides

Lukasz Skoczylas[1]; Marta Gawin[2]; Mateusz Smolarz[3]; Sonja Ludwig[4]; Nils Ludwig[5]; Piotr Widlak[6]; Theresa L. Whiteside[7]; Monika Pietrowska[2]
[1]Maria Sklodowska-Curie Memorial Cancer Center and Instytute of Oncology, Gliwice, Poland; [2]Maria Skłodowska-Curie National Research Institute of Oncology, Gliwice, Poland;, Gliwice, Poland; [3]Maria Sklodowska-Curie National Research Institute of

Oncology, Gliwice Branch, Gliwice, Poland; [4]Department of Otorhinolaryngology, Head and Neck Cancer Surgery, University Hospital Mannheim, Mannheim, Germany;, Mannheim, Germany; [5]Department of Oral and Maxillofacial Surgery, University Hospital Regensburg, Regensburg, Germany;, Regensburg, Germany; [6]Medical University of Gdansk, Gdansk, Poland; [7]UPMC Hillman Cancer Center, University of Pittsburgh, Cancer Institute Pittsburgh, Pennsylvania, USA, Pittsburgh, USA

Introduction: Human papillomavirus (HPV) is a risk factor for head and neck squamous cell carcinoma (HNSCC) associated with a favorable response to therapy. We hypothesized that tumor-derived exosomes (TEX) produced by HPV(+) or HPV(-) HNSCCs differentially modulate anti-tumor immune responses, which may be mediated in part by the presence of viral antigens in HPV(+) TEX.
Methods: TEX isolated from supernatants of HPV(+) SCC-90 or HPV(-) PCI-30 HNSCC cells by size exclusion chromatography were characterized according to the recent MISEV2018 guidelines. A comparison of proteome profiles was performed by high-resolution mass spectrometry (HRMS). Selected immunoregulatory proteins were analyzed by flow cytometry. Further, fluorescence microscopy with labeled antibodies was employed to verify the presence of selected viral proteins (E7 and E2) in TEX (co-staining with CD63).
Results: TEX produced by SCC-90 and PCI-30 cells contained 711 proteins overall, including 80 proteins specific for HPV(+)-derived vesicles and 77 proteins specific for HPV(-)-derived vesicles. Only HPV(+) TEX were enriched in immune effector cell-reactive CD47 and CD276 antigens, while only HPV(-) TEX contained tumor-protective/growth-promoting antigens, MUC-1 and HLA-DA. The proteome of HPV(+) TEX determined by HRMS contained three viral peptides derived from three different HPV proteins: Minor Capsid Protein L2, Replication Protein E1 and Probable Protein E5.
Summary/Conclusion: The distinct proteomic cargo composition of HPV(+) and HPV(-) TEX might contribute to forming the characteristic immune landscape of HPV(+) and HPV(-) HNSCC. Recent reports indicate that E2/E5 viral proteins, unlike E7/E6, are immunogenic and induce antigen-specific T cell responses in HPV(+) individuals. Therefore, TEX carrying immunogenic viral proteins as well as tumor-associated antigens may mediate anti-viral and anti-tumor immune responses in HPV(+) HNSCC patients.
Funding: This research has been financed from Preludium Bis grant, NCN Poland, grant no. UMO-2020/39/O/NZ4/02838 (LS, MP) and NIH grant U01-DE029754 (TLW).

PT06.09 | Identified distinctive miRNA profile of Menstrual Stem Cells-derived sEV repress angiogenesis-associated gene expression in breast cancer and endothelial cells

Hugo E Tobar[1]; Nicolas Georges[1]; Leonie Rosenberger[1]; Fernando Lillo-Vera[1]; Monica Kurte[1]; Dario Donoso Meneses[1]; Yessia Hidalgo[2]; Aliosha I. Figueroa-Valdés[3]; Francisca Alcayaga-Miranda[1]
[1]Universidad de los Andes, Santiago, Chile; [2]IMPACT, Center of Interventional Medicine for Precision and Advanced Cellular Therapy, Santiago, Chile, Santiago, Chile; [3]Laboratory of Nano-Regenerative Medicine, Centro de Investigación e Innovación Biomédica (CiiB), Universidad de los Andes, Santiago, Chile, Santiago, Chile

Introduction: Angiogenesis represents a rational therapeutic target critical for solid tumor growth. We isolated small extracellular vesicles (sEV) secreted by cultured menstrual stromal cells (MenSC) that act as inhibitory agents of tumor cell- and endothelial cell-derived angiogenic-secretome in prostate, breast, and head and neck cancer. Here, we identify the unique miRNA signature in MenSC-sEV compared to other tissue sources. We also profile the miRNA cargo implicated in the anti-angiogenic effect and identify the underlying pathways. Besides, we single out the putative gene targets of the selected miRNAs within the host cells.
Methods: MenSC-sEV were isolated by differential centrifugation, characterized following MISEV 2018 guidelines, and their miRNA profile was obtained using HTG EdgeSeq miRNA whole transcriptome assay. To assess the functional impact of MenSC-sEV treatment on human breast cancer cell lines and endothelial cells, we performed whole transcriptome RNA sequencing. The miRNA signature of sEV secreted by bone marrow (BM) and adipose tissue (AT) MSC was collected from public databases.
Results: The identity and purity of sEV were confirmed by the presence of CD63, CD9, CD81, Flotillin-1, Syntenin-1, and the absence of Calnexin and TOMM20. The miRNA signature of MenSC-sEV revealed that the top 15 common miRNAs enriched in MenSC-sEV regulate tumor growth by targeting multiple key oncogenic signaling pathways. Bioinformatic analysis of host cells treated with MenSC-sEV identified changes in the mRNA expression profile associated with various anti-tumor pathways, including angiogenesis. Besides, MenSC-sEV possesses a unique miRNA profile that differs qualitatively from the other MSC sources as BM and AT.
Summary/Conclusion: This analysis confers the first evidence to strongly support the data that miRNAs enriched in MenSC-sEV play a crucial role in the regulation of angiogenesis.
Funding: FONDECYT 1190411, IMPACT #FB210024, ANID, Chile.

PT06.10 | New strategies based on the use of protein-based drugs into sEVs to overcome resistance to BRAF/MEK inhibitors in tumours with a mutation in BRAF

Amanda Guitián-Caamaño[1]; Adrián Varela-Vázquez[1]; Paula Carpintero-Fernández[1]; Alexander Carneiro-Figueira[1]; Marta Varela-Eirín[2]; Susana B. Bravo[3]; Teresa Calleja-Chuclá[4]; María Varela-Quindós[5]; David Santamaría[6]; Eduardo Fonseca[7]; María D. Mayán[1]

[1]CellCOM Group, Instituto de Investigación Biomédica de A Coruña (INIBIC), Servizo Galego de Saúde (SERGAS), A Coruña, Spain; [2]European Research Institute for the Biology of Ageing (ERIBA);, University Medical Center Groningen (UMCG), University of Groningen, Antonius Deusinglaan, Groningen, Netherlands; [3]Instituto de Investigación Sanitaria de Santiago de Compostela IDIS, Proteomics laboratory, Santiago de Compostela, Spain; [4]Hospital Pharmacy Service. CH-Universitario A Coruña (XXIAC). Servizo Galego de Saúde (SERGAS), A Coruña, Spain; [5]Translational Cancer Research Group, Instituto de Investigación Biomédica de A Coruña (INIBIC). CH-Universitario A Coruña (XXIAC). Servizo Galego de Saúde (SERGAS), A Coruña, Spain; [6]University of Bordeaux, INSERM U1218, ACTION Laboratory, Bordeaux, France; [7]Dermatology Deparment, University Hospital of A Coruña, CellCOM group, Servizo Galego de Saúde (SERGAS), A Coruña, Spain

Introduction: The channel protein connexin43 (Cx43) shows tumour suppressive roles in melanoma. On the other hand, BRAF/MEK inhibitors (BRAF/MEKi) have become the standard therapy in patients with BRAF-mutated melanoma. However resistance to targeted agents is a frequent cause of therapy failure. Cx43 is a complex protein that has channel-dependent and channel-independent roles. This protein is also found in small extracellular vesicles (sEVs) but it is role in sEVs in cancer has not been explored yet.

Methods: Different human BRAF-mutated cell lines were used in this study. Expression vectors and sEVs-enriched in Cx43 were used to target tumor cells. sEVs were isolated by ultracentrifugation and characterized by NTA, electron microscopy and WB. IP was performed to study protein interactions and samples were analyzed by LC-MS/MS. RNA-seq was used to identified sRNAs in sEVs. Standard methods were used to study cellular senescence, cell proliferation and cell death by apoptosis.

Results: Cx43 overactivity, using a vector or sEVs containing Cx43, significantly decreases cell growth and proliferation and increases senescence and apoptosis in BRAF-mutant cell lines. The presence of Cx43 in sEVs changes their function and the content of proteins and sRNA detected by MS/MS and RNA-seq. In addition, restoration of Cx43 using sEVs for its delivery, increases the efficacy of the BRAF/MEKi and prevents drug resistance by reenforcing cellular senescence and enhancing cell death by apoptosis, alone and in combination with the senolytic drug navitoclax. These results were confirmed in ex vivo a in vivo models using xenotransplant of human melanoma cell lines and under BRA/MEK inhibitors treatments combined with sEVs enriched in Cx43.

Summary/Conclusion: Our results indicate that Cx43 radically changes the protein and RNA content of sEVs changing their function. In this study we have develop a new strategy based on the use of sEVs as drug delivery system to transport a transmembrane protein that increases the efficacy of the BRAF/MEK inhibitors and prevents drug resistance in BRAF mutated positive tumours. These results have been protected in one European patent and could impact in the manage and treatment of metastatic tumors with a potential clinical benefit in patients with a metastatic disease.

PT06.12 | Tissue factor-dependent and -independent blood coagulation activities of ovarian clear cell carcinoma cells and their extracellular vesicles

Hiroko Tadokoro; Shiro Koizume; Mitsuyo Yoshihara; Yukihide Ota; Shinya Sato; Yohei Miyagi

Molecular Pathology and Genetics Division, Kanagawa Cancer Center Research Institute, Yokohama-shi, Japan

Introduction: Cancer-associated thromboembolism (CAT) is the second leading cause of death after progression of tumors in cancer patients. Blood coagulation produces thrombin, which converts fibrinogen to fibrin, via independently activated endogenous and extrinsic pathways. Tissue factor (TF) is a type I membrane protein with a transmembrane domain and a short cytoplasmic domain and initiates the extrinsic coagulation pathway. TF is incorporated in extracellular vesicles (EVs) secreted by pancreatic cancer cells and believed as a major cause of CAT in the patients. Although ovarian clear cell carcinoma (OCCC) patients are prone to complicate CAT, it is not yet clarified the implication of TF, which is reported to be overexpressed in OCCC cells. Therefore, we aimed to investigate the effect of TF on the coagulation-promoting activity of OCCC cells and their EVs.

Methods: TF expression in human OCCC cell lines, ES-2, RMG-1, and TOV21G was examined by western blotting. TF-knockout (TF-KO) cell lines were prepared by the CRISPR-Cas9 genome editing approach. Effect of TF-KO on cell proliferation and EV production itself was evaluated, and then TF procoagulant activity (TF-PCA) of cells was accessed by factor Xa generation assay. EVs were prepared by ultracentrifugation of serum-free culture medium of cells. In addition, whole coagulation activity of cells and EVs was evaluated by an optical fibrin generation assay using human plasma.

Results: TF protein was identified in ES-2 and RMG-1 cells and in their EVs. TF was not identified in TOV-21G cells and EVs. Expectedly, TF-PCA was detected in ES-2 and RMG-1 cells but not TOV21G cells. TF-KO did not affect cell proliferation and EV production of ES-2 and RMG-1 cells. However, unexpectedly, optical fibrin generation assay revealed that all three cells and their EVs have fibrin generation activity.
Summary/Conclusion: Our data suggest that OCCC cells and their EVs have both TF-dependent and -independent coagulation activity, potentially contributing to OCCC-CAT.

PT06.13 | Proteomic profiles of small extracellular vesicles released by colon cancer cell line HCT116 with different p53 status

Monika Pietrowska[1]; Mateusz Smolarz[1]; Marta Gawin[1]; Urszula Strybel[2]; Marek Rusin[3]; Piotr Widlak[4]; Anna Wojakowska[2]
[1]Maria Sklodowska-Curie National Research Institute of Oncology, Gliwice Branch, Gliwice, Poland; [2]Institute of Bioorganic Chemistry Polish Academy of Sciences, Poznan, Poland; [3]Maria Sklodowska-Curie National Research of Oncology, Gliwice Branch, Gliwice, Poland; [4]Medical University of Gdansk, Gdansk, Poland, Gdansk, Poland

Introduction: Extracellular vesicles (EV) are important components of cellular secretome that play a key role in cell-cell communication, including immune signaling, angiogenesis, stress response, senescence, proliferation, and cell differentiation. p53 controls the expression and secretion of numerous extracellular factors, either soluble or contained within EVs. It has been postulated that p53 is responsible for the composition of small EVs (sEV), primarily exosomes, released by cancer cells. For example, a p53-dependent increase in EV secretion was observed and p53 protein was implicated in the upregulation of RAB27b (a key component of EV secretory pathways). Here we aimed to compare directly proteome profiles of sEV released by a colon cancer cell line with different p53 statuses.
Methods: sEV were isolated from supernatants of p53-proficient (wild-type) and p53-deficient (homozygous deletion) variants of HCT116 cell line. sEV were purified using size exclusion chromatography and characterized according to the recent MISEV2018 guidelines. Cell lysates were prepared by extraction with RIPA buffer. Proteins present in purified sEV and corresponding cell lysates were analyzed by high-resolution mass spectrometry (HRMS). The raw data obtained for each dataset (EV and cell lysates) were imported into Proteome Discoverer v.1.4 (Thermo Fisher Scientific) for protein identification and quantification.
Results: There were 374 proteins detected in sEV, including 44 proteins specific for HCT116p53(+) and 3 proteins specific for HPVp53(-) vesicles. The gene ontology analysis of 227 proteins upregulated in HCT116p53(+) vesicles revealed that over-represented processes associated with p53-upregulated sEV proteins included (i) viral process, (ii) symbiotic process, and (iii) translational initiation.
Summary/Conclusion: We identified the protein profile of EVs that discriminates vesicles released by cells with different p53 statuses.
Funding: This study was supported by the National Science Centre, Poland, Grant 2017/26/D/NZ2/00964 (for MP, US, AW).

PT06.14 | Pro-inflammatory role of EV in transplant lung rejection signaling

Alessandra M. Storaci[1]; Alessandro Palleschi[2]; Sara Franzi[2]; Valeria Musso[2]; Rodolfo Bruschi[2]; Letizia Corinna Morlacchi[2]; Valeria Rossetti[2]; Francesco Blasi[2]; Mario Nosotti[2]; Silvano Bosari[3]; Stefano Ferrero[2]; Valentina Vaira[3]
[1]Università degli studi di Milano, Milan, Italy; [2]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [3]Università degli Studi di Milano, Milan, Italy

Introduction: Lung transplantation (LT) is a life-saving treatment for patients (pt) with end-stage lung diseases. However, the survival is threatened by acute rejection (AR) that favours the onset of chronic lung allograft dysfunction (CLAD). Further, no early accurate biomarkers of graft rejection currently are known and its molecular signaling is unclear. Therefore, we study AR and CLAD lung microenvironment through a profiling of extracellular vesicles (EV) purified from broncho-alveolar lavage fluids (BAL) from LT pt.
Methods: EV were isolated from the BAL fluid (500ul) of pt with AR, CLAD and no rejection event (CTRL; n=3, 3, 6).
BAL were centrifuged 20min at 1000g to remove debris; supernatants were centrifuged for 30min at 10000g.
The expression of EV specific markers (presence of TSG101, ARF6, CD63 and absence of Calnexin, Ago2) was analysed by western blot.
Nanoparticles tracking analysis was used to assess size and concentration of EVBAL and PKH26 labelling for internalization assay. After the co-culture with Human epithelial bronchial cells, supernatants (48 and 72h) and cell lysates (72h) were collected and the cyto/chemokine contents were assessed using a cytokine array.
EVAR/CLAD array signals are compared to matched CTRL.

Results: Cells supernatant, after co-culture with MVCLAD showed the increase of 7 cytokines at 72h. EVAR, instead, induced a transient up-modulation at 48h, with 16 cytokines that remained higher than CTRL at 72h.
In cell lysates at 72h only 3 cytokines resulted as up-modulated after EVCLAD co-culture; on the contrary, EVAR induced a general up-modulation of the analysed cytokines.
KEGG analysis of up-modulated cytokines indicated their involvement in allograft rejection (IL10, IL12), pro-inflammatory processes (IL32) and IL17 signalling pathway (CXCL1, CXCL8, CSF), known to promote the damage of donor graft.
Summary/Conclusion: These preliminary data indicate that EVCLAD/AR are functional and carry molecules with a role in the onset and perpetuation of inflammatory processes. Further, targeting the pro-inflammatory IL32 might prevent the onset of an unfavourable environment for the lung allograft. This study could provide new information about the pathways involved in rejection events and potential therapeutic targets.

PT06.15 | Effects of tumor derived exosomes on T cells expression

Shahid Hussain; Shaukat Iqbal Malik
Cust islamabad, Islamabad, Pakistan

Introduction: Exosomes are 30-120nm bio particles transferred from donor to recipient cells leading to modification in their regulatory mechanisms depending upon the coded message in the form of loaded biomolecule. Cancer cells derived exosomes the true representatives of the parent cells have been found to modify the tumor surrounding/distinct regions and participate in metastasis, angiogenesis and immune suppression.
Methods: The current study was aimed to study the effects of tumor mice derived exosomes on the normal mice spleen isolated T cells by using co-culture experiments and flow cytometer analysis. We mainly focused on some of the T cells population and cytokines including IFN-γ, FOXP3+ regulatory T (Treg) cells and KI67 (proliferation marker).
Results: Overall results indicated random changes in different set of experiments where the cancer derived exosomes reduced the IFN-γ expression in both CD4 and CD8 T cells, similarly the Treg cells were also found decreased in the presence of cancer exosomes. No significant changes were observed on the Ki67 marker expression.
Summary/Conclusion: Such studies are helpful in understanding the role of cancer exosomes in immune cells suppression in tumor microenvironment. That needs further in vivo and in vitro validation on molecular level to provide a potential uses of exosomes clinically, in the development of vaccines, in targeting tumors, and in diagnosis and/or early detection.

PT07: Physiology and pathology: Cancer immunology and immunotherapy

Chair: Christian Preußer – Institute for Tumor Immunology, Center for Tumor Biology and Immunology (ZTI), Philipps University of Marburg, Marburg, Germany

Chair: Elke Pogge von Strandmann – Institute for Tumor Immunology, Center for Tumor Biology and Immunology (ZTI), Philipps University of Marburg, Marburg, Germany

PT07.01 | B cell targeting of extracellular vesicles by a novel fusion protein

Annemarijn Offens[1]; Loes Teeuwen[2]; Sander A. Kooijmans[3]; Gözde Güclüler Akpinar[2]; Hannah Weissinger[2]; Maria Eldh[1]; Pieter Vader[4]; Susanne Gabrielsson[1]
[1]Division of Immunology and Allergy, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden, Stockholm, Sweden; [2]Division of Immunology and Allergy, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden, Solna, Sweden; [3]CDL Research, University Medical Center Utrecht, Utrecht, The Netherlands, Utrecht, Netherlands; [4]CDL research, University Medical Center Utrecht, Utrecht, The Netherlands, Utrecht, Netherlands

Introduction: Allogeneic extracellular vesicles (EVs) loaded with ovalbumin (Ova) can induce an anti-tumor immune response in mice with Ova-expressing melanoma tumors. This anti-tumor immunity of Ova-loaded EVs relies on the activation of T cells, a response that is dependent on B cells. Additionally, previous research showed that EBV-derived GP350-containing EVs target human CD21 present on B cells. We hypothesized that EVs targeting B cells would induce stronger T cell responses to EVs. Therefore, we aim to examine if decorating Ova-loaded allogeneic EVs with a fusion protein that binds CD21 target the EVs to B cells and thereby improve antigen-specific immune responses.
Methods: A fusion protein containing the phosphatidylserine-binding domain (C1C2) of lactadherin and CD21-binding domain (D123) of GP350 was designed. Ultracentrifugation-isolated macrophage-like RAW264.7 EVs were dyed with cell tracker deep

red, washed by size exclusion and incubated with fusion protein C1C2-D123. Fusion protein-decorated EVs were incubated with splenocytes of human CD21+ mice or human PBMCs. In vitro targeting was assessed by flow cytometry.
Results: The results show that, depending on the amount of EVs used, the percentage of B cells that are positive for C1C2-D123 fusion protein-decorated EVs increased by more than 50% in mouse splenocytes and by more than 90% in human PBMCs compared to EVs in absence of the fusion protein.
Summary/Conclusion: In conclusion, C1C2-D123 fusion protein-decorated EVs improves B cell targeting in vitro. Future in vivo experiments will assess if Ova-loaded B cell targeted EVs improve antigen-specific immune responses as compared to non-targeted EVs.
Funding: This work was supported by grants from the Swedish Research Council Medicine, The Swedish Cancer Foundation, The Cancer Research Foundations of Radiumhemmet, The Swedish Heart-Lung Foundation, the Centre for Allergy Research Karolinska Institutet, and the KID grants of the Karolinska Institutet.

PT07.02 | Correlation between the immunological cell profile, clinical data, and the extracellular vesicle content in non-small cell lung cancer patients

Magdalena Dlugolecka[1]; Lukasz Zareba[1]; Joanna Domagala-Kulawik[2]; Malgorzata Polubiec-Kownacka[3]; Malgorzata Czystowska-Kuzmicz[4]
[1]Doctoral School, Chair and Department of Biochemistry, Medical University of Warsaw, Warsaw, Poland; [2]Department of Internal Medicine, Pulmonary Diseases and Allergy, Medical University of Warsaw, Warsaw, Poland; [3]Department of Surgery, Institute of Tuberculosis and Lung Diseases, Warsaw, Poland; [4]Medical University of Warsaw, Warsaw, Poland

Introduction: Establishing the connection between the patients' immunological profile and the extracellular vesicle (EV) profile and patients' clinical data is of great scientific interest and may help to choose the best therapy regimen and to monitor immune response during therapy. Here we investigated associations between the immunological cell profile, EV content, and clinical data of non-small cell lung cancer (NSCLC) patients.
Methods: Immune cells and EVs obtained from blood and bronchoalveolar lavage fluid (BALF) of 82 NSCLC patients were analyzed. Immune cells from the cancerous (cBALF) and the opposite lung (oBALF) as well as from blood were phenotyped by flow cytometry, to check their activation potential and immunosuppressive content. In parallel, EVs were isolated from the same samples. Plasma EVs were obtained from the patients' blood by centrifugation, filtration, and size-exclusion chromatography (SEC). The BALF EVs were isolated by ultracentrifugation (UC). The EVs were characterized by several methods following the MISEV2018 guidelines. The EV concentration, size, and tetraspanin content were measured by nanoparticle tracking analysis (NTA).
Results: Our analysis showed the association between tumor stage and the concentration of EVs in our patients' cohort. In the case of cBALF, we observed an increase in the total EV count along with tumor development at the early tumor stage (IA2, IA3, IB, IIB, IIA, IIIA). In addition, this increase was also visible in the amount of cBALF EVs labeled with membrane dye and some tetraspanin markers (CD63, CD9). Interestingly, in tumor stage IIB, the concentration of EVs was lower than in IIA and IIIA. We did not observe such association in the case of oBALF and plasma EVs. Additionally, there was a moderate correlation between the presence of some immunosuppressive markers on lymphocytes and the EV profile.
Summary/Conclusion: We have provided first hints that the molecular profile of BALF EVs may be a marker of immune suppression in lung cancer patients and that there is an association between tumor stage and the amount of EVs from the cancerous lung. However, its clinical impact still needs to be validated.
Funding: National Science Center - OPUS 14 2017/27/B/NZ6/01990; NAWA – PPI/APM/2019/1/00051/V/00001

PT07.03 | Ex vivo expansion and activation of Natural Killer cells by co-culture with exosomes derived from engineered K562 cells

Tayler J. Croom-Perez[1]; Jeremiah L. Oyer[1]; Robert Y. Igarashi[2]; Deborah A. Altomare[3]; Alicja J. Copik[1]
[1]University of Central Florida, College of Medicine, Orlando, USA; [2]Kiadis pharma, a Sanofi Company, Amsterdam, Netherlands; [3]Univeristy of Central Florida, College of Medicine, Orlando, USA

Introduction: Natural Killer (NK) cells are cytotoxic effector lymphocytes important in the anti-tumor response of the innate immune system and are of great interest as an emerging cellular therapy. However, NK cells are in low abundance in blood and to become feasible in adoptive immunotherapy, novel methods to enhance anti-tumor activity and expand numbers of NK cells ex vivo are needed. Our group developed a NK cell expansion method that utilizes plasma membrane nanoparticles derived from engineered K562 cells (CSTX-002) expressing 41BBL and membrane-bound IL21 (PM21). In this study, we

investigate the use of exosomes derived from these cells to expand NK cells as they are amenable to engineering for further enhancement of the proliferation and activation of NK cells and could be used to deliver agents to genetically engineer NK cells themselves.

Methods: Exosomes derived from CSTX-002 cells (EX21-exosomes) were isolated by ultrafiltration and characterized by Nanosight and TRPS Measurement. The presence of exosomal markers was confirmed by Western blot and flow cytometry. In vitro cytotoxicity of the NK cells was determined and comparative in vivo efficacy of EX21-NK and PM21-NK cells was assessed in SKOV-3Luc ovarian tumor-bearing mice. Mice were treated with vehicle, EX21-NK cells or PM21-NK cells and monitored for survival.

Results: CD3-depleted PBMCs cultured with EX21-exosomes expanded 530-fold over 14 days compared to a 735-fold change in the presence of PM21-particles. EX21-NK and PM21-NK cells had comparable in vitro cytotoxicity against K562 cells. Treatment of SKOV engrafted NSG mice with either EX21-NK cells or with PM21-NK cells allowed significantly ($p < 0.0001$) increased survival compared to untreated animals (41–44 vs 29 days post treatment).

Summary/Conclusion: EX21-exosomes efficiently expand NK cells in vitro, and are equally as effective as PM21-NK cell at anti-tumor activity, both in vitro and in vivo.

Funding: Florida Department of Health James and Ester King Program (Grant No. 9JK04), The Guillot-Henley Family AML Research Fund in loving memory of William L. Guillot, the University of Central Florida Preeminent Postdoctoral Program, and Kiadis pharma, a Sanofi Company.

PT07.04 | Extracellular vesicles transfer the NKG2D ligand MICA on multiple myeloma cells and increase their susceptibility to NK cell-mediated killing: role of two distinct MICA allelic variants

Luisa Loconte[1]; Elisabetta Vulpis[1]; Laura Masuelli[2]; Giulio Caracciolo[3]; Angela Santoni[1]; Alessandra Zingoni[1]

[1]1Laboratory affiliated to Istituto Pasteur Italia-Fondazione Cenci Bolognetti, Department of Molecular Medicine, "Sapienza" University of Rome, Italy, Rome, Italy; [2]Department of Experimental Medicine, "sapienza" University of Rome, Rome, Italy; [3]Department of Molecular Medicine, "Sapienza" University of Rome, Rome, Italy

Introduction: Natural Killer (NK) cells are innate cytotoxic lymphoid cells that play a crucial role in cancer immunosurveillance. NKG2D is an activating receptor that binds to MIC and ULBP molecules typically induced on damaged, transformed or infected cells. The secretion of NKG2D ligands (NKG2DLs) through protease-mediated cleavage or by extracellular vesicle (EV) is a mode to control their cell surface expression and a mechanism used by cancer cells to evade NKG2D-mediated immunosurveillance. The NKG2DL MICA is characterized by a high grade of polymorphism that could influence its biological properties.

Methods: Medium-size EV (mEVs) were isolated by ultracentrifugation from a MM cell line transfected with two different MICA allelic variants namely MICA*008 (GPI-anchored prototype) and MICA*019 (transmembrane prototype). mEVs were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The expression of MICA and other markers was evaluated by flow cytometry, western blot and ELISA. Polyclonal cultures of NK cells were obtained from PBMCs and used in functional assays.

Results: The MICA allelic variants, MICA*008 and MICA*019, were expressed on the surface of mEVs and were transferred from vesicle to target MM cells with different kinetics and efficacy suggesting a different transfer mechanism on the target cell plasma membrane. Interestingly, EV-treated target cells that had acquired MICA were able to downmodulate NKG2D on NK cells. We provide evidence that the EV-derived MICA on MM cells rendered those cells more susceptible to NK cell recognition and killing.

Summary/Conclusion: Our findings shed light on the role of EV-associated MICA allelic variants in the modulation of NKG2D-mediated NK cell immunesurveillance in the tumor microenvironment. Moreover, the EV-mediated transfer of MICA could suggest a novel therapeutic approach based on the usage of engineered nanoparticles aimed at increasing cancer cell immunogenicity

Funding: This work was supported by grants from AIRC and “Sapienza” University of Rome.

PT07.05 | Macitentan attenuate extracellular vesicle PD-L1 mediated immunosuppression

Chan-Hyeong Lee[1]; Ju-Hyun Bae[1]; Eun-Ji Choe[1]; Ju-Mi Park[1]; Seong-Sik Park[1]; Hee Jin Cho[2]; Byoung-Joon Song[3]; Moon-Chang Baek[1]

[1]Kyungpook National University School of Medicine, Daegu, Republic of Korea; [2]Department of Biomedical Convergence Science and Technology, Kyungpook National University, Daegu, Republic of Korea; [3]National Institute on Alcohol Abuse and Alcoholism, NIH, Bethesda, USA

Introduction: Extracellular vesicles (EVs) carrying tumor cell-derived programmed death-ligand 1 (PD-L1) interact with programmed death 1 (PD-1)-producing T cells, thus significantly lowering a patient's response to immune checkpoint blockade drugs. No drug that reinvigorate CD8+ T cells by suppressing EV PD-L1 has been approved for clinical usage. Here we have identified macitentan (MAC), an FDA-approved oral drug, as a robust booster of antitumor responses in CD8+ T cells by suppressing tumor cell-derived EV PD-L1.
Methods: EV was analyzed by the data from nanoparticle tracking, immunoblotting analyses, and nano-flow cytometry. Antitumor immunity were evaluated by luciferase assay and immune phenotyping using flow cytometry. Clinical relevance was analyzed using the cancer genome atlas database.
Results: MAC inhibited secretion of tumor-derived EV PD-L1 by targeting the endothelin receptor A (ETA) in breast cancer cells and xenograft models. MAC enhanced CD8+ T cell-mediated tumor killing by decreasing the binding of PD-1 to the EV PD-L1 and thus synergizing the effects of the anti-PD-L1 antibody. MAC also showed an anticancer effect in triple-negative breast cancer (TNBC)-bearing immunocompetent mice but not in nude mice. The combination therapy of MAC and anti-PD-L1 antibody significantly improved antitumor efficacy by increasing CD8+ T cell number and activity with decreasing Treg number in the tumors and draining lymph nodes in TNBC, colon, and lung syngeneic tumor models. The antitumor effect of MAC was reversed by injecting exogenous EV PD-L1. Notably, ETA level was strongly associated with the innate anti-PD-1 resistance gene signature and the low response to the PD-1/PD-L1 blockade.
Summary/Conclusion: These findings strongly demonstrate that MAC, already approved for clinical applications, can be used to improve and/or overcome the inadequate response to PD-1/PD-L1 blockade therapy.

PT07.06 | Macrophage education by melanoma cells-vesicles after radiotherapy

Silvina Odete Bustos[1]; Luciana Nogueira de Sousa Andrade[2]; Tiphany Coralie De Bessa[3]; André Zelanis[4]; Murilo Salardani[5]; Cristiane Damas Gil[6]; Francisco Laurindo[3]; Roger Chammas[7]
[1]Center for Translational Research in Oncology (LIM24), Instituto do Cancer do Estado de Sao Paulo (ICESP), Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo (HCFMUSP), Sao Paulo CEP 01246-000, SP, Brazil, São Paulo, Brazil; [2]Center for Translational Research in Oncology (LIM24), Instituto do Câncer do Estado de São Paulo (ICESP), Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HCFMUSP), São Paulo, Brazil, São Paulo, Brazil; [3]Laboratório de Biologia Vascular, LIM-64 (Biologia Cardiovascular Translacional), Instituto do Coração (InCor), Hospital das Clínicas HCFMUSP, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil., São Paulo, Brazil; [4]Functional Proteomics Laboratory, Department of Science and Technology, Universidade Federal de São Paulo (ICT-UNIFESP), São José dos Campos, SP, Brazil., São José dos Campos, Brazil; [5]Functional Proteomics Laboratory, Department of Science and Technology, Universidade Federal de São Paulo (ICT-UNIFESP), São José dos Campos, SP, Brazil., São Jose dos Campos, Brazil; [6]Department of Morphology and Genetics, Federal University of São Paulo (UNIFESP), São Paulo 04023-900, Brazil, São Paulo, Brazil; [7]Center for Translational Research in Oncology (LIM24), Instituto do Cancer do Estado de Sao Paulo (ICESP), Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo (HCFMUSP), Sao Paulo CEP 01246-000, SP, Brazil, Sao Paulo, Brazil

Introduction: Cutaneous melanoma is the most aggressive skin cancer that harbors a high mutational burden and potential to form metastases which is associated with acquired resistance. In this regard, tumor extracellular vesicles (TEVs) have become critical components in the melanoma microenvironment promoting drug resistance. TEVs can transfer their cargo to stromal cells, such as macrophages, modulating several processes influencing tumor progression. Herein, we investigated whether TEVs secreted by melanoma cells submitted to radiotherapy modulate tumor response through macrophage reprogramming.
Methods: B16F10 melanoma cells were irradiated and, after 24h, TEVs were isolated from cell culture media by differential ultracentrifugation and resuspended in filtered PBS. TEVs were characterized by nanoparticle tracking analysis, transmission electron microscopy and the detection of EVs markers by western blot. For proteomics, RIPA buffer was added to TEVs samples followed by sonication and digestion.
Results: A single irradiation dose caused an increase in TEVs secretion by melanoma cells after 24 hours. These TEVs were taken up by bone marrow-derived macrophages modulating their redox state through SOD2 and PDIA1 downregulation, which was accompanied by an increase in reactive oxygen species like H2O2. Moreover, the uptake of irradiated TEV by macrophages resulted in increased VEGF secretion and, after co-culture with B16F10 cells, we observed an increase in tumor cell migration. Proteomics analysis of TEVs released by non-irradiated and irradiated melanoma cells showed a different protein cargo between these 2 groups and an enrichment in proteins related to cell migration was found in TEVs from irradiated cells.
Summary/Conclusion: Our preliminary data indicates that the uptake of TEVs released by irradiated melanoma cells are able to reprogram macrophages towards a pro-tumoral phenotype characterized by altered redox state.
Funding: CNPq (305700/2017).

PT07.07 | Salinomycin-induced actin reorganization limits the presence of CD20 antigen on extracellular vesicles and augments the efficacy of lymphoma immunotherapy

Anna Torun[1]; Abdessamad Zerrouqi[1]; Andrzej Ciechanowicz[1]; Tony Ng[2]; Malgorzata Czystowska-Kuzmicz[1]; Beata Pyrzynska[1]
[1]Medical University of Warsaw, Warsaw, Poland; [2]King's College London, London, United Kingdom

Introduction: We discovered that salinomycin (previously shown to eradicate the breast cancer stem cells) strongly upregulated the surface levels of CD20 antigen on B-cell-derived malignancies, including a variety of non-Hodgkin lymphoma cell lines and patients-derived primary malignant cells. Consequently, the efficacy of therapeutic anti-CD20 antibody, rituximab, was significantly increased by salinomycin, both in vitro and in a xenograft mouse model. Here, we further tested the effect of lymphoma-derived extracellular vesicles (EVs) on the therapeutic efficacy of rituximab.
Methods: EVs obtained by ultracentrifugation from either wild-type or CD20-null (CRISPR/Cas9-modified) Burkitt's lymphoma cells were further characterized by Western blotting, nanoparticle tracking analysis and flow cytometry. The effect of EVs on rituximab efficacy was estimated in cytotoxicity assays. Additionally, proteomic analysis of EVs was performed and results were validated using Western blotting as well as CytoFlex and ImageStream analyzers.
Results: Lymphoma-derived EVs carried the CD20 antigen and therefore limited the efficacy of rituximab toward malignant cells. Surprisingly, the EVs deriving from salinomycin-treated tumor cells carried lowered levels of CD20 antigen and exhibited a less negative effect on rituximab efficacy. To understand the molecular mechanisms of this unexpected effect, we carried out a proteomic analysis of EVs deriving from salinomycin-treated tumor cells. The analysis revealed a dramatic drop in the levels of actin and proteins involved in actin nucleation and reorganization, such as WAVE2, cofilin, ezrin, and moesin. The drop in levels of b-actin and cofilin was detected only in EVs, but not in total cell extracts and correlated well with the drop in levels of CD20.
Summary/Conclusion: Salinomycin-induced actin reorganization limits the availability of CD20 antigen in EVs derived from lymphoma cells and positively influences efficacy of anti-CD20 therapeutic antibodies.
Funding: Polish National Agency For Academic Exchange (NAWA), grant no PI/APM/2019/1/00051/U/001;
National Science Centre (NCN, Poland), grant no 2019/35/B/NZ5/01445.

PT07.08 | Stimulation of PD-L1 expression on renal cell carcinoma-derived extracellular vesicles and its effect on CD8+ T cells

Greta Jaschkowitz[1]; Dirk Himbert[1]; Angela Zaccagnino[1]; Elfriede Noessner[2]; Michael Stöckle[1]; Kerstin Junker[1]; Philip Zeuschner[1]
[1]Department of Urology and Pediatric Urology, Saarland University, Homburg/Saar, Germany; [2]Helmholtz Center Munich, German Research Center for Environmental Health, IMA Immunoanalytics, Munich, Germany

Introduction: The expression of PD-L1 on EVs has been shown to carry prognostic, diagnostic and predictive value in various cancers. However, its role as an important target of checkpoint inhibition in renal cell carcinoma (RCC) is not yet well understood. This study aims to evaluate the PD-L1 expression on EVs secreted by RCC cell lines and the putative immunomodulatory effects of these EVs on CD8+ T cells.
Methods: We isolated EVs from cell culture supernatant of 6 RCC cell lines by serial ultracentrifugation. The cells had been repetitively stimulated with interferon γ (IFNγ) on day 5 and 3 before harvest of the supernatant. Quality and quantity of EVs were verified by nanoparticle tracking analysis, transmission electron microscopy and western blot. PD-L1 expression was analyzed semi-quantitatively by western blot. CD8+ T cells were isolated from blood of healthy donors, activated and co-cultured with RCC-derived EVs for 3 days. Proliferation and PD-1 expression were assessed by flow cytometry.
Results: All 6 cell lines expressed PD-L1 at low levels. Repetitive stimulation with IFNγ led to increased PD-L1 expression on cells and EVs of 4/6 cell lines. Highest PD-L1 expression occurred in RCC53 cells after stimulation, the upregulation was most successful in cell line KTCTL26. First results of co-cultures of CD8+ T cells with RCC-derived small EVs suggest an anti-proliferative effect on T cell proliferation and inhibition of early T cell activation. These effects appeared to be stronger with EVs from IFNγ-stimulated cells.
Summary/Conclusion: RCC cell lines show low PD-L1 expression in cells and EVs. IFNγ stimulation upregulates the PD-L1 expression in cells and EVs in a cell line dependent manner. First results of co-cultures suggest that RCC-derived small EVs could affect early CD8+ T cell proliferation and activation patterns, and therefore seem to be involved in immunomodulation.
Funding: This project was funded by HOMFORexzellent (2020-2023), Medical Faculty of Saarland University.

PT07.09 | T-cell derived extracellular vesicles prime the STING pathway in macrophages by a DNA independent mechanism

Aida S. Hansen[1]; Lea S. Jensen[2]; Anders Etzerodt[2]; Kristoffer G. Ryttersgaard[2]; Christian Krapp[2]; Yan Yan[3]; Jesper Just[2]; Kasper L. Jønsson[2]; Bent W. Deleuran[2]; Martin R. Jakobsen[2]
[1]Aarhus University, Slagelse, Denmark; [2]Aarhus University, Aarhus, Denmark; [3]Interdisciplinary Nanoscience Center, Aarhus University, Aarhus, Denmark

Introduction: Extracellular vesicles (EVs) are key players in shaping immune responses. It is believed that EVs can prime antigen-presenting cells for a more robust immune activation. T-cell derived EVs have been shown to provide a danger signal to dendritic cells by carrying mitochondrial DNA, but little is known about the DNA independent function of these EVs on antigen-presenting cells. Here, we hypothesize that CD4+ T-cell derived EVs enhance macrophage function by priming the signaling pathway regulated by STING independent of DNA.
Methods: We purified EVs from anti-CD3/anti-CD28 stimulated CD4+ T cells by differential ultracentrifugation and pre-treated macrophages with these prior to stimulation with the STING ligand cGAMP. Subsequent activation was measured by induction of type I Interferon and phosphorylation of signaling molecules within the STING pathway.
Results: Here, we demonstrate that EVs collected from activated T cells sensitize macrophages to respond more potently to STING ligands. Importantly, this is independent on both surface-associated and intravesicular DNA. Intriguingly, we find the EVs activate the NFkB pathway in macrophages dependent on TBK1. Furthermore, we show that EVs from activated T cells carry pro-inflammatory cytokines capable of enhancing type I IFN production in macrophages upon STING activation.
Summary/Conclusion: We propose that activated CD4+ T cells enhance macrophage function by releasing EVs containing a "package" of pro-inflammatory molecules that leads to pre-activation of TBK1 and NFkB signaling pathways. This results in a potent immune response towards STING activation and we speculate this could be exploited therapeutically to increase macrophage activation in cancer.
Funding: Independent Research Fund Denmark, Grant 8026-00018B
Danish Cancer Society, Grant R149-A10167-16-S47
Novo Nordic Foundation, Innovation Fellowship Program 1-2020

PT07.10 | Temsirolimus enhances anti-cancer immunity by inhibiting the secretion of small extracellular vesicles containing PD-L1

Seong-Sik Park; Chan-Hyeong Lee; Ju-Hyun Bae; Eun-Ji Choe; Moon-Chang Baek
Kyungpook National University School of Medicine, Daegu, Republic of Korea

Introduction: In immune checkpoint blockade therapy (ICBT), monoclonal antibodies such as Anti-PD-L1 and Anti-PD-1 are used to inhibit the binding of programmed death-ligand 1 (PD-L1) in tumor cells to programmed death-1 (PD-1) in immune cell. However, tumor-derived small extracellular vesicle (sEV) includes an immune checkpoint such as PD-L1 expressed within the tumor. Thus, inhibiting the biogenesis and secretion of tumor-derived sEV is important because it can increase the reactivity of ICBT. Here, we found temsirolimus (TEM), a drug that inhibits sEV containing PD-L1 secreted from MDA-MB-231 breast cancer cell line.
Methods: Temsirolimus was purchased from MedChemExpress and used for in-vitro and in-vivo study, All in-vivo experiments were conducted in accordance with the Declaration of Helsinki.
Results: We confirmed that TEM inhibits mTOR and activates autophagy inside tumor cells. Also, it was confirmed by immunocytochemistry that the synthesis of sEV is inhibited through fusion with autophagosome or lysosome in autophagy in where multivesicular bodies are activated during the sEV biosynthesis process. In addition, it was investigated that TEM suppressed the secretion of sEV containing PD-L1 and PD-L1 expressed in sEV. TEM showed an increase in the number of CD8+ T cells in tumor-infilterating lymphocytes and draining lymph nodes of 4T1 tumor model. In particular, it was confirmed that interferon-gamma indicating the activity of CD8+ T cells was increased in the combined administration of TEM and Anti-PD-L1. The anti-cancer effect by TEM showed a phenomenon of reversal when artificially injected with tumor-derived sEV from the outside.
Summary/Conclusion: TEM has the potential to be used not only as a known target anticancer agent, but also as an immune checkpoint inhibitor by increasing the activity of CD8+ T cells through inhibition of sEV secretion containing PD-L1 and inhibition of PD-L1 expressed in sEV.

PT07.11 | The Role of Extracellular Vesicles in Radiation-induced Myelopoiesis in Glioblastoma

Subhajit Ghosh[1]; Dennis Hallahan[2]; Jiayi Huang[2]; Dinesh Thotala[2]
[1]*Washington University in saint louis, MO, USA, Saint Louis, USA;* [2]*Washington University in saint louis, Saint Louis, USA*

Introduction: Severe and prolonged lymphopenia frequently occurs in glioblastoma (GBM) patients after standard chemoradiotherapy and has been associated with significantly worse survival. The radiation-induced lymphopenia is associated with increased circulating myeloid cell, but the underlying mechanism for the increased myelopoiesis is not well understood. This study evaluated the role of extracellular vesicles (EV) in radiation-induced myelopoiesis in GBM.
Methods: GBM patient plasma was collected before, and during the chemoradiotherapy and the EV was isolated using ultracentrifugation. The lymphopenic patients were those who developed systemic lymphopenia within 12 weeks of the start of chemoradiotherapy. The EV were characterized using western immunoblotting, flowcytometry, and nano particle tracking assay. The cytokines in the EV were evaluated using a multiplex beads array. The hematopoietic stem and progenitor cell (HSPC) differentiation assay is analyzed using standard methylcellulose media in presence of EV. Orthotopic GL261 and CT2A glioma mouse models were used to evaluate the effects of EV on HSPC from the bone marrow.
Results: We found the elevated level of myelopoiesis-specific cytokines including interleukin (IL)-1β, IL-33, IL-10, IL-6, and IL-3 in the EV of lymphopenic patients when compared to non-lymphopenic patients. The EV from lymphopenic patients were able to induce more HSPC myelopoiesis to produce myeloid cells when compared to non-lymphopenic EV in-vitro. Similar to human GBM patients, we found a significant increase of myelopoiesis-specific cytokines in the EV isolated from GBM-bearing mice and control mice after cranial irradiation. The murine EV after irradiation also induced HSPC to differentiate into myeloid cells in naïve mice.
Summary/Conclusion: Irradiation of GBM tumor and normal tissue appears to release EV that contain cytokines that would activate emergency myelopoiesis.

PT07.12 | Tumor-Derived Exosomes: Hidden Players in PD-1/PD-L1 Resistance

Jessica Gobbo[1]; Carmen Garrido[2]; Aude Chavanton[2]; Valentin Vautrot[3]
[1]*CGFL/ INSERM1231, dijon, France;* [2]*INSERM1231, dijon, France;* [3]*CGFL, dijon, France*

Introduction: Immunotherapy has garnered increasing importance in cancer therapy, leading to substantial improvements in patient care and survival. However, while patients generally respond well to immunotherapy such as anti-PD-1/PD-L1, a proportion of patients present tumors that resist these treatments. Exosomes, small nanovesicles secreted by tumor cells, could be key actors in this resistance. We identified immunosuppressive molecules (IM) expressed by tumor-derived exosomes (TEX) in different types of cancer (melanoma, lymphoma, lung and colon cancer).
Methods: Exosomes were isolated by ultracentrifugation and evaluated by nanoparticle tracking analysis (NTA technology) and TEM. Isolated exosomes were tested for the expression of exosomal markers (TSG101, CD9, CD63, CD81, Alix, Grp94). IM concentration in exosomes were measured using an ELISA. T-cell and MDSC activation/ proliferation were observed by flow cytometry.
Results: First, we explored in vitro and in vivo the action of exosomal molecules on the immune system. We found that TEX have immunosuppressive properties like the cancer cell from which they are derived at inhibiting T-cell activation determined by the expression of PD-1, Ki67, granzyme B and IFNϒ.
In addition, we observe that TEX can also activate myeloid-derived suppressor cells (MDSCs). MDSCs activation leads to global immunosuppression of immune cells (T-cell, macrophages, and dendritic cells) independent of the PD-1/PD-L1 pathway. We have developed an inhibitor of this TEX/ MDSC pathway and demonstrate in mouse models that the decrease in MDSC was associated with tumor regression and with intratumor infiltration of immune cells (T cells, dendritic cells and macrophages).
Finally, we conducted a clinical trial to observe these immunosuppressive TEX in cancerous patients.
Summary/Conclusion: Altogether, these results show that exosomes derived from cancer cells are able to mediate immunosuppression. Further study of TEX is needed to better understand resistance under anti-PD-1/PD-L1 therapy.

PT07.13 | Tumor-derived extracellular vesicle uptake by PBMCs

Davinia Arguedas[1]; Luisa Loconte[2]; Lea guyonnet[3]; Coralie Guerin[3]; Clotilde Théry[4]; Lorena Martin-Jaular[4]
[1]*INSERM U932, Institut Curie Centre de Recherche, PSL Research University, Paris, France, Paris, France;* [2]*1Laboratory affiliated to Istituto Pasteur Italia-Fondazione Cenci Bolognetti, Department of Molecular Medicine, "Sapienza" University of Rome, Italy,*

Rome, Italy; [3]Institut Curie, Cytometry Core, 75005, Paris, France, Paris, France; [4]Institut Curie, PSL Research University, INSERM U932, Paris, France

Introduction: Tumor-derived extracellular vesicles (TD-EVs) can modulate diverse immune responses, both suppressing and activating the immune system. This work aims to discern the role of EVs in immune regulation by first determining which immune cells preferentially uptake the TD-EVs and consequently what are the induced phenotypic changes in the selected immune cell subpopulations.
Methods: EV uptake by the different immune cell types within human PBMCs has been evaluated using two EV sources: MDA-MB-231 (triple negative breast carcinoma) and Jurkat (T lymphoma) and compared to the uptake of fluorescent beads. To track EV capture by the recipient cells, lipophilic MemGlow 488 was selected due to its ability to fluoresce only when incorporated in membranes. Multicolor spectral flow cytometry has been used to identify several immune cell populations. Identification of the immune cells uptaking EVs has been evaluated in 1) the whole population of immune cells or2) the specific cell types that uptake the TD-EV at different time points.
Results: EVs are uptaken to different levels by all immune cells whereas beads are only uptaken by CD14+ monocytes. We have identified the CD14+ population (including classical and intermediate monocytes) as the major uptaking cell type followed by dendritic cells, B-cells, non-conventional monocytes, NK cells, NKT cells and finally cytotoxic and helper T cells. Interestingly, incubation of PBMCs with TD-EVs, as opposed to beads, resulted in the appearance of an FSC-low CD14+ population with phosphatidylserine exposure
Summary/Conclusion: Although EVs and beads are mainly uptaken by CD14+ cells they lead to different effects on these cells. Moreover, we observed differences of some immune preferred cells for EVs from distinct sources Understanding the aspects and mechanisms involved in the EVs and immune system interactions will provide significant insights into immune modulation by cancer cells and the role of EVs in the tumor microenvironment.
Funding: This work was supported by the H2020-MSCA-ITN (722148, TRAIN-EV).

PT07.14 | Tumor-derived extracellular vesicles boost melanoma response to oncolytic therapy

Darshak Bhatt[1]; Silvina Bustos[2]; Toos Daemen[3]; Roger Chammas[4]; Luciana Nogueira de Sousa Andrade[4]
[1]University of Groningen/University of Sao Paulo, Groningen, Netherlands; [2]ICESP, Sao Paulo, Brazil; [3]University Medical Center Groningen/University of Groningen, Groningen, Netherlands; [4]Center for Translational Research in Oncology (LIM24), Instituto do Cancer do Estado de Sao Paulo (ICESP), Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo (HCF-MUSP), Sao Paulo CEP 01246-000, SP, Brazil, Sao Paulo, Brazil

Introduction: Tumor-derived extracellular vesicles (TEVs) are active players in cancer establishment, progression and therapeutic resistance. Recently, we demonstrated that TEVs released by melanoma cells upon chemotherapy promote tumor outgrowth through nuclear reprogramming of both tumor and immune cells. Based on this, we hypothesized that the biological action of TEVs may predict treatment outcome. Thus, our goal was to evaluate the possible pro- or anti-tumoral effects of melanoma TEVs secreted during the oncolytic virotherapy based on Semliki Forest Virus (SFV) replicons.
Methods: B16F10 murine melanoma cells were infected with SFV (MOI 10 of non-replicating virus replicons). After 24hs, TEVs were isolated by differential ultracentrifugation, according to Théry et al (2006), and characterized by the presence of CD9, CD63 and flotillin through WB, TEM and nanoparticle tracking analysis. TEVs uptake by naïve tumor cells was determined by fluorescent microscopy after PKH26 vesicle labeling. Splenocytes were extracted from mouse spleens and stimulated with PMA and ionomycin in the presence of TEVs from infected tumor cells.
Results: In response to SFV infection, we observed a significant increase in TEVs secretion by B16F10 cells which presented 150 to 350 nm diameter range. These vesicles were taken up by naïve tumor cells enhancing their clonogenic ability. Interestingly, the infectivity of SFV was augmented when naïve cells were pretreated with these TEVs. Concerning their role in immune cells, TEVs from infected cells promoted an enhancement in lymphocyte proliferation.
Summary/Conclusion: Our results suggest that TEVs released upon SFV infection can boost the oncolytic therapy promoting viral infectivity in naïve tumor cells and lymphocyte proliferation, remodeling the tumor microenvironment towards an antitumor state.
Funding: FAPESP (process n° 2020/09176-8).

PT08: Cancer II (Lessons Learned from Blood Samples)

Chair: Daniel Bachurski – University of Cologne, Faculty of Medicine and University Hospital Cologne, Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf, Mildred Scheel School of Oncology Aachen Bonn Cologne Düsseldorf, Center for Molecular Medicine Cologne, CECAD Center of Excellence on Cellular Stress Responses in Aging-Associated Diseases, Cologne, Germany

PT08.01 | A novel serum extracellular vesicle protein signature to monitor glioblastoma tumor progression

Katrin S. Reiners[1]; Theophilos Tzaridis[2]; Johannes Weller[3]; Daniel Bachurski[4]; Farhad Shakheri[5]; Christina Schaub[3]; Peter Hau[6]; Andreas Buness[5]; Uwe Schlegel[7]; Joachim-Peter Steinbach[8]; Clemens Seidel[9]; Roland Goldbrunner[10]; Niklas Schaefer[3]; Robert J. Wechsler-Reya[2]; Bjoern Scheffler[11]; Martin Glas[12]; Lothar Haeberle[13]; Ulrich Herrlinger[3]; Christoph Coch[1]; Gunther Hartmann[1]

[1]Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital, University of Bonn, Germany, Bonn, Germany; [2]Tumor Initiation & Maintenance Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, USA, La Jolla, San Diego, USA; [3]Division of Clinical Neurooncology, Department of Neurology, Center of Integrated Oncology Aachen-Bonn-Cologne-Düsseldorf, Partner Site Bonn, University of Bonn, Germany, Bonn, Germany; [4]University of Cologne, Faculty of Medicine and University Hospital Cologne, Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf, Mildred Scheel School of Oncology Aachen Bonn Cologne Düsseldorf, Center for Molecular Medicine Cologne, CECAD Center of Excellence on Cellular Stress Responses in Aging-Associated Diseases, Cologne, Germany, Cologne, Germany; [5]Institute for Medical Biometry, Informatics and Epidemiology, Institute for Genomic Statistics and Bioinformatics, Medical Faculty, University of Bonn, Germany, Bonn, Germany; [6]Department of Neurology and Wilhelm Sander NeuroOncology Unit, University Hospital Regensburg, Germany, Bonn, Germany; [7]Department of Neurology, University Hospital Knappschaftskrankenhaus, Ruhr–University Bochum, Germany, Bochum, Germany; [8]Dr. Senckenberg Institute of Neurooncology, University of Frankfurt, Germany, Frankfurt, Germany; [9]Department of Radiation Oncology, University of Leipzig, Germany, Leipzig, Germany; [10]Center for Neurosurgery, University of Cologne, Cologne, Germany, Cologne, Germany; [11]DKFZ-Division Translational Neurooncology at the West German Cancer Center, German Cancer Consortium, DKFZ Heidelberg & Partner Site University Hospital Essen, Germany, Essen, Germany; [12]Division of Clinical Neurooncology, Department of Neurology and West German Cancer Center, German Cancer Consortium, University Hospital Essen, Germany, Essen, Germany; [13]Erlangen University Hospital, Department of Gynecology and Obstetrics, Comprehensive Cancer Center Erlangen-EMN, Friedrich Alexander University of Erlangen–Nuremberg, Germany, Erlangen, Germany

Introduction: Detection of tumor progression in glioblastoma patients remains a major challenge for clinicians due to equivocal MRI results. Extracellular vesicles (EVs) are potential biomarkers and can be detected in the blood of tumor patients. In this study, we evaluated the potential of serum-derived EVs from glioblastoma patients to serve as a marker for tumor progression in adjunction with MRI assessment.

Methods: Glioblastoma patients from two independent cohorts, one from the multicenter Phase III CeTeG/NOA-09 trial (n=36) and the other from patients treated at the University of Bonn (n=31), were included in this study. EVs from serum of GB patients and healthy volunteers were separated by size exclusion chromatography and ultracentrifugation. EVs were characterized by multiple methods in accordance with MISEV2018. (EV Track ID: EV200097). Putative glioblastoma EV markers were defined by using a proximity-extension assay and bead-based flow cytometry. Tumor progression was defined according to modified RANO criteria.

Results: EVs from the serum of glioblastoma patients (n=67) showed an upregulation of CD29 (p=0.08), CD44 (p< 0.0001), CD81 (p< 0.0001), CD146 (p< 0.0001), C1QA (p=0.003), and histone H3 (p< 0.0001) as compared to serum EVs from healthy volunteers. For both independent cohorts of glioblastoma patients, we noted upregulation of C1QA, CD44, and histone H3 upon tumor progression, but not in patients with stable disease. Notably, six patients with worse survival compared to the median survival of the cohort did not fulfill RANO criteria at the time of suspected progression, yet showed an elevation of at least one out of these three markers. In a multivariable logistic regression analysis, a combination of CD29, CD44, CD81, C1QA, and histone H3 correlated with RANO-defined tumor progression with an AUC of 0.76.

Summary/Conclusion: Measurement of CD29, CD44, CD81, C1QA, and histone H3 in serum-derived EVs of glioblastoma patients, along with standard MRI assessment, could improve detection of true tumor progression and thus be a useful tool for clinical decision making.

Funding: This research was funded by the University of Bonn (scholarship awarded to TT) and the German Research Foundation (DFG) under Germany's Excellence Strategy, of which GH is a member.

PT08.03 | Effects of exercise-induced EVs on the progression of cancer

Aija Linē[1]; Lilite Sadovska[2]; Jānis Auders[3]; Laura Keiša[3]; Pawel Zayakn[3]; Alicia Llorente[4]; Zane Kalniņa[3]
[1]Latvian Biomedical Research and Study Centre, Riga, Latvia; [2]Latvian Biomedical Research and Study Centre, Riga, Latvia, Riga, Latvia; [3]Latvian Biomedical Research and Study Centre (BMC), Riga, Latvia; [4]Institute for Cancer Research, Oslo University Hospital, Oslo, Norway, Oslo, Norway

Introduction: Increasing evidence suggests that regular physical exercise not only reduces the risk of cancer but also improves functional capacity, treatment efficacy and disease outcome in cancer patients. We hypothesized that exercise-induced EVs may directly interact with cancer cells and alter their behavior and/or change the functional phenotype of circulating and tumor-infiltrating immune cells. Here, we report a pilot study investigating the effects of exercise-induced EVs on the progression of cancer in an F344 rat model of metastatic prostate cancer.
Methods: Plasma samples were collected before and after the exercise from rats subjected to regular forced wheel running exercise and sedentary rats. EVs were isolated using SEC and characterized by TEM and NTA. RNA content of EVs was studied by RNA sequencing analysis. The effects of exercise-induced EVs on the progression of cancer were studied in a syngeneic orthotopic prostate cancer model in rats.
Results: We did not observe a consistent increase in the circulating EV levels after the exercise, however, the RNA sequencing analysis demonstrated substantial changes in the RNA content of EVs collected before and immediately after forced wheel running exercise as well as differences between EVs from runners at resting state and sedentary rats. The major RNA biotype in EVs was mRNA, followed by miRNA and rRNA. Molecular functions of differentially expressed RNAs reflected various physiological processes including protein folding, metabolism and regulation of immune responses triggered by the exercise in the parental cells. Intravenous administration of exercise-induced EVs into F344 rats with orthotopically injected syngeneic prostate cancer cells PLS10, demonstrated reduction of the primary tumor volume by 35% and possibly – attenuation of lung metastases.
Summary/Conclusion: Our data provide the first evidence that exercise-induced EVs may modulate tumor physiology and delay the progression of cancer.
Funding: EEA and Norway Grant No. EEA-RESEARCH-164.

PT08.04 | Extracellular Vesicles GCC2 in Tumor-Draining Pulmonary Blood Plasma as an Informative Biomarker in Patients underwent Lung Cancer Surgery

Byeong Hyeon Choi[1]; Yu Hua Quan[2]; Jiyun Rho[2]; Kyungsu Kim[2]; Jun Hee Lee[2]; Yeonho Choi[3]; Yong Park[4]; Ji-Ho Park[5]; Sunghoi Hong[6]; Hyun Koo Kim[2]
[1]Korea Artificial Organ Center, Korea University, Seoul, Republic of Korea; [2]Department of Thoracic and Cardiovascular Surgery, Korea University Guro Hospital, College of Medicine, Korea University, Seoul, Republic of Korea; [3]Department of Bio-convergence Engineering, Korea University, Seoul, Republic of Korea; [4]Division of Hematology-Oncology, Department of Internal Medicine, Korea University College of Medicine, Korea University, Seoul, Republic of Korea; [5]Department of Bio and Brain Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Republic of Korea; [6]School of Biosystems and Biomedical Sciences, College of Health Science, Korea University, Seoul, Republic of Korea

Introduction: Early diagnosis is associated with a better prognosis and improved survival rates for lung cancer patients. Extracellular vesicles (EVs) of liquid biopsy are rapidly introduced in clinical practice, providing possibilities to offer accurate information. We previously reported that tumor-draining pulmonary vein blood (TDVP) derived EVs were increased than the periphery, and GRIP and coiled-coil domain containing 2 (GCC2) in EVs act as potential lung adenocarcinoma biomarkers. The purpose of this study is to evaluate the diagnostic value of EVs GCC2 in peripheral blood and TDPV in patients with lung cancer who underwent surgery.
Methods: The utilization of EVs GCC2 in the TDPV of lung cancer for clinically informative diagnosis was investigated using the rabbit lung cancer model (n = 4), healthy control rabbits (n = 4), patients with surgically resected lung adenocarcinoma (n = 70), and healthy controls (n = 30). EVs GCC2 levels were measured using an ELISA with ROC analysis were used to calculate the diagnostic efficiency of carcinoembryonic antigen (CEA), EVs CD63, plasma GCC2, and EVs GCC2.
Results: In the rabbit lung cancer model, the expression of EVs GCC2 from the TDPV significantly increased compared with that in the periphery, as determined by western blotting. The AUC values of CEA according to the blood sampling sites were not significant (all $p > 0.05$). By contrast, the AUC values of CD63 were 80.0% for TDPV ($p < 0.0001$) and 55.0% for the periphery ($p = 0.42$), respectively. The AUC values of plasma GCC2 in the periphery and TDPV were 68.0% ($p > 0.05$) and 74.0% ($p < 0.0001$), respectively. Importantly, the diagnostic accuracy values of EVs GCC2 in the periphery and TDPV were 81.0%

($p < 0.0001$) and 90.0% ($p < 0.0001$), respectively. Moreover, EVs GCC2 in the TDPV was associated with pathological stage ($p < 0.001$), lymph node metastasis ($p < 0.01$), and lymphatic invasion ($p < 0.05$).

Summary/Conclusion: EVs GCC2 in the TDPV were significantly correlated with pathological stages and accurate AUL value than that in the periphery. EVs GCC2 in TDPV may be a promising and clinically informative biomarker for patients with lung cancer who have undergone surgery.

Funding: This research was supported by the Korea Health Technology R&D Project (Grant No. HR14C0007) through the Korea Health Industry Development Institute, funded by the Ministry of Health & Welfare.

PT08.05 | Extracellular vesicles isolated from plasma of multiple myeloma patients treated with daratumumab express the myeloma marker CD38, the complement inhibitory proteins CD55 and CD59 and programmed dead ligand-1 (PD-L1)

kieran brennan[1]; Katrine Fladeland Iversen[2]; Alfonso Blanco[3]; Torben Plesner[2]; margaret mc Gee[4]
[1]University College Dublin, Dublin, Ireland; [2]Vejle Hospital, Vejle, Denmark; [3]Flow Cytometry Core Facility, Conway Institute, University College Dublin, Ireland, Dublin, Ireland; [4]University College Dublin, dublin, Ireland

Introduction: Extracellular vesicles (EVs) represent promising non-invasive biomarkers that may aid in the diagnosis and risk-stratification of multiple myeloma (MM), an incurable malignancy characterized by monoclonal expansion of plasma cells in the bone marrow. Daratumumab (DARA) is a CD38 antibody approved for treatment of MM, and despite the anti-tumour effects of DARA, the majority of patients eventually relapse. One mechanism of DARA resistance is the upregulation of complement inhibitory proteins CD55 and CD59, and programmed dead ligand-1 (PD-L1) on plasma cells. The aim of this study was to determine if EVs from peripheral blood plasma (PB) and bone marrow aspirates (BM) from patients treated with DARA also contained these resistance markers.

Methods: MM patients treated with DARA at Vejle Hospital or Odense University Hospital, Denmark participated in the study (n=50). EV isolation was performed on platelet free PB and BM samples by density gradient ultracentrifugation using a 50nm cut off for ultracentrifugations and collecting fractions within a density range of 1.08-1.2g/ml. EV size and number was determined by Cytoflex flow cytometry and expression of EV markers, the myeloma marker CD38, CD55 and CD59 and PD-L1 was determined by EV bead conjugated flow cytometry.

Results: Results reveal that all patient EV samples contain the EV markers and CD38, PD-L1, CD55 and CD59. The complement inhibitory proteins CD55 and CD59 are present at higher levels in MM EVs relative to healthy patient EVs. Moreover, patients with matched bone marrow samples had similar CD38 levels on their bone marrow and plasma EVs suggesting that peripheral blood EVs reflect MM bone marrow plasma cells following DARA treatment.

Summary/Conclusion: Overall, this study provides proof of concept that blood plasma EVs represent a minimally invasive approach for monitoring patient response to Daratumumab and contributes towards the implementation of liquid biopsy for personalised patient care.

PT08.06 | Fucosylated integrins expressed on serum derived-EVs enable detection of bladder cancer

Misba Khan; Khirul Islam; Janne Leivo; Urpo Lamminmäki
University of Turku, Turku, Finland

Introduction: Bladder cancer (BlCa) is the fifth most commonly occurring cancer in European countries. The current diagnosis of BlCa relies upon cytology and cystoscopy. However, these methods have low sensitivity and specificity to low-grade tumors. Therefore, there is a need for new and improved diagnostic tools that could help in the prognosis and management of BlCa. Extracellular vesicles (EVs) have the potential to act as a source of novel biomarkers for various malignancies. However, isolation of EVs from body fluids and their characterization is difficult and time-consuming. The aim of our study was to develop a simple EV-based assay for non-invasive detection of BlCa using a highly sensitive nanoparticle-aided time-resolved fluorescence immunoassay (TRFIA).

Methods: EVs from the serum of bladder cancer (BlCa), benign and healthy samples were captured with biotinylated anti-integrin-antibody (ITGA3) immobilized on streptavidin coated microtiter wells. The captured EVs were detected using europium doped nanoparticles (polysterene beads packed with ~30,000 Eu3+ chelates) conjugated with anti-integrin-antibody (ITGA3) and fucose binding lectin (UEA). Isolated EVs-derived from cancer cell lines were used as analytical standards in the TRFIA. Serum samples from BlCa (n=58), benign (n=53), and healthy (n=14) individuals were analyzed. This study was conducted following the guidelines of Helsinki Declaration.

Results: After profiling of several integrin antibodies and our lectin library, we have found that integrin (ITGA3) and fucose binding lectin (UEA) were most prominently expressed on cancer cell-lines derived EVs. The sensitivity of glycovariant ITGA3-UEA assay for the detection of EVs was 2-fold higher compared to total ITGA3-ITGA3 assay. Eventually, ITGA3-UEA assay enabled significant discrimination of BlCa patients from benign (2.3-fold, p= 0.0001) and healthy controls (70-fold, p= 0.00001).
Summary/Conclusion: This study suggests that TRFIA based ITGA3-UEA assay may help to discriminate BlCa patients from patients with clinically challenging benign conditions in a non-invasive manner directly from human serum. However, further studies with larger cohort of samples need to be conducted to validate the results.

PT08.07 | Multiplex phenotyping of extracellular vesicles for analysis of potential biomarkers in glioblastoma patients

Amanda Salviano-Silva; Kathrin Wollmann; Cecile Maire; Katharina Kolbe; Katrin Lamszus; Franz Lennard. Ricklefs
Department of Neurosurgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Introduction: Extracellular vesicles (EVs) carry biological information from their cell of origin that is useful for non-invasive detection of tumor biomarkers and disease monitoring. In glioblastoma (GBM), blood circulating EVs are elevated and carry GBM-associated proteins. However, it is still challenging to analyze tumor derived EVs for translational purposes. Here, we used imaging flow cytometry (IFCM) as a robust strategy to perform phenotyping of EVs with GBM related surface markers in human plasma.
Methods: EVs were isolated via differential ultracentrifugation from plasma of (a) 40 GBM patients, pre- and post-surgery, (b) 11 matched GBM relapses and (c) 12 healthy donors (HD). EV sizes and concentrations were evaluated by NTA. EV markers (CD9, CD63 and CD81) together with glioma-related markers (integrin beta-1 [ITGB1], tenascin C [TNC], Profilin-1 [PFN1], CD44, GPNMB, SPARC, HLA-II or CD133) were analyzed by IFCM. EV percentages and objects/mL plasma were compared among the groups and correlated with clinical parameters.
Results: CD9 was the predominant tetraspanin in all groups (15-96%), while CD63 had the lowest levels (0-33%) and the strongest decrease in GBM patients after surgery (fold change [FC] = -5.4, $p < 0.01$). Among the glioma-related markers, ITGB1 and TNC displayed the most significant differences between the analyzed groups, especially the double positives ITGB1+/CD63+ and TNC+/CD63+, which decreased in patients after tumor removal (FC = -3.5 and -12, respectively; $p < 0.001$). Meanwhile, ITGB1+/CD9+ and TNC+/CD9+ EVs exhibited the highest levels in GBM when compared to HD subjects (FC = 8.6 and 17.4; $p < 0.001$) and upon tumor recurrence (FC = 3.7 and 10.9, respectively; $p < 0.01$).
Summary/Conclusion: We identified EV surface antigens with potential clinical utility as GBM biomarkers. Among them, we highlight ITGB1 and TNC as the most promising markers, which are currently being further investigated by our group.
Funding: Deutsche Forschungsgemeinschaft.

PT08.08 | Quantitative proteomic analysis of pancreatic cancer tissue-derived extracellular vesicles to identify biomarker candidates

Nasibeh Karimi[1]; Caroline Vilhav[2]; Cecilia Lässer[1]; Jan Lötvall[1]
[1]*Krefting Research Centre, Institute of Medicine at Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, Gothenburg, Sweden;* [2]*Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy at the University of Gothenburg, Sahlgrenska University Hospital, Gothenburg, Sweden., Gothenburg, Sweden*

Introduction: Proteomic analysis of tissue-derived extracellular vesicles (EVs) has shown promising results in identifying biomarkers and in uncovering mechanisms of cell-cell communication in cancerous tissues. Our aim was therefore to firstly determine whether EVs could also be isolated from pancreatic tissue and if so secondly to determine the proteome of tissue-derived vesicles from pancreatic cancer (PC) patients.
Methods: EVs were isolated from both tumor and non-tumor tissue of eleven PC patients, undergoing pancreatic cancer surgery according to our previously published protocol (Crescitelli et al JEV 2020). EVs were further characterized by Nanoparticle Tracking Analysis (NTA) and Western blotting (Wb). Proteomics analysis was then performed on tissue-derived EV samples (a total of 22 samples divided into two sets; set 1 (6 samples) and set 2 (16 samples) by quantitative mass spectrometry analysis to identify potential biomarkers for PC. To identify pathways and functional annotation of differentially expressed proteins, pathway enrichment analysis using Gene Ontology, Kyoto Encyclopedia of Genes and Genomes, and Gene Set Enrichment Analysis was performed.
Results: The NTA analysis revealed a 1.5-fold higher concentration of particles in tumor tissue-derived EVs compared to non-tumor tissue-derived EVs, even though the size of the particles appeared to be similar. Western blot confirmed expression of

the EV-specific markers CD63, CD81, CD9, and TSG101. A total of 6113 and 4331 proteins were quantified in set 1 and set 2, respectively. The GO analysis for cellular colocalization indicated that "Extracellular Exosome" was the top GO term associated with the proteins identified in both set 1 and set 2. In total, 1010 and 399 were differentially expressed between tumor tissue-derived EVs and non-tumor tissue-derived EVs in set 1 and set 2, respectively ($p < 0.05$; fold change > 1.5). KEGG pathway analysis indicated that "Metabolic pathways" was the top pathway associated with the proteins quantified in both non-tumor tissue-derived EVs and tumor tissue-derived EVs in set 1 and set 2.

Summary/Conclusion: As a result of the comprehensive proteomic analysis, several potential protein PC-biomarkers have been identified. However, further validation in clinical trials is needed.

PT08.09 | Characterization of EV RNA cargo from primary and telomerase immortalized MSCs using a novel small RNA-seq workflow enabling absolute RNA quantification

Marianne Pultar[1]; Matthias Wieser[2]; Zahra Mazidi[2]; Johanna Gamauf[2]; Andreas Diendorfer[1]; Johannes Grillari[3]; Regina Grillari[2]; Matthias Hackl[1]

[1]TAmiRNA GmbH, Vienna, Austria; [2]Evercyte GmbH, Vienna, Austria; [3]Ludwig Boltzmann Institute of Traumatology in Cooperation With AUVA, Vienna, Austria., Vienna, Austria

Introduction: EVs released from MSCs can elicit anti-inflammatory effects and are therefore of high interest for therapeutic use. The production of therapeutic EVs requires well-characterized, robust and reproducible host cells. Telomerase-immortalized MSCs have the advantage to fulfill these criteria by continuous growth and maintenance of cell type specific characteristics. Small ncRNAs including miRNAs control gene expression and are required to maintain cell differentiation status. Small RNAs are loaded into EVs resulting in distinct EV-RNA profiles representative of their cellular origin and status. It is unclear whether telomerase immortalization elicits significant changes in EV-RNA profiles indicating divergent cellular or EV status. Here, we used a novel small RNA-seq assay for genome-wide characterization of the EV small RNA cargo of 2D cultivated Wharton´s Jelly derived primary and the corresponding telomerized MSCs (WJ-MSC/TERT273).

Methods: MSCs (n=3 per group) were cultivated in MesencultTM medium on ACF coating (StemCell Technologies) and EV sized particles were harvested after 48 hours followed by TFF enrichment. Exactly 109 particles were used for total RNA extraction. Small RNA-seq libraries were generated from 8.5 μl RNA to which a set of 7 spike-in oligonucleotides harboring a unique 13mer core and 4N-randomized ends was added at defined attomolar concentrations (0.01 – 20 amol) to enable extensive quality control and absolute normalization.

Results: On average 25 Mio reads were generated per MSC EV-sized particle sample. Read classification identified similar EV-RNA profiles for primary and hTERT MSCs (~1% miRNA, ~10% tRNA, ~2% piRNA, ~20% rRNA, ~1% lncRNA, ~3% mRNA, ~8% other small RNAs). On average 500 distinct miRNAs were detected per sample and copy numbers per 1000 particles were calculated using the spike-in calibrator. A similar distribution of miRNA copy numbers was observed in primary and hTERT MSC EV-sized particles ($p>0.05$). Differential expression analysis (edgeR, FDR< 0.05) was performed on 214 miRNAs that passed independent filtering (DESeq2). Eight up-regulated miRNAs were identified in hTERT MSC EVs, including miR-138 and miR-424-5p, which target hTERT and act as tumor suppressors.

Summary/Conclusion: Using small RNA-seq we established that the EV-RNA profiles between primary and hTERT-MSCs are highly similar suggesting comparable cellular state and EV identity. We identified two miRNAs to be elevated in hTERT-MSC EVs that are capable of silencing hTERT expression.

PT08.10 | Exosome gene expression technology to understand human health risks in space exploration: applications for SANS

Dulaney L. Miller[1]; Sudipto K. Chakrabortty[1]; Yevgenia L. Khodor[1]; Steven P. Lang[2]; Robert R. Kitchen[3]; Seth Yu[1]; Eric Bershad[4]; Johan Skog[5]; Susana Zanello[6]

[1]Exosome Diagnostics, a Bio-Techne brand, Waltham, USA; [2]Exosome Diagnostics, a Bio-Techne brand, Boston, USA; [3]Massachusetts General Hospital, Boston, USA; [4]Department of Neurology, Baylor College of Medicine, Houston, USA; [5]Exosome Diagnostics, a Bio-techne brand, Waltham, MA, Waltham, USA; [6]Johnson Space Center, National Aeronautics and Space Administration, Houston, USA

Introduction: Spaceflight-Associated Neuro-ocular Syndrome (SANS) consists of a set of ocular structural and visual manifestations associated with prolonged exposure to microgravity. While it has been hypothesized that elevation of intracranial pressure (ICP) may be associated with SANS, ICP has not been fully examined in astronauts. We performed a transcriptome-wide profiling of exosomal RNA obtained from high-ICP patients to uncover the underlying processes possibly associated with SANS.

Methods: Plasma, PAXgene whole blood, CSF, and urine were obtained from a group of high-ICP patients, in sample volumes of 2, 2.5, 3, and 20mL, respectively, for the isolation of EV RNA using an optimized ExosomeDx Exolution platform. Total RNA-seq library construction and sequencing were performed with a proprietary EV long RNA-seq workflow. A DEX analysis was conducted alongside a GSEA to see whether these genes could be grouped for further investigation.
Results: Between high-ICP and healthy control samples, three differentially expressed genes were detected in PAXgene whole blood and 185 genes were detected in plasma, likely due to more diversity of RNA targets in plasma EVs. We found 826 genes expressed at higher levels in healthy CSF and 143 more highly expressed in high-ICP CSF. In urine, 120 genes were over-expressed and 89 genes under-expressed in controls versus post-treatment high-ICP patient samples.
Summary/Conclusion: Profiling of the exosomal RNA in four biofluids revealed plasma as the most informative from a diversity of physiological systems, while urine and CSF carry a gene enrichment in the urinary tract and nervous and immune systems, respectively. Biological processes for genes highly expressed in CSF of high-ICP patients are heavily implicated in immune responses, suggesting that high-ICP patients have a significant involvement of immune-associated gene products. A greater sampling of subjects is underway to better understand the significance of these gene sets and their implication in SANS.

PT08.11 | Small RNA sequencing of amniotic fluid-derived extracellular vesicle cargo reveals diverse RNA types

Michael A. Bellio[1]; Cassie Bennett[2]; Maria Ines Mitrani[1]
[1]Organicell Regenerative Medicine, Miami, USA; [2]Organicell Regenerative Medicine, Inc., Miami, USA

Introduction: Amniotic fluid (AF) has recently been characterized to contain a reserve of extracellular vesicles (EVs) with anti-inflammatory properties. AF derived-EVs are actively under investigation in the clinic as a therapeutic for inflammatory diseases. Therefore, the identification of the molecular cargo of EVs is important to understand their mechanism of action(s) and thus clinical potential. Here we present the results of a small RNA sequencing experiment that identified several subclasses of RNA present in this EV type.
Methods: AF was collected from consenting adults during planned, full term cesarean sections. Raw AF was filtered and ultracentrifuged to precipitate the EV population. Small RNA sequencing was performed by SBI's EXO-NGS exosomal RNA sequencing service (SBI, Palo Alto, CA.). Quality assessment, read alignment, and abundance determination was performed using the Banana Slug Genomics center analysis. EVs isolated from 3 donors were sequenced in replicates (n=3) to determine the expressed RNA types and IDs. The total read counts of each RNA type were calculated to determine their proportion relative to the total read counts of that sample. The proportions of each RNA type were calculated from the mean proportions (n=3) calculated from each 3 unique donors.
Results: The RNA sequencing analysis identified 21 different classes or types of RNA, such as miRNA, lnRNA, tRNA, rRNA, and piRNA. The tope 4 most abundant types of RNA included RefSeq introns (14.9%), miRNA (14.3%), anti-sense to introns (14.2%), and anti-sense to repeat elements (13.9%). Smaller proportions of RNA types included tRNA (3.6%), rRNA (1.0%), and piRNA (0.7%). Examples of top expressing miRNA include miR-30d-5p, let7a-5p, and miR-26a-5p.
Summary/Conclusion: AF-derived EVs contain different RNA types that could contribute to its mechanism(s) of action. Further research is underway to determine the primary RNA molecules involved in its anti-inflammatory properties.

PT09: Therapeutics: EVs for drug delivery/EV production

Chair: Dimitrios Tsiapalis – School of Pharmacy and Pharmaceutical Sciences & Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland

Chair: Alex Forterre, EVORA Biosciences

PT09.01 | Biodistribution of colostrum exosomes and tumor targeting of exosomal-paclitaxel formulation against lung cancer

Farrukh Aqil[1]; Raghuram Kandimalla[2]; Jeyaprakash Jeyabalan[3]; Disha Moholkar[2]; Wendy Spencer[3]; Ramesh C. Gupta[4]
[1]University of Louisville, Louisville, USA; [2]University of Louisville, Louisville, KY 40202, Louisville, USA; [3]3P biotechnologies Inc., Louisville, KY 40202, Louisville, USA; [4]3P biotechnologies and Department of Pharmecology & Toxicology and Brown Cancer Center, University of Louisville, Louisville, KY 40202, Louisville, USA

Introduction: Bovine milk and colostrum contain abundant exosomes and these small extracellular vesicles have been used for oral delivery of small molecules and nucleic acids. In this report, we demonstrate biodistribution and targeting of orthotopic lung tumors by folic acid-functionalized (FA) colostrum exosomes, enhanced sensitivity of drug-resistant lung cancer cells to exosomal formulation of paclitaxel (ExoPAC), and inhibition of lung tumors by oral administration of FA-functionalized ExoPAC.
Methods: Exosomes were isolated from bovine colostrum powder by rehydration and differential centrifugation and characterized for size by Zetasizer and TEM and hallmark protein markers by Western blot. For biodistribution, orthotopic lung tumor-bearing mice were treated orally with exosomes and FA-exosomes labeled with Alexa Fluor-750 dye and various tissues were imaged ex vivo. Drug-sensitive and drug-resistant A549 lung cancer cells were treated with ExoPAC and free PAC and cell survival was measured by MTT assay. For antitumor activity, orthotopic lung tumor-bearing mice were treated orally with three doses a week of ExoPAC, FA-ExoPAC or vehicle; free PAC was given intravenously once weekly. Tumor growth was monitored weekly.
Results: We found 1) AF750 signals in all tissues including the lung embedded with the tumors; however, a significantly higher (>2-fold) accumulation occurred in the tumor-bearing lung tissue with the functionalized exosomes compared with non-functionalized exosomes; 2) while the drug-sensitive A549 lung cancer cells were equally sensitive to free PAC and ExoPAC, the drug-resistant A549 cells showed sensitivity only to ExoPAC; and 3) the lung tumor growth inhibition was in the following descending order: FA-ExoPAC (55%; $p < 0.001$), ExoPAC (36%) and PAC (24%).
Summary/Conclusion: Orally delivered FA-functionalized exosomes accumulate in lung tumors and FA-ExoPAC formulation given orally exhibit significantly higher inhibition of lung tumors compared with non-functionalized ExoPAC and free drug. Thus, oral delivery of paclitaxel, a widely used chemotherapeutic drug against various cancers, provides a user-friendly, cost-effective alternative of the traditional bolus dose intravenous therapy which carries severe side effects.
Funding: Supported from the USPHS grant CA221487-01.

PT09.02 | Breast cancer extracellular vesicles transfer immune cargo following oncolytic virotherapy

Hyfa Alzahrani; munitta Muthana
university of sheffield, Sheffield, United Kingdom

Introduction: Breast cancer (BC) is the most common type of cancer in females in the UK. Novel treatments are required to treat radio-/chemo-resistant BC as well as advanced disease. Oncolytic viruses (OV) are naturally cytotoxic and infect and kill tumour cells whilst sparing healthy tissues. The full mechanism by which this occurs remains to be elucidated, but it may in part be mediated by extracellular vesicles (EVs). EVs are nanosized, membrane-enclosed vesicles that contain molecular cargo. EVs can be taken up by cells, for instance immune cells, at local or distant sites, causing phenotypic changes in the recipient cells.
Methods: The breast cancer cells lines MCF-7 and MDA-MB-231 were infected with the herpes simplex virus (HSV1716). EVs were isolated from the OV conditioned medium of infected cells and control cells by differential centrifugation. Nanoparticle tracking analysis (NTA) was used to detect the overall size and concentration of EVs. Transmission electron microscopy (TEM) was used to confirm the size and shape of the EVs and western blotting to detect well known EV protein markers as well as oncolytic viral cargo.
Results: So far, our study revealed that exposure of the BC cell lines to HSV1716 resulted in increased release of EVs in comparison to the control untreated cells as detected by NTA. Released EVs were typically 50-150nm in diameter and appeared to be typically cup-shaped structures as revealed by TEM. Western blotting showed the presence of the EV biomarkers, CD9 and CD63 in both the untreated and HSV1716 treated cells, whilst the presence of HSV released antigens was only detected in the EVs from infected cells.
Summary/Conclusion: In summary, HSV1716 induced increased EV release from infected breast cancer cells suggesting OV influences EV shuttling. Current studies are aimed at characterising the contents of the EVs by real time PCR and mass spectrometry. This will help identify the presence of immunological and viral cargo. Future studies will aim to investigate the antitumour properties of EVs of from infected cells.

PT09.03 | Defining the effects of electroporation drug loading protocols on extracellular vesicle integrity

Malika Singh; Ghazaleh Mazaheri-Tehrani; Ignacio Martin-Fabiani; Mark P. P. Lewis; Owen G. Davies
Loughborough University, Loughborough, United Kingdom

Introduction: Extracellular vesicles (EVs) are potentially efficient drug delivery systems. Electroporation has been applied for active EV-drug loading. However, protocols applied often lack standardisation and their effects on EV integrity is unknown. Defining these effects may determine the effectiveness of electroporation for EV loading and identify optimal protocols.
This study investigated the effects of varying electroporation parameters (voltage, pulse number and width) on zeta potential, total and surface protein concentration and EV morphology. This study aimed to optimise an effective EV-drug loading protocol whilst maintaining particle integrity.
Methods: EVs were isolated from C2C12 murine myoblasts using ultracentrifugation. EV recovery was validated per MISEV guidelines using Nanoparticle Tracking Analysis (NTA), Bicinchoninic Protein Assay (BCA), transmission electron microscopy and Western Blotting. The Neon Transfection System (Thermo Fisher Scientific) was used to electroporate EVs at voltages 500V and 1000V for 20 milliseconds using 3 pulses. Varied pulse numbers and pulse widths were also explored. Zeta potential was measured to identify surface charge variations. Surface and internal protein concentrations were measured using the BCA assay. Nano flow cytometry was applied to determine changes in surface tetraspanins.
Results: Preliminary results suggest a charge increase from -12.97mV (control) to -6.06mV and -4.75mV for voltages 500V and 1000V, respectively. Surface protein concentration variations suggest an average 59% decline from 1281.49μg/mL, to 515.87μg/mL and 516.37μg/mL for voltages 500Vand 1000V, respectively.
Summary/Conclusion: These results suggest that electroporation has a negative impact on common EV parameters which may affect the downstream capacity of EV-drug complexes to induce a therapeutic response. Hence, identifying a need for optimising effective exogenous loading protocols and evaluating EV integrity following electroporation.

PT09.04 | Development of a red blood cell nanoparticle -derived drug delivery platform

Juha Prittinen[1]; Kai Härkönen[2]; Heikki Saari[2]; Ulla Impola[2]; Petra Ilvonen[3]; Saara Laitinen[2]
[1]University of Eastern Finland, Finnish Red Cross Blood Service, Helsinki, Finland; [2]Finnish Red Cross Blood Service, Helsinki, Finland; [3]Finnish Red Cross Blood Service, University of Helsinki, Helsinki, Finland

Introduction: The use of artificial EV-like particles instead of natural EVs offers an easier way to gain enough material for drug-delivery purposes. We are utilizing red blood cells (RBCs), which provide a plentiful and easily processable starting material for EV-like nanoparticle production. EV-like nanoparticles combined with peptide targeting ligands could serve as an efficient tool for drug delivery. The main goal is to develop a biocompatible RBC-based drug carrier product with a homing ability through the vascular endothelium to specific tissues and cancer types.
Methods: Ghosted RBCs are isolated by centrifugation. The nanoparticles are produced by 200 nm extrusion and analysed with NTA and CryoEM. Nanoparticles are loaded by either hypotonic explosion or electroporation. Peptides will be linked with copper-free click chemistry to the nanoparticle surface. Surface modifications will be analysed with thin layer chromatography, Raman spectroscopy and flow cytometry.
Results: Extrusion seems to be better than sonication for generation of uniform nanoparticles out of ghosted and non-ghosted RBCs. Loaded RBCs with lumen specific fluorescent probes are retained through extrusion. Unmodified RBC-derived nanoparticles do not readily penetrate the blood-brain barrier (BBB) in vitro.
Summary/Conclusion: RBC nanoparticles could be utilized for drug delivery, but it seems that surface modification of RBC nanoparticles will be necessary for penetrating the vascular endothelium and the BBB. RBC membrane could potentially protect delicate cargo from degradation. RBC nanoparticles may be a more biocompatible system for drug delivery than current alternatives.
Funding: Business Finland ecosystem funded project EVE – Ecosystem Consortium with four research organizations and nine companies
Academy of Finland – GeneCellNano Flagship
Orion Research Foundation – Production and systemic effect mechanisms of red blood cell derived vesicles

PT09.06 | Engineering nanoexosomes for targeted drug delivery for ovarian cancer

Aayushi Ghodasara[1]; Carlos Salomon Gallo[2]; Amirali Popat[1]
[1]School of Pharmacy, University of Queensland, Brisbane, Australia; [2]The University of Queensland, Brisbane, Australia

Introduction: Ovarian cancer is the 8th most common cause of cancer mortality in women globally, the high mortality rate owing to unfavourable biodistribution, low penetration and rapid clearance of therapeutics. Biomimetic extracellular vesicle (EV)-encapsulated mesoporous silica nanoparticles (MSNs) can be ideal therapeutics by combining target-homing capacity of

EVs with high drug loading capacity of MSNs. Here, we propose construction of EV-coated MSNs as drug carriers for ovarian cancer therapy.
Methods: EVs were derived from SKOV-3 cell lines using differential ultracentrifugation. SKOV-3 cells were subjected to serum starvation and the serum-free cell conditioned media was collected after 24 hours. The cell conditioned media was subjected to differential centrifugation at increasing speeds for prolonged duration, i.e. 2000xg for 20 minutes to remove cell debris, 10000xg for 40 minutes to remove larger vesicles and apoptotic bodies, and finally at 100000xg for 90 minutes to pellet the EVs.
Amino-functionalized mesoporous silica nanoparticles (MSNs); in three different sizes (50 nm, 85 nm , and 100 nm); were synthesized using previously established methods. The MSNs were loaded into the EVs using three different methods, double extrusion, extrusion followed by incubation: and sonication. The morphology of the EV-coated MSNs was observed with transmission electron microscopy (TEM) and the physical properties were characterized using Nanoparticle tracking analysis (NTA), zeta potential measurement. Biomarker analysis of EVs, MSNs and EV-coated MSNs was performed using Exoview.
Results: EV-coated amino functionalized MSNs were successfully synthesized using the stated methods. Double extrusion improved the association of EVs with NPs compared to sonication of extrusion followed by incubation, resulting in more evenly sized EV-associated amino functionalised silica NPs, as evidenced from NTA measurements. TEM images showed complete association of the EVs around the nanoparticles. Zeta potential measurements demonstrated the reversal of positive charge of amino-functionalized MSNs to a negative charge imparted by the associated EV membrane. Exoview analysis showed that the EV-coated MSNs were CD63 positive indicating EV association around the MSNs.
Summary/Conclusion: We report the successful synthesis of EV-coated amino-functionalized mesoporous silica nanoparticles using three different methods. These biomimetic particles could significantly enhance the delivery of therapeutics to ovarian cancer tumours owing to their low immunogenicity, biocompatibility, and target homing capacity. Future studies will assess their gene delivery potential and efficacy of the particles in vitro.
Funding: Funding from Medical Research Future Fund (MRFF), under the National Health and Medical Research Council (NHMRC), Australia

PT09.07 | EV isolated from human dental stem cells as drug delivery vehicles for neurological diseases

Anne des Rieux[1]; Viridiane Gratpain[1]; Ludovic d'Auria[1]; Pauline Bottemanne[1]; Romano Terrasi[1]; Vincent van Pesch[2]; Giulio G. Muccioli[1]
[1]UCLouvain, Bruxelles, Belgium; [2]UCLouvain_Saint Luc Hospital, Bruxelles, Belgium

Introduction: Most central nervous system (CNS) affections are unmet medical needs, one reason being the limited accumulation of drugs in the CNS. Nose-to-Brain administration provides a direct access to the brain while encapsulation in nanomedicines protects drugs and increases their half-live. As drug delivery systems, EV are able to cross epithelial barriers, are non-immunogenic and have an intrinsic activity. In this work, our objective was to isolate and characterize EV from human dental mesenchymal stem cells (SCAP) to later use them as nanomedicines in the scope of multiple sclerosis.
Methods: EV were isolated from SCAP culture medium by centrifugation, ultrafiltration and size exclusion chromatography (SEC). Impact of ultrafiltration unit cut-off (30 and 100 kDa) and SEC (Izon qEV 70 and 35 nm) on EV yield (NTA, ZetaView) and separation from proteins (DELFIA immunoassay) was evaluated. MiRNA content (small RNAseq) and lipid composition (HPLC-MS) of EV produced by SCAP in pro-inflammatory condition (activated SCAP) vs steady-state were compared. Finally, the influence of EV on pro-inflammatory marker gene expression of microglial cells (BV2 cells) was evaluated (RT-qPCR).
Results: Using a cut-off of 30 kDa and the qEV 35 nm SEC provided the highest number of EV while eliminating most of the contaminating proteins. EV were negative for calnexin and positive for CD9, CD63, CD81 and flotillin. 236 miRNAs, associated mainly with MAPK, neurotrophins and cancer pathways, were identified in steady-state EV while 44 miRNAs were significantly affected in EV produced by activated SCAP. Lipidomic analysis is ongoing. EV obtained from activated SCAP induced a slight reduction of IL-6 and iNOS in LPS-treated BV2 cells. Incorporation of bioactive drugs in SCAP EV is ongoing.
Summary/Conclusion: We optimized the isolation of EV from SCAP, analyzed how their composition was influenced by the activation state of SCAP, and studied their impact on neuroinflammation. Then, bioactive molecules will be encapsulated in these EV and delivered to the CNS.
Funding: This work has been funded by the FNRS, the Fondation Charcot Stichting and by the Belgian French Community (ARC; EViMS).

PT09.10 | Isolation of extracellular vesicles from the HEK293F cell line using CD81 affinity for their future use as nanodrug carriers

Berglind E. Benediktsdóttir[1]; Einar Gunnlaugsson[2]; Jens G. Hjörleifsson[3]
[1]Faculty of Pharmaceutical Science, School of Health Sciences, University of Iceland, Reykjavík, Iceland; [2]Industrial Biotechnology, School of Engineering and Natural Sciences and School of Health Sciences, University of Iceland, Reykjavik, Iceland; [3]Industrial Biotechnology, School of´Engineering and Natural Sciences and School of Health Sciences, University of Iceland, Reykjavik, Iceland

Introduction: Extracellular vesicles (EVs) have attractive properties as nanodrug carriers, namely the ability to carry labile molecules and relatively long circulation half-life. Additionally, their parent cell line can be engineered to express surface ligands for active targeting in cancer therapies, subsequently conveyed to their EVs. The suspension adapted HEK293F cell line is interresting for EV isolation, as it can be engineered to express desired surface ligands and cultured in serum free medium. The aim of this study was to determine if CD81 Fab-TACS affinity capture was applicable in isolating nanocarrier-grade EVs from this cell line
Methods: The HEK293F cells were cultured in serum free medium (FreeStyle™ 293) supplemented with antibiotics in tubespin bioreactors. EVs were isolated with CD81 Fab-TACS affinity capture (IBA) according to manufacturers protocol. NanoSight (NS300) was used for particle concentration and size determination: pump speed 100, camera level 15, 60 sec capture, four repeats. The sample was then concentrated with an Amicon filter (10 kDa) for subsequent capillary western blotting (WB) analysis with anti-Alix (1:50, Novus NBP1-49701).
Results: Media originating from 1.04*10^8 cells (viability 93%) was collected and isolated via the CD81 affinity column. This resulted in 1,2 ml of medium containing particles with mean size of 131.0 ± 1.5 nm and 3.34 ± 0.20 * 10^8 particles/ml. WB of the particles showed a band at 62 kDa corresponding with the EV protein marker Alix, suggesting positive isolation of EVs.
Summary/Conclusion: The affinity-based capture of EV based sample is promising although quantification of impurities from the elution stage in the affinity capture needs to be carried out for downstream use. Yet, this cell line and this isolation strategy will be useful in future work isolating engineered EVs as nanosized drug delivery vesicles.
Funding: University of Iceland Research Fund and "Göngum saman" Research Fund is gratefully acknowledged.

PT09.11 | mRNA cancer vaccines based on monocytes extracellular vesicles exogenously loaded with antigen mRNA

Cristina Fornaguera[1]; Cristina P R Xavier[2]; Mònica Guarro[3]; Sara Peixoto da Silva[4]; Martí Lecina i Veciana[5]; Salvador Borrós[6]; M. Helena Vasconcelos[7]
[1]GEMAT- Grup Enginyeria de Materials. Institut Químic de Sarrià. Universitat Ramon Llull; Cancer Drug Resistance Group, IPATIMUP—Institute of Molecular Pathology and Immunology and i3S—Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Barcelona, Spain; [2]Cancer Drug Resistance Group, IPATIMUP—Institute of Molecular Pathology and Immunology and i3S—Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal; [3]1Grup d'Enginyeria de Materials (Gemat), Institut Químic de Sarrià (IQS), Universitat Ramon Llull (URL), Barcelona, Spain; [4]Cancer Drug Resistance Group, IPATIMUP—Institute of Molecular Pathology and Immunology, and i3S—Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal; [5]GEMAT- Grup d'Enginyeria de Materials, Institut Químic de Sarrià, Universitat Ramon Llull, Barcelona, Spain; [6]GEMAT- Grup Enginyeria de Materials. Institut Químic de Sarrià. Universitat Ramon Llull, Barcelona, Spain; [7]Cancer Drug Resistance Group, IPATIMUP—Institute of Molecular Pathology and Immunology, i3S—Instituto de Investigação e Inovação em Saúde, and Department of Biological Sciences, FFUP—Faculty of Pharmacy of the University of Porto, Porto, Portugal

Introduction: In addition to prophylaxis of infectious diseases, vaccines could play a role in cancer immunotherapy. Our work proposes the design of mRNA therapeutic cancer vaccines based on extracellular vesicles (EVs) from monocytes. EVs have advantages over synthetic carriers: they occur naturally; transfer their cargo to recipient cells; and are naturally loaded with biological molecules.
Methods: EVs were isolated from human and murine monocyte cell lines, by differential centrifugation, and characterized by transmission electron microscopy (TEM), Western blot (WB), and nanoparticle tracking analysis (NTA). EVs were loaded with two model mRNAs (GFP as a reporter or with a model antigen) by passive diffusion. The mRNAs loading and stability, after EVs incubation with RNases, were assessed by fluorescence measurement of labelled mRNA. Finally, in vitro preliminary studies to evaluate the uptake of EVs and mRNA expression by recipient cells were performed, by flow cytometry and fluorescence microscopy.
Results: EVs were characterized using three techniques. NTA and TEM confirmed EVs sizes below 200 nm. TEM showed an EVs homogeneous population, with the expected morphology. WB confirmed the presence of EV-related proteins. Importantly, mRNA loading efficiencies into EVs higher than 75% were found, which enabled stable mRNA levels for longer than 6h, in the

presence of nucleases. Finally, in vitro experiments confirmed that fluorescently-labelled mRNAs loaded into EVs can penetrate and be expressed in recipient cells.
Summary/Conclusion: Preliminary results support the idea of using EVs as mRNA vaccines, given the ability of mRNA artificially-loaded EVs to penetrate recipient cells, which can then express the delivered mRNAs.
Funding: European COST Action- STRATAGEM CA17104 (short-term mission fellowship) and Grupo Español de Investigación en Vesículas Extracelulares (GEIVEX mobility fellowship) allowed CF's work at i3S, Portugal.The MHV group is supported by FEDER through COMPETE 2020 and by FCT (POCI-01-0145-FEDER-030457).

PT09.12 | Sorafenib-Loaded MSC-Derived Exosomes as a Versatile Platform to Target Breast Cancer Spheroids

zahrasadat hashemi[1]; Mahlegha Ghavami Nodeh[2]; Ramin sarrami forooshani[1]
[1]1. ATMP Department, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran, tehran, Iran; [2]Slovac academy of sciences, Bratislava, Slovakia

Introduction: Mesenchymal stromal cells (MSC) are the most professional source of extracellular vesicles in addition to their regenerative potency. The properties of MSC-derived exosomes are considered as their parent cells. Exosomes (Exos), the natural nanovesicles, have gained tremendous interest recently due to their ability to deliver drugs. On the other hand, chemotherapeutic agents for breast cancer, such as sorafenib, have significant negative side effects on other organs.
Methods: MSC cells were isolated from Wharton's jelly. MSC exos were isolated and their morphology, particle size, zeta potential, and expression of specific markers were evaluated. Sor-MSC exos were prepared by incubation. The cytotoxic effect of exos in human breast cancer spheroids (3D culture) was monitored by MTT, AO /EtBr, DAPI, Annexin/ PI, scratch and migration assays, colony formation and real-time PCR for P53, VEGF-A and caspase 3.
Results: The loading efficiency was 35.6% and the release rate was 14.7% after 24 hours. Uptake of PKH-67-labeled Sor-MSC exos was detected by flow cytometry at 74.6%. All viability assays confirmed the inhibitory effect of Sor-MSC-Exos compared with free sorafenib. The results indicate that Sor-MSC-Exos selectively reduces tumor cell viability and spare fibroblasts and MCF -10A as non-cancerous cells. The remarkable effect of Sor-MSC-Exos was maintained for a long time after spheroid treatment.
Summary/Conclusion: Sor-MSC-Exos showed a potent inhibitory effect on proliferation and induced apoptosis in human breast cancer mass (tumor mimic) compared to the same dose of free sorafenib. Thus, the exosomes containing the cargo showed significantly higher uptake than the free drug in both 2D and 3D. Thus, they can be used as drug carriers with selective toxicity to the normal cells to reduce the side effects of chemotherapeutic drugs.

PT09.13 | Surface modifications of extracellular vesicles for plasma stabilization

Marie Morille[1]; Laurianne Simon[2]; Vincent Lapinte[1]; Tarek Benkhaled[1]; Farida Djouab[3]; Claudia Terraza[3]; Céline Elie-Caille[4]; Geetika Raizada[5]; Jai Prakash[6]; Kunal Pednekar[6]; Julie Constanzo[7]; Jean-Pierre Pouget[7]; Jade Berthelot[1]; Jean-Marie Devoisselle[1]; Joel Chopineau[1]
[1]ICGM, Montpellier University, CNRS, ENSCM, Montpellier, France, Montpellier, France; [2]Montpellier University, montpellier, France; [3]IRMB, INSERM U475, Montpellier, France, Montpellier, France; [4]Institut FEMTO-ST, UMR 6174 CNRS, 25030 Besancon cedex, France, Besancon, France; [5]Institut FEMTO-ST, UMR 6174 CNRS, 25044 Besancon cedex, France, Besancon, France; [6]Department of Biomaterials Science and Technology Targeted Therapeutics Section Technical Medical Centre University of Twente7500 AE Enschede, the Netherland, Twente, Netherlands; [7]IRCM, Inserm U1194, Université de Montpellier, Institut Régional du Cancer de Montpellier (ICM), Montpellier, F-34298 France, Montpellier, France

Introduction: Despite the proof of concept of their efficiency as drug delivery systems (DDS) compared to synthetic nanoparticles, the rationale of using extracellular vesicles (EVs) in therapy still requires improvements. Among them, their plasma instability (t1/2=4 min) when intravenous administration in a non-autologous host is a major issue avoiding reaching targeted organs by passive accumulation. In this context, our team aims at overcoming this hurdle by transiently functionalizing EVs surface to increase their plasma stability while maintaining their cell internalization capacity. Our strategy relies on the post-insertion of fine tuned bio-inspired polymers: the poly(2-oxazoline)s (POx). Known for their excellent biocompatible properties, POx also constituted an excellent alternative to poly(ethylene glycol) (PEG) as clinical awareness has risen around its overuse (e.g. anti-PEG Abs). Therefore, we designed amphiphilic POx with different lipid anchors to insert in EVs membrane: cholesterol hemisuccinate (CHEMS) and hexadecane (C16).
Methods: EVs were produced from murine MSC, isolated and characterized by nanoparticle tracking analysis. EVs were extruded through a membrane of 50 nm to obtain narrower sizes, as a reproducible basis for evaluation of surface modification. The

two POx were synthesized by cationic ring-opening polymerization, purified by membrane dialysis and fully characterized as CHEMS(POx)52 and C16(POx)54. Different conditions (time, buffer, temperature) were screened to obtain both POx association onto extruded EVs. Non associated chains were discarded by ultrafiltration. Thus obtained EVs were thereafter characterized at the physico-chemical (size, charge, SPR-AFM) and biological level (macrophage uptake, complement activation, internalization in complex organoids composed of pancreatic cancer cells, in vivo administration in mice).
Results: The conditions of post-insertion were first determined on liposomes as synthetic model and then transposed to EVs. Overall, the grafting density was determined of respectively 0.5 for C16(POx) and 1 POx/nm^2 for CHEMS(POx) and confirmed by SPR and AFM measurements. The macrophages uptake of EVs was evaluated on primary mice cells and we demonstrated a decrease of 50% with post-inserted species compared to non modified species. To verify the transient functionalization, both EVs species were tested on organoids cells to measure their penetration capacity. The preliminary results indicated no difference in cell internalization and identical penetration to the organoids core from non-extruded EVs to extruded one and with or without POxylation and significantly higher than with liposomes.
Summary/Conclusion: With these promising results, we are currently evaluating the behavior of the post-inserted EVs in vivo after IV injection in mice. We propose here to expose our latest results concerning the interest of EV surface functionalization.

PT09.14 | This abstract is about engineering extracellular vesicles for the treatment of viral diseases

rui zhang[1]; zhiwei Wu[2]
[1]NanJing university, Nanjing, China (People's Republic); [2]Nanjing university, Nanjing, China (People's Republic)

Introduction: Zika virus (ZIKV), a flavivirus associated with neurological disorders, constitutes a global health threat. During pregnancy, ZIKV traverses the placenta and causes congenital diseases such as microcephaly and Guillain-Barré syndrome in newborns. To develop a specific antiviral therapy against ZIKV-induced microcephaly that could cross placental and blood-brain barriers, we designed targeted small extracellular vesicles (EVs) encapsulating antiviral siRNA (small interfering RNA) to inhibit ZIKV. The neuro-specific targeting was achieved by engineering EVs membrane protein lamp2b fused with a neuron-specific rabies virus glycoprotein derived peptide (RVG). Intravenous administration of the RVG-engineered EVs loaded with siRNA (ZIKV-specific siRNA) protected pregnant AG6 mice against vertical transmission of ZIKV. Particularly, sEVsRVG-siRNA traversed placental and blood-brain barriers and suppressed ZIKV infection in fetal brains. Moreover, sEVsRVG-siRNA alleviated the neuroinflammation and neurological damage caused by ZIKV in the fetal mouse model. In general, we developed a EVs-based targeted system of antiviral therapy for brain and fetal brain infections
Methods: Isolation of sEVs、 EVs encapsulation of siRNA by electroporation、 Quantification of siRNA loading into EVs by electroporation、Flow cytometry、Western blot analysis、Nanoparticle tracking analysis
Results: By expressing a neurotropic RVG peptide sequence on the surface of EVs, the current study demonstrated that the antiviral siRNA-loaded sEVsRVG was selectively targeted to brain tissues and inhibited virus in fetuses. This therapeutic approach expanded the application of EVs to treat a viral infection of brains by intravenous injection of sEVsRVG-siRNA
Summary/Conclusion: we engineered EVs expressing RVG on the surface and demonstrated that intravenous administration of RVG-modified EVs loaded with siRNA (ZIKV-specific siRNA) protected pregnant AG6 mice against the vertical transmission of ZIKV. Moreover, sEVsRVG-siRNA protected the fetuses from ZIKV infection and alleviated the neuroinflammation and neurological damage caused by ZIKV. Therefore, sEVsRVG-encapsulated siRNA can provide an ideal method to enhance the delivery of cargo such as nucleic acids, achieving targeted treatment of brain viral infection and control of related neurological damage

PT09.16 | Efficient Production of Extracellular Vesicles using Cell Spheroids

Seokhwan Yun[1]; sang-Hyun An[2]; Young Cheol Kim[3]; Keunsun Ahn[4]
[1]Sphebio Co.Ltd, Seoul, Republic of Korea; [2]Daegu-Gyeongbuk Medical Innovation Foundation, Seoul, Republic of Korea; [3]Kyungpook National University, Seoul, Republic of Korea; [4]Sphebio, Seoul, Republic of Korea

Introduction: Cell-derived extracellular vesicles(EVs) contain miRNA, mRNA, and various proteins, and many studies have been conducted on the therapeutic potential due to their intracellular communication capabilities. However, for commercial use, it is necessary to improve the production efficiency and quality control standards of existing EV production technology.
Methods: Here, we have developed EV production technology using cell aggregates/spheroids to improve EV production efficiency. Since the EV production environment changes according to the size of the cell spheroid used for EV production, we developed cell spheroid production automation technology to produce uniform cell spheroids. The production of EVs derived from cell spheroids significantly increased the production index of number of EVs per cell(# of EVs/cell) compared to the conventional 2D cell culture.

Results: The results of this study could suggest mass production technology for EV commercialization. We used mesenchymal stem cells in out experiments. There was no morphological difference between 2D cell culture derived exosomes and Cell spheroids derived exosomes. Exosomal biomarkers(CD63, CD9, HSP90) showed no difference between 2D cell culture derived exosomes and Cell spheroids derived exosomes. However, exosome production per cell was increased. In addition, in Cell spheroid derived exosomes showed enhanced angiogenesis ability compared to 2D cell culture derived exosomes in vitro.
Summary/Conclusion: 3D Cell spheroid secret exosomes export more exosomes compared to conventional 2D culture. There are no significant difference in morphology and biological marker expression of exosomes. However, the ability of exosomes apprear to be different. We expect this to be a difference in exosomal cargo such as miRNA or proteins. Further, NGs for microRNA and LC-MS/MS proteomics for protein will be performed to elucidate the substance and its mechanism of therapeutic effect of 3D Cell spheroid derived exosomes.

PT09.17 | Targeting extracellular vesicles to heart

Alena Ivanova[1]; Andreia M. Silva[2]; Niek Dekker[2]
[1]Discovery Biology, Discovery Sciences, BioPharmaceuticals R&D AstraZeneca, Gothenburg, Sweden, Mölndal, Sweden; [2]Discovery Biology, Discovery Sciences, BioPharmaceuticals R&D AstraZeneca, Gothenburg, Sweden, Molndal, Sweden

Introduction: Extracellular vesicles (EVs) have come into focus of extensive research during the last decade, because of their broad biological functions and roles in various diseases. Myocardial infarction is a leading cause of death worldwide. Following injury, the organ function is impaired, and the adult human heart is lacking regenerative capability. EVs offer an exciting, novel therapeutic approach for treatment of heart failure.
Methods: In our studies as a first step we are developing gene engineering technologies to engineer the surface composition of EVs to guide these nanomedicines to the infarcted heart tissues.
Results: We have developed methods to put antibodies against cardiac target candidates on the surface of EVs. We have screened of range of anchor proteins and identified: nanobody against kappa chain of IgG antibody, Protein A and Protein G as successful candidates for antibodies EV display. Additionally, we developed EV based nanomedicines decorated with cardiac targeting peptides. We have optimized the fusion protein for functional display of these peptides at the surface of EV in the presence of blood plasma.
Summary/Conclusion: Our data demonstrate that we can display both antibodies and peptides on EVs surface, that these are available for interaction with their binding partners in presence of the blood serum. This opens the possibilities to use targeting potential of the designed EVs for delivery of therapeutic molecules to the heart tissues.

PT09.18 | Well controlled cell mechanical stimulation in bioreactors by a turbulent flow induces massive extracellular vesicles production

Julien Branchu[1]; Camille Simon[1]; Shony Lemieux[1]; Max Piffoux[1]; Jeanne Volatron[1]; Amanda Silva[2]
[1]EVerZom, Paris, France; [2]Laboratoire Matière et Systèmes Complexes, Paris, France

Introduction: Extracellular Vesicles (EVs) are one of the most interesting alternatives to cell therapy in regenerative medicine. However, large scale EV manufacturing methods are costly and time consuming. We discovered that shear stress induces massive EV production and transformed this unexpected discovery in an industry-compatible and easy to scale-up technology to achieve large scale EV production. Shear stress is exerted on cells after their 3D culture in bioreactors thanks to well controlled turbulent flows. This method was proven to be effective for all tested cell types (>10) with both adherent and suspended cells.
Methods: As an example, adherent cells like human adipose derived stem cell (hASC) may be cultivated in bioreactors on microcarriers (MC), 200 μm polymer beads that have a high surface/volume ratio and can be handled easily. This old and well-described technology allows to culture adherent cells just like if they were in suspension. Bioreactors may be viewed as bottles of cell culture media agitated by an impeller where parameters like temperature, pH, metabolic activity or gas input are controlled. When the desired cell-confluence on MC is reached, a precise mechanical stimulation in a serum-free media is applied for a few hours to induce high yield vesiculation.
Results: On overall, this process achieves a ≈10-fold increase in yield in 4 hours compared to classical production by starvation in 2D our 3D. Using this technology, hASC in a bioreactor produce approximately 20,000 EVs/cell in 4 hours compared to about 4,000 EVs/cell in 2D starvation for 72 hours. This method preserves a good cell viability at the end of the process (>90%) when monitored by both Nucleocounter and immunostaining. EVs are produced within a culture media free of any contaminating human or animal protein, limiting the need for complex purification. The batches may be clarified by centrifugation or filtration and concentrated by tangential flow filtration with a global harvesting yield above 70%. Furthermore, the turbulence-generated

EVs conserve the regenerative properties of their parental cells and have been shown to be effective in various small and large animal models of regenerative medicine or drug delivery.

Summary/Conclusion: We describe a GMP/clinical grade EV production and purification platform to bring EV therapeutics to patients and are open to collaboration on this subject.

PT09.19 | Therapeutic extracellular vesicles: from drug discovery to manufacturing process design

Olga Shatnyeva[1]; Simone Strauch[1]; Nora Olszok[1]; Denise Hartung[1]; Farah Aqel[1]; Philipp Themann[1]; Jonas Vogt[1]; Anna J. Vesting[1]; Katrin Ridders[1]; Julia D. Knopf[1]; Alexandra Schmitter[1]; Alfia Latfullina[1]; Andrei Shchebet[1]; Susann Santag[1]; Christoph Weber[2]; Carleen Kluger[2]; Mandy Richter[1]; Stefanie Pfaender[1]; Gregory Antonios[1]; Melanie Pichery[3]; Michael Esquerre[3]; Audrey Hotzinger[1]; Matthias Austen[1]; Andreas Scheel[1]; Cord Dohrmann[1]

[1]Evotec International GMBH, Göttingen, Germany; [2]Evotec International GMBH, Munich, Germany; [3]Evotec (France) SAS, Toulouse, France

Introduction: Extracellular vesicles (EV) are emerging as a safe alternative to cell therapeutics. Full realization of their potential requires a new paradigm where technology development and future large-scale manufacturing are considered already during the earliest stages of research. Our approach aims to combine optimization of culturing conditions for efficient EV production, purification, quality control and design of the manufacturing process. During our study, we focus on 1) use of cell lines and iPSC-derived cells for EV production and 2) detailed quality control analysis of purified vesicles.

Methods: Expi293 and different iPSC derived cells were used for EV production. We developed optimal cell culture protocols and differentiation protocols for cell types of interest. Tangential flow filtration (TFF) in combination with differential ultracentrifugation were used for EV purification. EVs were characterized by nanoparticle flow cytometry (NanoFCM), Exoview, transmission electron microscopy, western blotting and mass spectrometry (MS). EV functionality was analyzed in Incucyte-based cellular assays (e.g. viability, proliferation and functional uptake)

Results: We found GMP compatible cultivation conditions for efficient EV production for all cell types analyzed. During the development of our set of EV characterization procedures, we found that a combination of "state-of-the-art" methods, such as NanoFCM, TEM, MS in combination with cell based assays provides extensive and reproducible information about quality and functionality of EV batches.

Summary/Conclusion: Overall, using EVs produced by Expi293 and iPSC-derived cells, we could generate proof of principle data to demonstrate that our established EV platform supports identification of optimal EV production conditions along with in depth qualitative, quantitative and functional characterization of EV production batches. We envision that this platform will be useful for a variety of therapeutic applications.

PT09.20 | Bead to bead transfer as scale-up method for mesenchymal stem cells expansion

Bastien Thauvin[1]; Camille Simon[2]; Julien Branchu[2]; Latifa Chebil[3]; Eric Olmos[3]

[1]EVerZom, Nancy, France; [2]EVerZom, Paris, France; [3]Laboratoire Réactions et Génie des Procédés, Nancy, France

Introduction: Extracellular vesicles (EVs) are nano-sized subcellar particles involved in intercellular communication. Because they reflect their parental cell phenotype, EVs produced by mesenchymal stem cells (MSC) have regenerative properties and are promising as cell-free therapy to repair damaged tissues. However, lack of large-scale GMP compatible EVs production processes limits clinical translation. EV production yield can be improved by mechanical stress induced by turbulence but a large number of cells is still required. Conventional 2-Dimensional systems are time consuming and allows insufficient control of cell culture parameters. Development of 3D culture systems tend to give interesting solutions to these issues. We designed a microcarrier-based (MC) cell culture process in stirred tank bioreactors using cell bead-to-bead transfer (Btb, i.e. adding fresh MC in the media) as a scale-up method for EV production without the need for enzymatic detachment step.

Methods: The effect of MC addition on Human adipose derived stem cells (hADSC) cultivated on MCs in spinner flasks was evaluated based on daily cell count and metabolites analysis. After each addition, an intermittent agitation was applied to promote cell migration.

Results: Cells can migrate on newly added MC thanks to intermittent agitation. Btb lead to prolong cell expansion, delay aggregate apparition and increase final harvest compared to control. Nevertheless, drop of mean cell/bead ratio below 3 inhibit cell growth and late addition of MC when cell/bead ratio is above 7 leads to aggregate formation which cannot be dissociated.

Summary/Conclusion: BtB is a promising scale-up method for MSC large-scale production but the optimal cell per bead ratio and time addition need to be precised to maintain a high expansion rate.

Funding: Private, European, ANRT fundings.

PT09.21 | High-throughput media screening for EV production

Ken Naruse[1]; Cassandra Bellver[1]; Alison Fujii[1]; Aaron Fulton[1]; Richard A. Festa[1]; Matthew Tenorio[1]; David T. Ho[1]; Nisa Renault[2]; Michele Hamrick[2]; Jacquelyn Wong[2]; Robert Newman[1]
[1]*FUJIFILM Irvine Scientific, Santa Ana, USA;* [2]*Fujifilm Cellular Dynamics, Madison, USA*

Introduction: Extracellular vesicles (EVs) such as exosomes and microvesicles serve as messengers of an intercellular network, allowing exchange of cellular components between cells. EVs carry lipids, proteins, and RNAs derived from their producing cells, and have a potential to be used as a cell-free therapy for a variety of diseases including cancer, heart disease and inflammation. However, there are major challenges to applying EVs in these areas: overcoming low yields of EVs from producing cells and the lack of established production and purification processes for therapeutic EVs. Here, we report the establishment of a process to optimize the formulation of chemically-defined EV production media.
Methods: We have developed semi-automated high-throughput cell culture and EV ELISA methods with a automated liquid handler to allow us to test more media conditions simultaneously and reduce experiment time. Single EV characterization was performed with super resolution microscopy to characterize subtypes of EVs.
Results: Using iPSC-derived cells as a model, we identified prototype media that promote cell growth and stimulate EV secretion. By utilizing our media development and EV characterization capability, we have demonstrated a platform by which we can implement high-throughput approaches to study media effects on EVs and subsequently optimize media.
Summary/Conclusion: This study underscores the powerful influence cell culture media has on a cell's microenvironment. Demonstrated here by its effect was on not only cell expansion and quality, but also EV yield, quality and purity. Our high throughput compatible approach for media development will enable us to develop specialized EV production media for a variety of cell types.

PT09.22 | A human-cell-based potency assay for the screening, development, and final product release of small extracellular vesicle-enriched compositions for therapeutic use

Michele Hamrick; Megan K. Livingston; Coby Carlson; Jacquelyn Wong; Nisa Renault
Fujifilm Cellular Dynamics, Madison, USA

Introduction: To advance novel extracellular vesicle (EV)-based therapeutics, there is a need for screening tools and functional release assays that are small-scale, reproducible, human-relevant, and have a meaningful product dosing strategy with appropriate controls. We have developed a cell survival assay using human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes (CM) to assess the potency of therapeutic candidates containing small EV-enriched (sEV) compositions.
Methods: Cardio-protective sEV candidates were isolated by ultracentrifugation from primary and iPSC-derived cell types. Media without cells were "cultured" and ultracentrifuged for "virgin media" (MV) negative controls. CM were plated, chemically stressed, and treated with sEV or MV in standard or serum-free media. Dosing strategies based on particle number (nanoparticle tracking analysis) or the number of secreting cells producing the conditioned media were assessed. Assay controls were unstressed or stressed CM, each treated with DPBS only (vehicle control). Cell survival was assessed by quantifying viable cell number (imaging / microscopy), ATP (chemiluminescence), and cell adherence (electrical impedance).
Results: Our assay consistently displayed a dose-dependent improvement in survival of CM with the addition of therapeutic sEV over controls for all readout methods. Potency results with CM paralleled data from human endothelial cells in scratch wound healing assays when using the same dosing strategies. Dosing by mother cell number was a superior strategy for product development for both assays, in part due to highly variable particle counts in sEV compositions from different donors or culture methods.
Summary/Conclusion: We have developed a useful dosing strategy for product screening and development of sEV-containing compositions featuring a reproducible, human cell-based, CM-survival assay relevant to the testing, development, and final product release of sEV-containing compositions for the treatment of cardiac diseases.
Funding: FUJIFILM Cellular Dynamics, Inc.
FUJIFILM

PF01: Technologies and methods: EV separation from blood

Chair: Marija Holcar – Institute of Biochemistry and Molecular Genetics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

Chair: Olivier P. BLANC-BRUDE – Paris Center for Cardiovascular Research, Institut National de la Santé et de la Recherche Médicale U970, Université de Paris, France

PF01.02 | Applying the MISEV guidelines (2018) to EVs from feline plasma: A "comparative medicine" approach to EV isolation and characterisation

Jane Howard[1]; Kieran Wynne[2]; Evelin Moldenhauer[3]; Paul Clarke[4]; Ciaran Maguire[5]; Stephanie Bollard[6]; Xiaofei Yin[6]; Lorraine Brennan[6]; Louise Mooney[6]; Stephen Fitzsimons[6]; Melinda Halasz[6]; Ester Rani Aluri[6]; Dermot F. Brougham[6]; Walter Kolch[6]; Róisín M. Dwyer[7]; Shirley Potter[6]; Pamela Kelly[6]; Amanda McCann[6]
[1]UCD School of Medicine, University College Dublin., Dublin 4, Ireland; [2]Systems Biology Ireland, Dublin 4, Ireland; [3]Postnova Analytics GmbH, Landsberg, Germany; [4]Postnova Analytics UK Ltd, Worcestershire, United Kingdom; [5]Particular Sciences Ltd, Dublin, Ireland; [6]University College Dublin, Dublin, Ireland; [7]University College Galway, Galway, Ireland

Introduction: Extracellular vesicles (EVs) are nanoparticles found in all biological fluids, capable of transporting biological material around the body. Research into the physiological role of EVs has led to the MISEV framework in 2018 guiding the standardisation of protocols in the EV field. To date, these guidelines have focused on EVs of human origin. As the importance of comparative medicine progresses, there has been a drive to study similarities between diseases in humans and animals. To research EVs in felines with pathologies, we must initially validate the application of the MISEV guidelines in this group.
Methods: EVs were isolated from the plasma of healthy humans and healthy felines by size exclusion chromatography and characterised according to the MISEV guidelines. Nanoparticle tracking analysis, total protein concentration, western blot analysis of known EV markers and transmission electron microscopy were carried out. Further EV characterisation by mass spectrometry, asymmetrical flow field blow fractionation and metabolomic profiling was also undertaken.
Results: Human and feline plasma showed a similar concentration of EVs, comparable expression of EV markers and analogous particle to protein ratios. Mass spectrometry analyses showed a similar proteomic signature of EVs from humans and felines. Asymmetrical-flow field flow fractionation showed two distinct subpopulations of EVs isolated from human plasma, whereas one subpopulation was isolated from feline plasma. The EVs from humans and felines were metabolically similar.
Summary/Conclusion: Isolation and characterisation of EVs from humans and felines show that MISEV2018 guidelines may also be applied to EVs from felines. Potential comparative medicine studies of EVs provide a model for studying naturally occurring diseases in both species.
Funding: Work was supported by the UCD PhD Advance Core Funding Scheme, and the Mater Foundation, Mater Misericordiae University Hospital (MUUH) Dublin, Ireland.

PF01.03 | Asymmetric flow field-flow fractionation for blood plasma extracellular vesicle and other extracellular particle separation

Olesia Gololobova[1]; Wyatt Vreeland[2]; Kenneth W. Witwer[1]
[1]Johns Hopkins University School of Medicine, Department of Molecular and Comparative Pathobiology, Baltimore, USA; [2]NIST, Gaithersburg, USA

Introduction: Circulating extracellular vesicles (EVs) are potentially promising biomarkers for many diseases and pathological conditions. However, EVs are in very low abundance compared with non-vesicular extracellular particles (EPs) such as lipoprotein particles (LPP) and protein complexes, and some of these non-EV components overlap in size and density with EVs. Therefore, the separation of vesicles from blood is challenging. Currently, the most used plasma EV separation methods are ultracentrifugation (UC, with or without density gradient), size exclusion chromatography (SEC), and combinations thereof. However, alone, these methods do not fully purify EVs. Here, we apply SEC and asymmetric flow field-flow fractionation (AF4) to plasma EV separation.
Methods: Gel size separation with IZON qEV70 original columns was used to deplete abundant plasma proteins such as albumin and the very small high-density lipoprotein particles (HDL) from 0.5 mL of platelet-depleted plasma. EV-containing fractions were pooled together and concentrated with Amicon 10 kDa filters. Concentrated fractions were separated using asymmetric flow field flow fractionation (AF4) with the long channel which allows separating large particles at low crossflow to minimize membrane (10 kDa) interaction. Collected fractions were probed by Western blot (WB) for lipoprotein markers ApoB100, ApoE,

and ApoA1, as well as free proteins such as albumin, fibrinogen, and transferrin, and EV markers such as CD9 and CD81. Single-particle analyses were performed with nano-flow cytometry and single-particle interferometric reflectance imaging sensing (SP-IRIS).
Results: Even after SEC, EV-containing fractions analyzed via WB showed high abundant low-density lipoprotein (LDL) and HDL markers and sizing peaks corresponding with these particles (AF4 fractogram). Following AF4, a separate population particle with a size greater than 30 nm was observed. WB and SP-IRIS confirmed the separation of LDL and HDL from EVs.
Summary/Conclusion: AF4 is a powerful tool to isolate highly pure extracellular vesicles from blood plasma with minimal time and input requirements.
Funding: This work was supported by the National Cancer Institute (NCI) and the Office of the Director (UG3/UH3CA241694).

PF01.04 | Automated proteomics sample preparation of Extracellular vesicles from human plasma and serum

Jun Adachi[1]; Satoshi Muraoka[2]; Masayo Hirano[1]; Junko Isoyama[1]; Satoshi Nagayama[3]; Takeshi Tomonaga[4]
[1]National Institute of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan; [2]National Institutes of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan; [3]The Cancer Institute Hospital of the Japanese Foundation for Cancer Research, Tokyo, Japan; [4]National Institute of Biomedical Innovation, Health and Nutrition, ibaraki, Japan

Introduction: Extracellular vesicles (EVs) are ubiquitously secreted by almost every cell type and present in all body fluids. The blood-derived EVs can be used as a promising source for biomarker monitoring in disease. Current development in EVs proteomics have analyzed in clinical subjects. To date, researcher have developed the EV isolation methods, including differential centrifugation, sucrose gradient ultracentrifugation, size exclusion chromatography, affinity capture and asymmetric-flow field-flow fractionation. However, their isolation methods are limited in throughput for human subjects. Here, we introduced a novel automated EV isolation and sample preparation method for EV proteomics analysis that can be started with low volume of multiple clinical samples.
Methods: EVs were automatically separated from both EDTA plasma and serum of six healthy subjects (n=3) by an affinity capture isolation method using Magcapture isolation kit, and we applied them in Mass spectrometry, data-independent acquisition. In addition, the sample preparation for EV proteomics performed using combination single-pot, solid-phase-enhanced sample-preparation (SP3) technology with Flex system in 96 well format.
Results: The automation of EV purification and protein digestion made it possible to complete the pretreatment of 96 samples within one day. In particular, the automation of EV purification reduced the variability of protein identification by 70% compared to manual purification, enabling the quantification of 1400 proteins in one hour of MS analysis.
Summary/Conclusion: We have successfully isolated EVs from blood using an automated isolation method and developed an automated method for EV proteomic sample preparation. This method is attractive for processing large cohort samples for biomarker development, validation and routine testing.

PF01.05 | Development of chromatographic methods to isolate Extracellular Vesicles (EV) from blood products

Reetta Pusa[1]; Heikki Saari[2]; Heli Marttila[3]; Petra Ilvonen[1]; Kai Härkönen[2]; Ulla Impola[2]; Hanna Oksanen[3]; Minna Poranen[3]; Saara Laitinen[2]
[1]Finnish Red Cross Blood Service, University of Helsinki, Helsinki, Finland; [2]Finnish Red Cross Blood Service, Helsinki, Finland; [3]University of Helsinki, Helsinki, Finland

Introduction: The requirements for the EV isolation methods for materials poor or rich in contaminating particles such as protein complexes or lipoproteins are not the same. Well-accepted isolation method, size exclusion chromatography (SEC) separates impurities smaller in size by porous matrix. This is not an optimal choice to separate pure EVs from donated whole blood rich in EV-sized lipoproteins: chylomicrons, VLDL and LDL. Hence development of alternative or combinatory chromatographic techniques are needed to isolate large quantities of pure EVs. Here we report results from different chromatographic techniques: SEC and ion exchange chromatography with material derived from platelet concentrates (PC).
Methods: Standard leukocyte-reduced PCs were derived from buffy coats of four anonymous ABO RhD-matched whole blood donations, were obtained from the FRC Blood Service as accepted by the Finnish Supervisory Authority for Welfare and Health. Isolations were done using selected anionic/cationic ion exchange and size exclusion chromatographic techniques. Yield and purity were estimated by NTA, imaging flowcytometry, SDS PAGE, WB, Agarose electrophoresis of lipoproteins and EM.

Results: Our results show that a simple SEC column is not enough for separating larger lipoproteins from EVs. With anion exchange methods, enhanced separation of EVs from lipoproteins was possible with the addition of chaotropic, but the yield was poor due to loss of EVs into the column. Cation exchange methods seem to result in similar purity and yield as with anion exchange.
Summary/Conclusion: While chromatographic EV-purification is a promising scalable approach, lipoproteins pose a particularly challenging contaminant for such methods. Nevertheless, our results show that lipoproteins and EVs can be separated with carefully selected chromatographic approaches for large-scale blood derived EV purification.
Funding: Business Finland ecosystem funded project EVE – Ecosystem Consortium
Orion Research Foundation

PF01.06 | Electrochemical detection and EMT evaluation of extracellular vesicles from whole blood by using an integrated microfluidic device

Kyung-A Hyun[1]; Gurudatt NG[1]; Hogyeong Gwak[2]; Kyungyeon Lee[1]; Sunyoung Park[2]; Seong-Eun Kim[3]; Hyo-Il Jung[2]
[1]Yonsei University, Seoul, Republic of Korea; [2]School of Mechanical Engineering, Yonsei University, Republic of Korea, Seoul, Republic of Korea; [3]Korea Electronics Technology Institute, Seoul, Republic of Korea

Introduction: Tumor-derived extracellular vesicles (tdEVs) are one of the most promising biomarkers for liquid biopsy-based cancer diagnostics due to the expression of specific membrane proteins of their cellular origin. The investigation of Epithelial-to-mesenchymal transition (EMT) in cancer using tdEVs is an alternative way of evaluating the metastatic risk. An ultra-sensitive selection and detection methodology is an essential step to develop a tdEVs-based cancer diagnostic device.
Methods: In this study, we developed an electrochemical sensor integrated microfluidic chip, consisting of a multi-orifice flow fractionation (MOFF) channel for eliminating blood cells and a micro-vortex for enhancing the collision between tdEVs and ITO electrodes. The transparent ITO electrodes provide an advantage for dual electrochemical and fluorescent monitoring of the tdEVs interaction. The micro-vortex and the ITO sensors are assembled with a 3D printed magnetic housing to prevent sample leakage and to attach/detach the sensors into the microfluidic channel.
Results: The tdEVs were successfully captured on the specific antibody modified ITO surfaces in the integrated microfluidic channel. The integrated sensors showed an excellent linear response between 103 and 109 tdEVs/mL. Simultaneous evaluation of the epithelial and mesenchymal markers on the tdEV surfaces successfully revealed the EMT index of the corresponding cancer cells.
Summary/Conclusion: Our microfluidic device-integrated ITO sensors show excellent detection in the clinically relevant concentration range for tdEV detection in blood. Hence, this system is expected to open a new avenue for the liquid biopsy-based cancer diagnostics.
Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No.2020R1A5A1018052) and (No. 2021R1A2C301125411) and the Technology Innovation Program (20008829) funded By the Ministry A1A01067448of Trade, Industry & Energy (MOTIE, Korea).

PF01.07 | EV preparation from plasma and serum by size exclusion chromatography: 35 nm qEV columns allow higher EV recoveries than 70 nm qEV columns

Oumaima Stambouli[1]; Monika Wolny[2]; Tobias Tertel[1]; Simon Staubach[1]; Ingvild Birschmann[2]; Bernd Giebel[1]
[1]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany; [2]Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabeteszentrum Nordrhein-Westfalen, Universitätsklinik der Ruhr-Universität Bochum, Bad Oeynhausen, Deutschland, Bad Oeynhausen, Germany

Introduction: Extracellular vesicles (EVs) residing in various body fluids including plasma represent promising biomarkers for several diseases. However, EV preparation with fair purities in short time intervals remains challenging. Plasma and serum samples for example contain a high load of lipoprotein particles such as high density (HDL) and low density lipoproteins (LDL) that are often co-isolated with EVs. In recent years, size exclusion chromatography (SEC) emerged as a frequently used method for EV preparation. In fact, SEC efficiently allows separation of EVs from a high proportion of soluble components. In addition to self-loaded SEC columns, commercially available qEV columns (IZON) are frequently used. Originally, qEV columns were provided being filled with 70 nm pore-sized resin, more recently, qEV columns being filled with 35 nm pore-sized resin, were introduced to the market.
Methods: Here, we compared the performance of both qEV column types in preparing EVs from plasma and serum of healthy donors. EVs were prepared according to the manufacturer's instructions. The particles and protein concentration were

determined by nanoparticle tracking analysis (NTA) and the bicinchoninic acid assay, respectively. LDL and albumin were measured using Clinical Chemistry Analyser Architect C8000 by photometric determination method and immunoturbidimetry assay, respectively . In addition, fractions were investigated by imaging flow cytometry (IFCM) and by western blot, which we also probed for the lipoprotein ApoB.

Results: According to our analysis, 35 nm qEV columns provided higher EV yields but did not separate plasma-derived EVs and ApoB as efficiently as the 70 nm qEV columns. Noteworthy, both columns failed to efficiently separate EVs and ApoB from serum samples. Furthermore, our data confirm our published findings that NTA detects far more particles than just EVs and that in its traditional form NTA should not be considered as an appropriate method for the reliable evaluation of given EV preparation protocols.

Summary/Conclusion: To conclude, 35 nm qEV column have an increased EV yield compared to 70 nm for both plasma and serum. However, both columns were not able to separate EVs and LDL for sera samples. Moreover, our data further confirm our recent publication that NTA is insufficient for EV characterization as its measures other particles in addition to EVs.

PF01.08 | Heat inactivation of fetal bovine serum increases protein contamination of extracellular vesicles

Marta Moschetti[1]; Cecilia Lässer[2]; Roger Olofsson Bagge[3]; Rossella Crescitelli[4]

[1]University of Palermo, Dep. of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), palermo, Italy; [2]Krefting Research Centre, Institute of Medicine at Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, Gothenburg, Sweden; [3]Sahlgrenska Center for Cancer Research and Wallenberg Centre for Molecular and Translational Medicine, Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; [4]Sahlgrenska Center for Cancer Research and Wallenberg Centre for Molecular and Translational Medicine, Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, Gothenburg, Sweden

Introduction: Extracellular vesicles (EVs) released in cell cultures are influenced by the cell culture conditions, such as the use of fetal bovine serum (FBS). FBS contains EVs and it is usually depleted of EVs by ultracentrifugation (UC) and/or heat inactivated (HI). Several studies have evaluated the effect of different UC protocols for FBS by evaluating both cells and EVs. However, less is known about the effect of HI on the cells and the released EVs. The aim of this study was therefore to evaluate the effect of HI on EV purity.

Methods: To determine the effect of heat inactivation, three different protocols were applied based on different combinations of: 1) UC at 118,000 × g for 18h and 2) HI at 56°C for 30 min. The three conditions tested were: FBS ultracentrifuged but not heat inactivated (no-HI), FBS heat inactivated before UC (HI-before EV-dep), and FBS heat inactivated after EV depletion (HI-after EV-dep). The FBS was add to the media of three melanoma cell lines (MML1, UM22Ctr and UM22BAP1) at a final concentration of 10%. After 72h, large and small EVs were isolated by differential UC. The EV purity was determined by protein quantity, electron microscopy (EM) and nanoparticle tracking analysis (NTA).

Results: The protein quantity ($\mu g/\mu l$) of large EVs was similar in the three conditions analyzed. On the contrary for small EVs, the protein amount was higher when the HI was performed after EV depletion as compared to HI before the UC and UC alone. However, significantly more particles were not detected in the HI-after EV-dep which resulted in a lower purity of small EVs in HI-after EV-dep illustrated by calculating the ratio of number of particles/μg proteins. Presence of contaminants (indicated by strong background) was observed in EM pictures of small EVs isolated in HI-after EV-dep condition differently from large EV samples.

Summary/Conclusion: The HI of FBS induces release of contaminating elements that end up in small EV pellets if not previously removed.

PF01.09 | Hydrophobic effects in membrane sensing peptide for small extracellular vesicle isolation and analysis

Roberto Frigerio[1]; Alessandro Strada[2]; Greta Bergamaschi[3]; Lucio Barile[4]; Gabriella Andriolo[4]; Alfonso Gautieri[5]; Alessandro Gori[2]; Marina Cretich[2]

[1]Consiglio Nazionale delle Ricerche, Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC), Milan, Italy, Milano, Italy; [2]Consiglio Nazionale delle Ricerche, Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC), Milan, Italy, Milano, Italy; [3]SCITEC-CNR, milano, Italy; [4]Istituto Cardiocentro Ticino, Ente Ospedaliero Cantonale, Lugano, Switzerland; [5]Politecnico di Milano, Milano, Italy

Introduction: Small extracellular vesicles (sEV) present distinctive lipid membrane features in the extracellular environment. These include high curvature, lipid-packing defects and lipids composition. sEV membrane could be then considered as a "universal" marker, alternative to traditional surface proteins. Our recent work has identified a class of membrane-sensing peptide, derived from Bradykinin protein, as a class of molecular ligands for integrated small EV. The membrane recognition and binding mechanisms are based on complementary electrostatic interactions between the peptide and the phospholipids on the outer membrane leaflet, that subsequently can lead to the insertion of hydrophobic residues into the membrane defects.
Methods: In the present work, we investigate the role of hydrophobic aminoacids on the membrane recognition efficiency. To achieve this aim, we synthetized a small library of membrane sensing peptide derived from Bradykinin protein by replacing phenylalanine with residues with different hydrophobicity. Phenylalanine residues were replaced by halogen-aminoacids (Phe-I, Phe-Br, Phe-Cl), isoleucine and alanine; to evaluate the contribute of hydrophobic effects of Phe in membrane sensing peptides. To assess peptide binding capacity, we used peptide microarray for capturing EV-mimicking liposomes and EVs isolated by cell culture.
Results: These substitutions showed how the hydrophobic component of peptide sequence play an important role in membrane sensing. In addition, the same profile of binding for both types of particles demonstrated the possibility to use synthetic liposomes as model for biological extracellular vesicles. Finally, a preliminary computational approach was used to determine the free-energy profile of these peptides in order to assess the interaction with the phospholipid bilayer.
Summary/Conclusion: These results represent a step forward to the development of a new generation of membrane-sensing peptides for EV isolation and analysis.
Funding: Supported by European Union's Horizon 2020 research and innovation program under grant agreements No. 951768 (project MARVEL).

PF01.10 | Parallel characterization of total extracellular vesicle populations with current technologies

Johanna Puutio[1]; Petteri Parkkila[2]; Saara Laitinen[3]; Tapani Viitala[4]; Mari Palviainen[4]; Pia Siljander[4]
[1]University of Helsinki, helsinki, Finland; [2]Chalmers University, Gothenburg, Sweden; [3]Finnish Red Cross Blood Service, Helsinki, Finland; [4]University of Helsinki, Helsinki, Finland

Introduction: Human cells release heterogenous extracellular vesicles (EVs) into the bloodstream as a response to pathophysiological stimuli. Due to a lack of technologies capable of detecting all EVs, accurate quantification of the total EVome (size and surface markers) remains a challenge. We selected a panel of current technologies and measured the total platelet EVome to investigate their strengths and limitations in EV detection.
Methods: Human platelets were collected from clinical grade platelet concentrates and EVs were generated and fractionated with differential ultracentrifugation, ultrafiltration and asymmetric flow field-flow fractionation (AF4). The presence of EVs was confirmed by electron microscopy (EM) and subpopulation enrichment by western blotting and Single particle interferometric reflectance imaging sensing (SP-IRIS). EV sizing was carried out with light scatter (LS, e.g. UV, MALS, DLS and NTA), capture methods (surface plasmon resonance (SPR) and SP-IRIS), and resistive pulse sensing (RPS) to determine their effective detection ranges.
Results: Western blotting and SP-IRIS showed that fractions enriched in large EVs (>150 nm) and small EVs (sEVs, 100-150 nm) displayed EV markers (Hsp-70, Alix, CD9, CD63) and platelet marker CD41, whereas those enriched with very small EVs (< 80 nm) carried markers associated with exomeres. The reference size range and morphology of the total EVome was confirmed with EM and multi-detector AF4. LS-based techniques failed to find EVs below 100 nm, whereas capture methods became saturated with sEVs, leaving EVs above 150 nm underrepresented. Combining "bridge methods", such as EM and AF4, with LS, RPS and capture techniques improved the characterization and quantification of the total EVome.
Summary/Conclusion: A systematic combination of the current technologies comprising LS, RPS and surface-capture methods is essential for capturing the total EVome. Knowledge of the detection limits and analytical range of each technology needs to be established.
Funding: This project is funded by the EVE consortium of Business Finland.

PF01.11 | Polyethylene Glycol Precipitation is the Best Method to Obtain Extracellular Vesicles Depleted Fetal Bovine Serum for stem-cell culture

Peng Wang[1]; Onno Arntz[2]; Fons van de Loo[2]
[1]Experimental Rheumatology, Radboud university medical center, Nijmegen, Netherlands; [2]RadboudUMC, Nijmegen, Netherlands

Introduction: Stem cell-EVs are therapeutics having immunoregulatory and anti-inflammatory properties. To maintain growth of stem cells the culture medium must contain FCS but free of EVs, so pure stem cell-EVs can be obtained. In this study, we used

three EV depletion techniques: ultracentrifugation, polyethylene glycol precipitation, and ultrafiltration to obtain EV depleted FBS (EVdFBS) and compared them to untreated (UT) FBS and commercial EVdFBS (Com) on in vitro stem cell growth, differentiation, and EV-production.

Methods: EVs were depleted from FBS in parallel by, 18-hour ultracentrifugation at 100,000g (UC), 100kda centrifugal filtration (UF), or o/n 10% PEG precipitation (PEG). In all isolated batches, the amount of EVs and their protein content were determined by NTA and micro-BCA. Loss of serum growth factor TGFb, was determined by CAGA-LUC reporter assay. By western blot, CD81 and HSP-70 were detected in the isolated FBS EVs. Next, differentiation (alizarin red staining), proliferation (XTT), and EV release (NTA) of cultured human adipose stem cell (hADSCs) in the EVdFBS (5%) batches was studied

Results: Compared to UT FBS, the amount of EV depletion was almost comparable between UC (82%), PEG (95.6%), and UF (96.6%) but the protein loss was markedly different between UC (51%), PEG (47%), and UF (87%). Only in the PEG precipitated FBS EVs, CD81 and HSP-70 were detectable by WB does showing the highest selectivity of EV depletion. By UF, a significantly reduction of TGFb was observed, showing the lowest selectivity of all techniques. Culturing hADSCs in the five different EVdFBS batches for 96 hours, had no obvious influence on cell differentiation, but remarkable, proliferation was the lowest in UF and Com EVdFBS, but not in UC and PEG EVdFBS. Moreover, the EV release of hADSC was highest when cells were cultured in PEG EVdFBS.

Summary/Conclusion: This study showed that PEG precipitation is the most optimal method to obtain EVdFBS for support of growth and EV production by hADSCs.

PF01.12 | Proteomic and metabolomic composition of serum-derived small extracellular vesicles could discriminate rectal cancer patients with different responses to neoadjuvant radiotherapy

Urszula Strybel[1]; Lukasz Marczak[1]; Marcin Zeman[2]; Krzysztof Polański[3]; Łukasz Mielańczyk[4]; Mateusz Smolarz[2]; Piotr Widlak[5]; Monika Pietrowska[2]; Anna Wojakowska[1]

[1]Institute of Bioorganic Chemistry Polish Academy of Sciences, Poznan, Poland; [2]Maria Sklodowska-Curie National Research Institute of Oncology, Gliwice Branch, Gliwice, Poland; [3]Wellcome Sanger Institute, Hinxton, United Kingdom; [4]3 Department of Histology and Cell Pathology, Faculty of Medical Sciences in Zabrze, Medical University of Silesia, Katowice, Poland, Katowice, Poland; [5]Medical University of Gdansk, Gdansk, Poland

Introduction: Molecular predictors of the response to neoadjuvant radiotherapy (neo-RT) in locally advanced rectal cancer are not known yet. Here we used a combined proteomic and metabolomic approach aimed to search for such potential biomarkers among molecular components of small extracellular vesicle (sEV) present in serum.

Methods: Blood samples were collected directly before surgery from 40 rectal cancer patients treated with neo-RT. Patients were classified as good and poor responders based on tumor regression grading. sEV were isolated from serum using SEC and characterized according to MISEV2018 criteria. Proteomic and metabolomic profiling was performed using LC-MS/MS and GC-MS approaches, respectively. Furthermore, the composition of serum-derived sEV and a whole plasma was analyzed in parallel to assess the biomarker potential of both specimens.

Results: Applied multi-omics approach allowed to reveal several proteins and metabolites, which levels discriminated patients with different responses to neo-RT. These molecules were associated with a few common pathways relevant to response to the treatment, including immune system response, complement activation cascade, platelet functions, metabolism of lipids, and cancer-related signaling pathways. Moreover, the proteome component of sEV has the highest capacity to discriminate samples of good and poor responders (compared to the metabolome of sEV and both components of the whole serum).

Summary/Conclusion: Molecular components of sEV are associated with the response of rectal cancer patients to neo-RT and could be used for the prediction of such response. Besides, the integration of metabolomic and proteomic data reveals novel insights into the role of sEV in the response to cancer treatment.

Funding: This study was supported by the National Science Centre, Poland, Grant 2017/26/D/NZ2/00964 (for US, LM, MP, AW).

PF01.13 | Purity of plasma EVs isolated by size exclusion chromatography is affected by column length and resin

Jillian Bracht[1]; Mandy Los[1]; Edwin van der Pol[2]; Rienk Nieuwland[3]

[1]Department of Clinical Chemistry, Vesicle Observation Center, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Amsterdam, Netherlands; [2]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Amsterdam, Netherlands; [3]Department of Clinical Chemistry; Vesicle Observation Center; Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands

Introduction: Extracellular vesicles (EVs) are a promising source of biomarkers. Size exclusion chromatography (SEC) is now the second most used EV isolation method. SEC isolates particles based on size, but when blood plasma is used as a starting material, platelets, lipoproteins (LPPs) and chylomicrons co-migrate with EVs and thus are confounders for downstream analyses. SEC columns can differ in pore size, cross-linking ability, column length, etc. The aim of this study was to compare the EV yield and purity of different SEC columns.
Methods: We tested four SEC-2B (70 nm cut-off) and four SEC-4B (35 nm cut-off) columns, including home-made-, commercially available 10 mL- (IZON, generation 1 and 2) and commercially available 14 mL (IZON, generation 2) columns. The starting material for each column was 1 mL platelet-free human pooled plasma. Per column, the three fractions (each 0.5 mL) containing the highest concentration of EVs were selected by flow cytometry (Apogee A60; size detection range: 150-1,000 nm), and pooled. We compared the EV yield, LPPs (ApoB ELISA) and protein concentration (Bradford Assay) in the pooled fractions.
Results: The EV yield of SEC-4B was up to 1.8-fold higher compared to SEC-2B, but at the expense of purity, since up to 30-fold higher concentrations of LPPs and proteins were detected. The EV enriched fractions of the generation 2 columns have a lower concentration of proteins and LPPs (up to 2.4- and 17-fold, respectively) compared to the generation 1 columns, but also a 12% lower EV concentration.
Summary/Conclusion: There is a trade-off between EV yield and purity when enriching for EVs, and the most appropriate SEC column for EV purification depends on the downstream application. The generation 1 SEC-4B (10 mL) column has the highest EV yield, whereas the generation 2 SEC-2B (14mL) column yields the purest EVs (1.7- and 1.5-fold higher EV to protein- and EV to LPP ratio compared to SEC-4B, respectively).
Funding: This study was funded by Health Holland (AQrate project)

PF01.14 | Quantitative and reproducible EV immuno-enrichment and depletion

Erika Duggan; John P. Nolan
Scintillion Institute, San Diego, USA

Introduction: Introduction. EVs released from cells are heterogeneous and this can challenge conventional bulk biochemical analysis. Immunofractionation is useful to enrich (or deplete) particular EV sub-sets, but most protocols lack quantitative rigor, and even qualitative assessments of reproducibility are rare. We aim to develop EV immunocapture protocols that are both quantitatively rigorous and reproducible by incorporating single vesicle flow cytometry and bead-based flow cytometry into an optimized immunocapture protocol.
Methods: Methods. EVs preparations were obtained from cell culture supernatants or citrated plasma by pelleting cells (2x 2500xg, 15') and concentration by ultrafiltration where necessary. EV concentration, size and surface cargo expression were measured by single vesicle flow cytometry (vFC). Magnetic beads bearing validated capture antibodies were used to capture EVs bearing specific surface markers. Bead and vesicle fluorescence was measured using qualified and calibrated commercial flow cytometers.
Results: Results. EVs in plasma were measured using vFC to determine the concentration of PLT-derived (CD41 positive) and RBC-derived (CD235ab positive) EVs. Magnetic beads bearing anti-CD41 were used to capture PLT-derived EVs from plasma, and vFC was used to confirm the quantitative depletion of CD41-positive EVs from the plasma. The beads bearing the captured EVs are analyzed directly measured by flow cytometry after staining for CD41 and additional surface markers, provide additional characterization of the immuno-enriched fraction. The combination of pre- and post-immunocapture vFC analysis and direct analysis of the captured EVs on-bead allows the input to downstream molecular or biochemical analyses to be quantitatively defined.
Summary/Conclusion: Conclusions. EV immunocapture can be a useful approach to simplify sample composition for bulk biochemical or molecular analysis, but demonstrating quantitative and reproducible EV capture is generally lacking. Here, we combine immunomagnetic beads with quantitative single vesicle and single bead measurements to provide efficient and scalable EV immunoisolation of EV sub-sets that is both rigorously quantitative and reproducible. Current efforts are directed towards release and recovery of EVs from immunocapture supports in an unperturbed form to downstream functional studies.

PF01.15 | Reproducible automated isolation of extracellular vesicles using high-performance liquid chromatography (HPLC) for clinical applications

Andrew Lai[1]; Dominic Guanzon[2]; Flavio Carrion[3]; Andreas Obermair[2]; Andreas Moller[4]; Carlos Salomon Gallo[2]
[1]*Exosome Biology Laboratory, University of Queensland Centre for Clinical Research, The University of Queensland, Brisbane, Australia;* [2]*The University of Queensland, Brisbane, Australia;* [3]*Universidad del Alba, Santiago, Chile;* [4]*QIMR Berghofer Medical Research Institute, Brisbane, Australia*

Introduction: Current method of isolating extracellular vesicles, such as ultracentrifugation, can be time-consuming, requiring specialised equipment, labour intensive, and quality control can be challenging to achieve. Here we describe the development of a reproducible, automated method to isolate extracellular vesicles using high-performance liquid chromatography (HPLC) without human intervention, targeted for clinical use for which high-throughput and reproducibility are essential.
Methods: A modular HPLC system with a fraction collector coupled with either a multimodal chromatography or size-exclusion column was used for this study. To optimise the HPLC method, multiple replicates of fetal bovine serum or human serum were processed (200 μL), with multiple elution fractions collected from each starting sample. Reproducibility was calculated based on Nanoparticle Tracking Analysis (NTA) and protein quantification data. To further demonstrate the utility of this isolation platform, the isolated EVs from human plasma were subjected to both next-generation sequencing (NGS) and mass-spectrometry proteomic analysis.
Results: A highly reproducible method of isolating extracellular vesicles from bovine and human serum was achieved. The average inter CV for among the technical replicate was below 10% for the particle mean (3.75 %), mode (7.68 %) and protein concentration (8.45 %). This suggests that this method is reproducible with low technical variations. In addition, from one biological sample, using the optimised automated method, most of the total vesicles can be purified into only one tube. Furthermore, the purity of the isolated vesicles was comparable to current isolation techniques and can be used for downstream analyses such as NGS and proteomics.
Summary/Conclusion: The use of automated systems to isolate extracellular vesicles could have important implications in the field of diagnostics, where high-throughput and reproducibility is essential.
Funding: This research was funded by The Lions Medical Research Foundation (2015001964), The Medical Research Future Fund (MRF1199984), National Health and Medical Research Council (NHMRC 1195451), The Donald & Joan Wilson Foundation Ltd (2020000323), and Ovarian Cancer Research Foundation (OCRF, 2018001167).

PF01.17 | Microfluidic Size Exclusion Chromatography (μSEC) for Automated Extracellular Vesicles Purification from Human Plasma

Sheng Yuan Leong[1]; Hong Boon Ong[2]; Jia Jia Lim[3]; Hui Min Tay[4]; Fang Kong[5]; Megha Upadya[5]; Lingyan Gong[3]; Ming Dao[6]; Rinkoo Dalan[7]; Han Wei Hou[8]
[1]School of Mechanical and Aerospace Engineering, Nanyang technological University, Singapore, Singapore; [2]School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore, Singapore; [3]School of Mechanical and Aerospace Engineering, Nanyang technological University, singapore, Singapore; [4]School of Mechanical and Aerospace Engineering, Nanyang Technological University, singapore, Singapore; [5]School of Biological Science, Nanyang Technological University, singapore, Singapore; [6]Department of Material Science and Engineering, Massachusetts Institute of Technology, Cambridge, USA; [7]Endocrine and Diabetes, Tan Tock Seng Hospital, Singapore, Singapore; [8]School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore 639798, Singapore, Singapore, Singapore

Introduction: Extracellular vesicles (EVs) are recognized as next generation diagnostic biomarkers due to their importance in cell-cell communications and the presence of disease-specific biomolecular cargoes. A major bottleneck in EV sample preparation is the inefficient and laborious isolation of EVs from biological samples. Herein, we report an automatable microfluidic platform for EVs purification from human plasma based on the principle of size exclusion chromatography (SEC).
Methods: Using a novel rapid (~20 min) replica molding technique, an fritless microfluidic SEC device (μSEC) with on-chip sample injector was fabricated using commercial UV glue (NOA81) and resin extracted from commercial SEC columns (IZON, cutoff: 75 nm). Sample and sheath flow were introduced into μSEC using two syringe pumps and a simple 3-way valve for sample plug generation.
Results: We first demonstrated on-chip nanoliter sample plug injection (600 nL) with rapid response time (< 1.5 s) using a modified T-junction sample injector. Device performance was validated using fluorescent nanoparticles (50, 200, 500 nm), FITC-albumin, and breast cancer cells (MCF-7)-derived EVs. As a proof-of-concept for clinical applications, we directly isolated EVs (~50 to 200 nm) from undiluted human platelet-poor plasma using μSEC, and showed distinct elution profiles between EVs and proteins based on nanoparticle particle analysis, western blot, bicinchoninic acid protein assays and flow cytometry analysis. The miniaturised μSEC reduced sample volume in commercial SEC from ~100 μL to ~5 μL, and EV elution volume from ~600 μL to ~10 μL.
Summary/Conclusion: We have developed a novel μSEC device for EVs isolation from human plasma. With its modular design and continuous-flow processing, the μSEC device enables easy coupling with downstream EV-based assays for "sample in-answer out" EV profiling or real-time EV screening applications (e.g. EV cargo).
Funding: SMART Innovation Centre (ING-000539 BIO IGN and ING-001058 BIO IGN)
Nanyang Technological University Startup grant and Research Scholarship

PF01.18 | Electrokinetic Isolation of Extracellular Vesicles from Pancreatic, Ovarian and Bladder Cancers Enables Early Detection

Juan P. Hinestrosa; Jean M. Lewis; David Searson; Heath Balcer; Gregor Schroeder
Biological Dynamics, San Diego, USA

Introduction: Extracellular vesicles (EVs) carry cancer biomarkers and are central to emerging liquid biopsy technologies; however, current EV isolation methods balance purity against efficiency. We investigated an alternating current electrokinetic (ACE) method to isolate EVs directly from plasma. We compared the ACE method against differential ultracentrifugation (DU) to study yield, purity and utility. EVs isolated by ACE were then used for detection of early-stage pancreatic, ovarian, and bladder cancers.
Methods: Plasma from control donors (N=532, 54.5% female) and stage I-II cancer patients (N=224, 55.3% female, pancreatic N=78, ovarian N=70, bladder N=76) were obtained from a biorepository. For ACE, EVs were captured onto microarray chips, plasma contaminants washed away, then the current stopped to release purified EVs. For UC, plasma underwent 3 low-speed, then 2 high-speed spins (100,000 g, 70 min). EV particle size and concentration (nanoparticle tracking analysis) and total protein content (bioanalyzer, qubit) were compared. Concentrations of cancer-related proteins were measured using bead-based immunoassay kits, and EV expression patterns analyzed using a machine learning approach for selection of relevant biomarkers that can distinguish cases from controls.
Results: DU had similar particle characteristics (138 nm, ACE; DU, 120 nm), but ACE-purified EVs had less plasma protein contamination. Analysis of biomarker levels (CA19-9, CA125) for ACE EVs showed discrimination between a subset of cancers and controls unlike DU EVs. Expression patterns for ACE EVs were analyzed for all samples, and the multi-cancer classifier had an overall area under the curve (AUC) of 0.93 (95% CI: 0.89-0.97) with an overall sensitivity of 52.2% at a 99.1% specificity. The stage I sensitivity was 42.9% and the stage II sensitivity was 62.9% at the same specificity.
Summary/Conclusion: ACE isolation of EVs is streamlined and provides greater separation from blood proteins than DU. EVs isolated via ACE may facilitate detection of biomarkers and enhance their utility for liquid biopsy assays.
Funding: Funding provided by Biological Dynamics

PF01.19 | Strategies using plasma extracellular vesicles for the discovery of non-invasive biomarkers for neurodegenerative diseases

Hubert Aviolat[1]; David Holzinger[1]; Christoph Kleinert[2]; Liang Jin[3]; Xue Wang[3]; Astrid Wachter[1]; Ameya Kulkarni[4]; Simone Giaisi[1]; Bryan Frey[1]; Chenqi Hu[3]; Michael Schulz[2]; Janina Ried[1]; Yu Tian[3]; Stefan Barghorn[1]; Roland Heym[1]
[1]AbbVie Deutschland GmbH & Co. KG, Neuroscience Discovery, Knollstrasse, Ludwigshafen, Germany, Ludwigshafen, Germany; [2]AbbVie Deutschland GmbH & Co. KG, Drug Metabolism and Pharmacokinetic, Knollstrasse, Ludwigshafen, Germany, Ludwigshafen, Germany; [3]AbbVie Inc., AbbVie Bioresearch Center, 100 Research Drive, Worcester, MA, United States of America, Worcester, USA; [4]AbbVie Inc., Genomics Research Center, 1 N Waukegan Road, Lake County, IL, United States of America, Lake County, USA

Introduction: Analysis of cargo from blood circulating extracellular vesicles (EVs) originating from the central nervous system (CNS) has recently gained great interest for biomarker discovery for neurodegenerative diseases (NDD). Here, we present methods for purifying (1) total EVs and (2) neuron-, astrocyte- and oligodendrocyte-derived EVs from plasma and preview their clinical utility as disease biomarker for Alzheimer's (AD) and Parkinson's disease (PD).
Methods: We developed and validated (1) a workflow for total EV purification from human plasma using size-exclusion chromatography (SEC) followed by EV marker detection, particle size and concentration measurement (TRPS), and lipoprotein contamination assessment. Detection of CNS-enriched or NDD-related protein cargo were achieved by high-throughput, multiplex immunoassays (Olink® Explore) in AD and PD. Deeper analysis was done on α-synuclein (αSyn) in PD to characterize its inside or outside location by protease protection assay, and separation by ultrafiltration (UF) and ultracentrifugation (UC).
We also developed (2) a workflow combining in-silico and mass spectrometry analysis of CSF EVs for the identification of membrane CNS-enriched protein present on EV surface for their immunoprecipitation (IP).
Results: We show (1) that targeted proteomics identified potential biomarker candidates only detectable in EV fraction and not in crude plasma and that a small fraction of αSyn in human plasma mirror EV markers profile elution during SEC. Protease protection assay indicated that the majority of αSyn in the plasma EV fraction is present outside of EVs. UC as well as UF of SEC-purified plasma EVs separated αSyn from EV markers suggesting no or transient interaction between αSyn and plasma EVs. Quantification of αSyn levels in the plasma EV fraction showed no significant difference between 25 PD and 25 healthy control individuals, even after normalization to different EV markers. Finally, αSyn was significantly correlated with CD9 and Flotillin-1 and with αSyn in raw plasma.

In parallel, we also show (2) that our workflow identified two new promising candidates for IP of plasma EVs of neuron- and astrocyte- and one of oligodendrocyte-origin.
Summary/Conclusion: EVs may have great potential as NDD biomarkers but careful characterization is required. Moreover, by the identification and validation of CNS-enriched membrane protein candidates, IP of cell-type specific EVs appears as promising and feasible strategy.
Funding: All authors are employees of AbbVie. The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

PF01.20 | Isolation of Tim4-reactive EVs from cell culture medium of bone marrow derived MSCs and their biological activities

Vi Tran[1]; Takamasa Ishidome[2]; Yuki Marutani[2]; Masayuki Yamane[2]; Sadamu Ozaki[3]; Kodai Sasamoto[4]; Naoko Imawaka[2]; Ryo Ukekawa[2]; Takahiro Nishibu[2]
[1]FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany; [2]FUJIFILM Corporation, Amagasaki-shi, Japan; [3]FUJIFILM Corporation, Minato-ku, Japan; [4]FUJIFILM Wako Pure Chemical Corporation, Amagasaki-shi, Japan

Introduction: Extracellular vesicles (EVs) released from mesenchymal stem cells (MSCs) are expected to be used for the treatment of various diseases. Ultracentrifugation and size exclusion chromatography (SEC) are commonly used for EV purification. However, these methods are not suitable for mass purification of EVs. The phosphatidylserine (PS)-Tim4 affinity method enables to purify PS-positive EVs with high purity and minimum damage. In this study, we verified the usefulness of the PS affinity method for the isolation of EVs from MSCs by using the PS affinity method and evaluated their biological activities. We also developed a Tim4 column-based mass purification method.
Methods: Bone marrow MSC-derived EVs were recovered with Tim4 protein-immobilized magnetic beads and then eluted with buffer containing EDTA. The amount of EVs was evaluated by using ELISA capturing and detecting the EV markers CD9, CD63, or CD81. The anti-inflammatory and anti-fibrotic activities of the EVs were evaluated by the in vitro system. Tim4 column was prepared by immobilizing Tim4 on the resin and packed in a column.
Results: More than 80% of EVs secreted in the medium of the MSCs was reactive to Tim4 beads and were isolated in high purity. Only a smaller portion (c.a. 10%) of the EVs were detected in the Tim4 non-reactive fraction. The anti-inflammatory and anti-fibrotic activities of the Tim4 reactive EVs fraction was higher than those of the EVs obtained by the ultracentrifugation method. The column purification technique also recovered EVs with a similar efficiency as the magnetic bead-based technique.
Summary/Conclusion: The Tim4 protein based PS affinity method was a convenient and effective technique to isolate biologically active EVs from MSC culture supernatant. We are aiming to apply it to the purification of EVs derived from therapeutic MSCs in a larger scale.

PF02: Immune diseases or viral infection

Chair: Maja Kosanović – Institute for the Application of Nuclear Energy, INEP, University of Belgrade

PF02.01 | Detection of inflammatory cytokines released by in vitro 3D cultures of human monocytes stimulated with SARS-CoV-2 spike protein

Alexandra Van Driessche[1]; An Jacobs[1]; Jeroen Sajdak[2]; Pascale berckmans[1]; Inge Nelissen[1]
[1]Flemish Institute for Technological Research (VITO), Health Unit, Boeretang 200, 2400 Mol, Belgium, Mol, Belgium; [2]VITO, Mol, Belgium

Introduction: The most severe outcome of SARS-CoV-2 infection causing COVID-19 lies in its ability to trigger a cytokine storm leading to acute respiratory distress syndrome (ARDS) with high mortality. Timely detection of the onset of this syndrome could significantly impact the treatment and clinical outcome of critically ill patients. Cytokines can be released in soluble form and/or associated with extracellular vesicles (EVs), which might interfere with accurate detection. In this in vitro study, we aimed to mimic a time-kinetic cytokine release response induced by COVID-19, and evaluate the detection of relevant cytokines that are present in different formats.
Methods: We grew a 3D culture of the human Mono Mac 6 cell line in a FiberCell hollow-fiber bioreactor and stimulated these monocytes with SARS-CoV-2 S1 spike protein. Spike protein is responsible for cell entry and inflammatory response of the SARS-CoV-2 virus via ACE2 and TLR4 receptors, which were confirmed to be highly expressed on the cell membrane using flow cytometry. After stimulation, conditioned cell medium (CCM) was harvested at regular intervals for 48 hours, and partially

used for EV isolation using size exclusion chromatography. The EV fractions were characterized for their size, concentration, and protein markers using fluorescent NTA, high-sensitivity flow cytometry and western blotting. We determined the concentration of TNF-α, IL-1β and IL-6 in full CCM, EV-depleted CCM, on isolated EVs' membranes and in the lysate of isolated EVs via an MSD V-PLEX Human Pro-Inflammatory Panel assay.

Results: The bioreactor system allowed us to cultivate a dense cell culture and generate CCM rich in EVs and cytokines at levels suitable for detection and quantification. Quantification using the MSD assay revealed stark contrast between the release formats of the different cytokines, and their levels in unprocessed CCM.

Summary/Conclusion: This research highlights the possibilities of bioreactor systems in EV research and the challenges related to accurate quantification of EV associated cytokines.

Funding: This research was funded by the European Union's H2020 Safe-N-Medtech project (grant number 814607) and VITO nv.

PF02.02 | Differential exosome profiles impact on T cell function in COVID-19

Anna Schurich[1]; Molly George[2]; Jenifer Sanchez[2]; Katie Flaherty[2]; Christina Rollings[3]; Matthew Fish[2]; David Fear[2]; Manu Shankar Hari[2]; Linda Sinclair[3]; Peter Irving[2]

[1]King's College London, School of Immunobiology and Microbial Sciences, London, United Kingdom; [2]King's College London, School of Immunology and Microbial Sciences, London, United Kingdom; [3]University of Dundee, Division of Cell Signalling and Immunology, Dundee, United Kingdom

Introduction: As important mediators of intercellular communication alterations in exosomes have been reported in various diseases, including in cancer and viral infections. Here we explore the profile of COVID-19 patient-derived exosomes and their possible impact on the crucial T cell mediated immune response.

Methods: Our cohort included healthy donors, patients with severe acute infection requiring hospitalisation and donors recovered from mild or severe COVID-19. Cell free plasma was used to purify exosomes by Miltenyi MACS exosome isolation kit for CD63+ selection. Exosome origin was established by staining exosomes for more than 30 cell-specific surface markers and profiles were assessed by flowcytometry. For functional experiments human T cells from healthy donors were pre-activated, rested and co-cultured with 2.5ug patient-derived exosome on two consecutive days, before restimulation for assessment of phenotypic, metabolic and functional changes.

Results: Exosome profiles related to disease severity, indicating that different subsets of cells were contributing to the anti-viral response in mild and severe infection, while vaccination mimicked aspects of viral infection. Changes were maintained for several weeks post recovery and after vaccination. Interestingly, alterations in CD8 T cell-derived exosomes were amongst the most pronounced detected. Patient-derived exosomes influenced cytokine production, activation status and metabolic profile of T cells in vitro, with exosomes derived from patients recovered from mild disease overall inducing immune suppression, while those from patients recovered from severe disease led to increased T cell activation and corresponding metabolic reprogramming.

Summary/Conclusion: We find that exosomes in the circulation sustain distinct profiles both post vaccination and during an immune response, making them interesting candidates as biomarkers. Furthermore we show that patient derived exosomes influence T cell function indicating their contribution to shaping anti-viral immunity and clinical outcome.

Funding: British Society of Antimicrobial Chemotherapy (BSAC) and Miltenyi Biotec

PF02.03 | Proteomic characterization of extracellular vesicles recovered from plasma and bone marrow from a patient with active leishmaniasis in the context of HIV co-infection

Inês Costa[1]; Ana Isabel Pinto[1]; Sofia S. Esteves[2]; Nuno Santarém[1]; Cátia Caldas[3]; Carmen Fernandez Becerra[4]; Anabela Cordeiro-da-Silva[1]

[1]Parasite Disease Group, Institute for Molecular and Cell Biology, Instituto de Investigação e Inovação em Saúde, Universidade do Porto & Serviço de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia da Universidade do Porto, Portugal, Porto, Portugal; [2]Parasite Disease Group, Institute for Molecular and Cell Biology, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal & Serviço de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia da Universidade do Porto, Porto, Portugal; [3]Serviço de Doenças Infeciosas do Centro Hospitalar Universitário de São João, Porto, Portugal e Faculdade de Medicina da Universidade do Porto., Porto, Portugal; [4]ISGlobal IGTP, Barcelona, Spain

Introduction: Leishmania-HIV co-infection has been an emergent problem in the last twenty years and has been reported in 35 endemic countries. HIV-infected people are especially vulnerable to visceral leishmaniasis (VL), and VL accelerates HIV replication and advancement to AIDS. VL and HIV co-infection is characterized by significantly lower cure rates, higher relapse

risk and mortality when compared with HIV-negative VL patients. The current disease management options are limited and require improvement to control infection progression. Therefore, new biomarkers are required to address complex situations like asymptomatic disease, co-infection and treatment assessment. In this context, extracellular vesicles (EVs) are still an untapped resource with great potential for biomarker development. Thus, EVs from an HIV+ patient with active Leishmania infection for more than 8 years were recovered from plasma and bone marrow, characterized by proteomics and compared to non-infected patients, six healthy samples and five HIV+ samples.
Methods: The purification and characterization of EVs were performed by SEC. EVs were characterized by bead-based flow cytometry assay, using specific EVs markers, such as CD5L, CD71 and CD9. The proteins present in these EVs were compared against databases for HIV, Homo sapiens sapiens and Leishmania infantum.
Results: The proteomic analysis confirmed the presence of several EVs biomarkers, in all samples, confirming the capacity to detect exosomal content. Two peptides matching Leishmania proteins were detected only in the infected patient and absent in non-infected controls. An uncharacterized Leishmania protein and tubulin beta chain were detected in the EVs recovered from plasma and bone marrow, respectively.
Summary/Conclusion: The potential of these proteins as biomarkers is undergoing evaluation. We will be following up and analysing further plasma and bone marrow samples recovered from this patient at the same time point.
Funding: This work was financed by the Portuguese Foundation for Science and Technology (FCT) through the PhD scholarships SFRH/BD/140177/2018, SFRH/BD/140119/2018 and by Individual Support Grant CEECIND/04304/2017 awarded to Inês Costa, Sofia Esteves, and Ana Pinto, respectively.
The full legal and ethical requirements have been considered in close collaboration with the Research Integrity Unit of i3S. The participants consents were designed to cover the potential usage of theirs samples and data in other Biomedical studies, the participants can withdraw at any moment, the data is anonymized and treated in compliance with the data protection measures imposed by European data protection legislation constituted, namely, by the Regulation (EU) 2016/679, already implemented in i3S.

PF02.04 | Evaluation of extracellular vesicles (EVs) and antibodies from chronic Chagas disease patients after infection reactivation

Rafael Madeira Madeira[1]; Paula Meneghetti[1]; Nicholy Lozano[1]; Vera Lucia Pereira-Chioccola[2]; Ana Claudia Torrecilhas[3]
[1]UNIFESP, Sao Paulo, Brazil; [2]Instituto Adolfo Lutz (IAL), Sao Paulo, Brazil; [3]Federal University of Sao Paulo, Sao Paulo, Brazil

Introduction: Chagas disease is a neglected zoonosis characterized as a tropical infection caused by Trypanosoma cruzi. This infection is widely distributed in the Americas, with an estimated 7 million people infected worldwide. The immigration of infected people from endemic countries contributes to the spread of Chagas disease in non-endemic continents. The research for biomarkers using extracellular vesicles (EVs) circulating in samples of chronic chagasic patients is still incipient. Our aim was to evaluate and characterization of the specific markers express in circulating EVs isolated from the blood of chagasic patients with immunosuppressive treatment after transplantation, cancer or patients with HIV co-infection
Methods: Patients' blood samples were collected using EDTA (ethylenediamine tetraacetic acid). Then, it was centrifuged for 15 minutes and PCR (polymerase chain reaction) was performed to detect the parasites. This was followed by the purification of EVs for the analysis by NTA, chemiluminescent enzyme immunoassay (CL-ELISA) to detect the parasite-specific glycoconjugate, such as Transialidase (TS), alpha-Gal epitopes.
Results: The cohort of chronic chagasic patients (PCC) were composed the according to clinical forms; Group I was composed of 52% patients with recurrence of infection by immunosuppressive treatment after transplantation or cancer; Group II consisted of 40% patients with the chronic cardiac form of Chagas disease; Group III consisted 8% of the patients with HIV/Chagas disease co-infection. The EVs mean size were 150 nm for all patients samples. The concentration of EVs was around 1x108 to 1x109 particles/mL for all samples. The CL-ELISA results showed an increase of α-Gal epitopes than TS in samples from EVs isolated from CCP
Summary/Conclusion: The isolation of the total circulating extracellular vesicles from CCP can serve as an important basis for further studies on biomarker research
Funding: FAPESP2020/07870-4; FAPESP2019/15909-0 and CNPq Universal 408186/2018-6

PF02.05 | Analysis of Extracellular Vesicles isolated from patients with SARS-CoV-2

Jaques Novaes de Carvalho[1]; Flávio Freitas[2]; Reinaldo Salomão[1]; Ana Claudia Torrecilhas[3]
[1]UNIFESP, Sao Paulo, Brazil; [2]UNIFESP and Setor de Medicina Intensiva, Hospital Sepaco, Sao Paulo, Brazil; [3]Federal University of Sao Paulo, Sao Paulo, Brazil

Introduction: In Brazil, 22,246,276 cases of COVID-19 were confirmed with 619,000 deaths with this having an unprecedented economic and public health impact of the COVID-19 pandemic. The virus causes severe acute respiratory syndrome 2 (SARS-CoV-2) and infected host cells by binding the spike protein (S) to its receptor angiotensin-2 converting enzyme (ACE2) and subsequent membrane fusion. The EVs transfer of viral components and contributing in the spread of the human infection, but little research has been done on the role of EVs during viral infection. Our aim is to analyze the EVs released in the blood of patients infected with SARS-CoV-2 and characterization this particles in the role in the human infection
Methods: We analyzed plasma samples from 42 patients with COVID-19 from Hospital Sepaco in São Paulo, Brazil (positive RT-PCR tests for SARS-CoV-2) and 19 controls samples healthy individuals. The EVS were isolated by ultracentrifugation at 100,000x g for 16 hours. The EVs were analyzed using the Nanoparticle Tracking Analysis (NTA) to verify the size and the concentration, correlating them with the conditions of the patients with a COVID-19.
Results: NTA analyses showed that EVs from patients and controls are of similar size, 200nm diameter. In the other hand, EVs concentrations were 3.6+109 particles/mL(COVID patients) and controls 1.5+1010 particles/mL. The age of patients older than 60 years had a higher concentration of 1.7 + 1010 particles/mL. the group of the 40 years and 59 years with 1.62 + 1010 particles / mL and those under 39 years of age 9, 9 +109 particles/mL. Regarding outcome, those who died because of COVID-19 had the highest concentration of EVs 1.6 + 1010 particles/mL and those who were discharged from hospital 1.5 + 1010
Summary/Conclusion: EVs isolated from patients with COVID-19 may be a new candidate biomarker during virus infection openning a new perspective for the prognosis and diagnosis of human infections
Funding: FAPESP, CAPES and CNPq

PF02.06 | Extracellular vesicles released by picornavirus-infected cells trigger an antiviral response in immune cells

Xinyi Pei[1]; Kyra A.Y. Defourny[1]; Frank J.M. Van Kuppeveld[2]; Esther N.M Nolte-'t Hoen[1]
[1]Division of Cell Biology, Metabolism & Cancer, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands; [2]Virology Section, Division Infectious Diseases & Immunology, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands

Introduction: Naked viruses of the picornaviridae family commonly spread via lysis of infected cells. However, recent studies indicate that, prior to cell lysis, naked viruses can be packaged and released inside extracellular vesicles (EVs). The EV membrane protects enclosed virus particles against neutralizing antibodies, thereby facilitating virus spread. However, it is unknown how immune cells respond to virus-induced EVs. Previously, we demonstrated that the picornavirus Encephalomyocarditis virus (EMCV) induces the release of various virus-carrying and other EV subsets. Here, we investigated how virus-induced EVs affect the antiviral response of immune cells.
Methods: EVs were isolated and purified from the supernatant of EMCV-infected cells, via differential ultracentrifugation and Optiprep density gradient. EVs were characterized by western blot, high resolution flow cytometry and TCID50 assay. Human peripheral blood mononuclear cells (PBMC) were incubated with EVs from (non-)infected cells or with naked virus, after which cytokine production was assessed by RT-qPCR, ELISA, and flow cytometry.
Results: EVs from EMCV-infected cells, but not those from non-infected cells, stimulated the immune cells to produce the antiviral cytokines IFN type I, II, III IP-10 and inflammatory cytokines IL-6, IL-8 and TNF-a. Intriguingly, we observed that EVs from infected cells, of which a subset contained naked virus, were more potent in triggering overall IFN type I production and IP-10 production in CD14+ monocytes than a similar amount of naked virus.
Summary/Conclusion: Although EVs can function as pro-viral factor by protecting enclosed naked virus particles from neutralizing antibodies, our data indicates that virus-induced EVs may also alarm the immune system by triggering the production of antiviral/inflammatory cytokines. These findings shed light on the diverse effects of virus-induced EVs on the antiviral immune response.
Funding: This work was funded by The Netherlands Organisation for Scientific Research (NWO-ALW grant number ALWOP.351) and China Scholarship Council.

PF02.07 | MSC-EV preparations with and without immunomodulatory capabilities reveal comparable, detergent resistant Ecto-5'-nucleotidase (CD73) activities

Fabiola Nardi Bauer[1]; Tobias Tertel[1]; Oumaima Stambouli[1]; Robin Dittrich[1]; Simon Staubach[1]; Verena Börger[1]; Sven Brandau[2]; Bernd Giebel[1]

[1]*Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany;* [2]*Department of Otorhinolaryngology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany*

Introduction: Extracellular vesicles (EVs) derived from conditioned media of human mesenchymal stem/stromal cells (MSCs) reveal immunomodulatory capabilities in a number of different in vitro assays and preclinical models. Recently, we compared the immunomodulatory potential of independent MSC-EV preparations in a multi-donor mixed lymphocyte reaction (mdMLR) assay and in an optimized steroid refractory acute Graft-versus-Host Disease (aGvHD) mouse model. We observed, only a proportion of our applied MSC-EV preparations revealed immunomodulatory capabilities. In more detail, only MSC-EV preparations with mdMLR immunomodulating activities were able to suppress GvHD symptoms in vivo and vice versa. Since the mdMLR assay is quite complex and depends on primary human cells of different donors, we sought to establish a quantitative assay that is much easier to standardize and fulfills requirements for becoming qualified as a potency assay.
Methods: To this end, we adopted an enzymatic assay that monitors the 5' ectonucleotidase activity of CD73, one of the bona fide MSC marker proteins that is also prominently recovered on MSC-EVs. The enzymatic activity of CD73 was evaluated by the quantification of free phosphate that is formed upon CD73-mediated cleavage of adenosine-monophosophat into adenosine and free phosphate.
Results: Here, we report that that recorded 5' ectonucleotidase activities of different MSC-EV preparations does not correlate with the immunomodulatory capabilities of the MSC-EV preparations that had been monitored in the mdMLR assay or the aGvHD mice, respectively. Furthermore, this activity is hardly affected by EV-destroying detergents.
Summary/Conclusion: Thus, for the evaluation of the immunomodulatory activities of independent MSC-EV preparations produced under our standardized procedure, including their integrity, the CD73 enzyme activity is not indicative. Defining, setting up and validating an appropriate assay as potency assay for the evaluation of independent MSC-EV products remains challenging.

PF02.08 | Extracellular vesicles isolated from human plasma as potential diagnostic tool for osteoarthritis

Jana Janockova[1]; Jana Matejova[2]; Marko Moravek[1]; Lucia Slovinska[1]; Denisa Harvanova[1]; Timea Spakova[1]; Jan Rosocha[1]
[1]*Associated Tissue Bank, Faculty of Medicine, P. J. Šafárik University in Košice, Slovakia, Kosice, Slovakia;* [2]*Associated Tissue Bank, Faculty of Medicine, P. J. Šafárik University in Košice, Slovakia, Košice, Slovakia*

Introduction: Extracellular vesicles (EVs) are membranous structures derived from cells and play an essential role in intercellular communication via transfer of bioactive proteins, lipids and RNAs. Both, normal and pathological cells are able to release various types of EVs with different physiological properties, functions and composition. In general, EVs are classified based on their biogenesis to the three main classes – apoptotic bodies, microvesicles and exosomes, which have different size and biogenesis. The proper definition and characterization of the subtypes of EVs varies in the literature, therefore in this study the collective term EVs is used. One of the great sources of EVs is peripheral blood plasma, as it is easily collected and does not usually cause excessive discomfort for patients. However, blood plasma contains large amounts of soluble proteins and aggregates which may cause a problem concerning of clinical grade EVs production in sufficient quantity, high efficiency and without impurities. This work is focused on the isolation of EVs from human plasma from healthy donors and osteoarthritis patients. Osteoarthritis (OA) is a disease of the entire joint including structural changes in articular cartilage, subchondral bone, synovial membrane and muscles around the joint. It is known that EVs reflect the physiological and pathological condition of cells and may operate as an effective tool for biomarkers diagnosis of OA.
Methods: qEV Izon columns based on size exclusion chromatography were used for EVs isolation. Isolated exosomes were further identified by vesicle size using NTA analysis. Subsequent qualitative and quantitative analysis using flow cytometry, western blotting and ELISA methods were applied for identification of typically surface markers (CD63, CD9, CD81, TSG101, HSP70) expression.
Results: Results showed the successful preparation and characterization of EVs from human plasma which were confirmed in terms of their size and protein components. NTA analysis showed isolated particles in the size range of 30–150 nm. The presence of EVs markers was different between studied groups.
Summary/Conclusion: Obtained results suggested potential usage of EVs in the OA diagnostic.
Funding: Acknowledgment: This publication is the result of the project implementation: "Open scientific community for modern interdisciplinary research in medicine (OPENMED)", ITMS2014+: 313011V455 supported by the Operational Programme Integrated Infrastructure, funded by the ERDF. This research was also supported by the Slovak Research and Development Agency under the contract No. APVV-17-0118 and the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and Slovak Academy of Sciences (VEGA, No. 1/0229/20).

PF02.09 | Phenotypic characterization of small extracellular vesicles in synovial fluid of osteoarthritic patients

Álvaro Compañ Bertomeu[1]; María Isabel Guillén[1]; María Luisa Ferrándiz[1]; María José Alcaraz[1]; Ignacio Muñoz[2]
[1]*Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València, Universitat de València, Valencia, Spain;* [2]*Instituto Musculoesquelético Europeo (IMSKE), Valencia, Spain*

Introduction: Osteoarthritis (OA) is the most common joint problem. In OA, oxidative stress and inflammation occur, which causes destruction of the articular cartilage with an increase in the volume of synovial fluid (SF). Extracellular vesicles (EV) are lipid vesicles which contain a bioactive cargo of RNAs, proteins and lipids. In OA, EV have immunomodulatory functionality and chondroprotective effect. However, much remains to be discovered about the contributions of SF EV to OA pathophysiology. Our objective was to characterise small EV contained in SF of OA patients as a possible biomarker.
Methods: The SF was obtained from 25 patients diagnosed with OA undergoing knee replacement with ages between 50 and 79 years old. SF is processed following the differential centrifugation standard to get rid of intact cells, debris, and organelles. For small EV isolation we used Exo-spinTM midi columns Purification Kit, and IZON's qNano to quantify them. Finally, for the phenotypic characterisation we used CD9 and CD81, as transmembrane markers, PDCD6IP (ALIX) and ESCRT-I/II/III (TSG101) as cytosolic protein markers and Calnexin (CNX) as a negative control.
Results: All patients presented leukocyte values and sedimentation rate within normal ranges, indicating that there is no inflammation due to other causes. The colorimetric study of the SF indicated that all the samples had a similar appearance: yellowish colour and the same viscosity. The results of qNano study showed a mean size of 185,44±34,26 nm of particle and a raw concentration of 7,71E+8±1,87E+8 EV/mL. The phenotypic study showed that isolated EV were positive for CD9, CD81, ALIX, TSG101 and negative for CNX.
Summary/Conclusion: This preliminary study indicates that small EV of OA SF have phenotypic markers characteristic of this type of EV. These small EV could be a diagnostic tool to know the severity and evolution of OA.
Funding: Projects SAF2017-85806-R (Ministerio de Economía, Industria y Competitividad).

PF02.10 | Biomarkers analysis of extracellular vesicles (EVs) derived from Peripheral Blood Mononuclear Cells (PBMCs) of patients with Inflammatory Bowel Disease (IBD)

Emilia Emilia Scharrig; Claudio Giraudo
Thomas Jefferson University, Philadelphia, USA

Introduction: IBD is a hyperinflammatory disorder that manifests with a severely compromised intestine, combined with dysregulation of the immune system. Although several factors have been associated with the disease, the pathophysiological mechanism is still not well-understood. Recent research suggests that EVs may be involved in the progression of IBD inflammation, still little is known about the contribution of immune cells-derived EVs to this pathology.
Methods: To inquire about potential biomarkers of IBD we evaluate the protein composition of PBMCs-derived EVs. They were obtained by differential ultracentrifugation, filtration, and washing from the supernatant of PBMCs (3 IBD Patients and 3 Healthy Controls -HC-) cultured in serum-free media. Size and concentration were analyzed using a NanoSight instrument. The presence of EVs markers (CD63, CD81, Hsp70) was analyzed by immunoblotting. The proteomic profile was executed by MS/MS. Bioinformatics analysis was performed using Perseus. String database was used to predict relations between proteins, and functional-enrichment analysis was done with FunRich.
Results: More than 820 proteins were identified in each sample. The patient EVs shared a core of 754 proteins. Several proteins were under-expressed in patients like SLC1A5, SHMT2, ARF3, SAR1A, and others were more abundant than in controls: VIM, ORM1, RPL6, A1BG. Interestingly, the absent proteins in patients were implicated in lipid metabolism (HADH) and proteosome (PSMA4) between others. The unique ones were implicated in cell adhesion (FLG2) and others.
Summary/Conclusion: In conclusion, our studies show that the EVs from PBMCs of IBD patients contain a specific protein composition that differs from those of HC, having the potential to be used as a biomarker for diagnostics and progression of the disease. Additionally, these data provide new insights into our understanding of the cellular pathways that might be involved in the pathogenesis of IBD. The studies were performed with corresponding IRB approvals.

PF02.11 | Protein Analysis of Plasma Extracellular Vesicles Isolated by Optimized Size Exclusion Chromatography Revealed ADAM10 as an EV-marker for Rheumatoid Arthritis

Onno Arntz[1]; Rogier Thurlings[1]; Michiel Vermeulen[2]; Peter van der Kraan[1]; Marije Koenders[1]; Fons van de Loo[1]
[1]RadboudUMC, Nijmegen, Netherlands; [2]Radboud Institute for Molecular Life Sciences, Nijmegen, Netherlands

Introduction: Isolation of pure extracellular vesicles (EVs) from plasma is still a challenge. Size exclusion chromatography (SEC) has become the common method to isolate EVs from plasma but recently, we discovered that conventional SEC columns does lead to impure plasma EVs (pEVs)(1). In this study, protein profiles of optimized SEC isolated pEVs from rheumatoid arthritis (RA), osteoarthritis (OA) patients, and healthy controls (HC) were determined to discover potential pEV biomarkers.
1. Arntz O.J. PLoS One. 2020
Methods: pEVs were isolated from well-defined RA- and OA-patients, and HC by optimized SEC. After characterization by Nanosight-track-analysis, exosome markers were determined by western blot (WB) and protein content and composition by respectively, microBCA and mass spectrometry (MS).
Results: Concentration, size, and protein content of RA- (9.63 x109 particles/ml, 120 nm, 0.30 fg protein/particle), OA- (9.25 x109 particles/ml, 114 nm, 0.31 fg protein/particle), and HC-pEVs (9.30x109 particles/ml, 114 nm, 0.26 fg protein/particle) donors were similar. Exosome markers CD9, ALIX, and HSP-70 were present on isolated pEVs, determined by WB. Other exosome markers like CD81, CD63 were detected by MS. The proteomic profile of pEVs revealed high overlap (417 proteins, 76% of total), while 12 proteins seems unique for RA-pEVs and 4 proteins for OA-pEVs. Eight proteins were only detectable in HC-pEVs. One protein was significantly present in RA-pEVs (ADAM10) and of three proteins (SPN, AQP1, COL6A2), the levels were statistically significant higher. No significant differences were observed in OA-pEVs compared to RA and HC. The significant RA-pEV proteins were linked to muscle diseases by FunRich proteomics software analysis, with ADAM10 as central protein in the functional interaction network.
Summary/Conclusion: The observed different protein profile in RA-pEVs compared to HC and OA patients, could serve as a fingerprint for this disease and ADAM10 seems to be a specific pEV marker for RA.

PF02.12 | Characterisation of Circulating Extracellular Vesicles from Rivaroxaban-Treated VTE Patients Reveals an Altered Inflammatory State

Luisa Weiss[1]; Paulina Szklanna[1]; Tadhg Prendiville[2]; Karl Egan[1]; Sarah Kelliher[2]; Áine Lennon[2]; Eugene Dillon[3]; Alfonso Blanco[4]; Barry Kevane[2]; Seán Murphy[5]; Fionnuala Ní Áinle[2]; Patricia Maguire[1]
[1]UCD Conway SPHERE Research Group, Conway Institute, University College Dublin, Ireland, Dublin, Ireland; [2]Department for Haematology, Mater Misericordiae University Hospital, Ireland, Dublin, Ireland; [3]Mass Spectrometry Core Facility, Conway Institute, University College Dublin, Ireland, Dublin, Ireland; [4]Flow Cytometry Core Facility, Conway Institute, University College Dublin, Ireland, Dublin, Ireland; [5]Department for Stroke Medicine, Mater Misericordiae University Hospital, Ireland, Dublin, Ireland

Introduction: Venous Thromboembolism (VTE) remains a significant cause of morbidity and mortality worldwide. Rivaroxaban, a direct oral factor Xa inhibitor, mediates anti-inflammatory and cardioprotective effects besides its well-established anticoagulant properties; yet these remain poorly characterized. Extracellular vesicles (EVs) are important messengers regulating a myriad of (patho)physiological processes and, as considered proinflammatory entities, may be highly relevant to the pathophysiology of VTE. The effects of rivaroxaban on circulating EVs in VTE patients remain unknown and we hypothesized that its anti-inflammatory properties are reflected upon differential molecular profiles of circulating EVs.
Methods: VTE patients anticoagulated with either Rivaroxaban or warfarin were recruited following informed written consent. We used single vesicle analysis by nanoparticle tracking and flow cytometry to comprehensively characterise the concentration and size of small (0-200nm) and large (200-1000nm) EVs, respectively. Proteomic cargoes of enriched vesicular fractions were assessed using a label-free quantification mass spectrometry approach.
Results: While total small and large EV counts did not differ, we found a statistically significant, selective reduction in the proportion of 400-700nm large EVs in Rivaroxaban-treated patients. Proteomic profiling of circulating EV cargoes from these patients robustly quantified 182 proteins, with a cluster of proteins involved in negative feedback regulation of inflammatory and coagulation pathways found to be enriched in Rivaroxaban-treated patients.
Summary/Conclusion: Our results indicate an ameliorated baseline pro-inflammatory state of VTE patients anticoagulated with Rivaroxaban and establish that EV proteomic signatures may be powerful biological sensors of rivaroxaban's anti-inflammatory potential. These findings are of translational relevance towards characterizing the underlying anti-inflammatory and cardioprotective mechanisms associated with this therapy.
Funding: Bayer AG

PF02.13 | Anti-viral Effect of MSC-derived Extracellular Vesicles in models of Virus Induced Acute Respiratory Distress Syndrome

Byong Seung Cho; Jun Ho Lee; So Jeong Kim; Hyeong Tae Yu; Yong-In Yoo; Hyungtaek Jeon
ExoCoBio Inc., Seoul, Republic of Korea

Introduction: Both corona- and influenza viruses have the capacity to induce severe respiratory symptoms, and even lung injury with significant mortality, as illustrated by the current COVID-19 pandemic. In this study, we aimed to determine whether adipose tissue-derived mesenchymal stem cell EVs (ASC-EVs) can attenuate acute lung injury (ALI) induced by either H1N1 influenza A virus and SARS-CoV-2, and by which mechanisms this may occur.
Methods: EVs were isolated from ASC conditioned media by tangential flow filtration, and were characterized according to MISEV recommendation. Influenza A/Puerto Rico/08/1934 (H1N1) and SARS-CoV-2 (NCCP43326) were used to model highly pathogenic human influenza A and SARS-CoV-2 virus infection, respectively, in mice and Syrian hamsters respectively. All animal experiments were performed under animal biosafety level 2 or 3 conditions.
Results: Treatment of ASC-EVs, from 0.15 x 10^9 to 5.0 x 10^9 particles/mL, showed inhibitory activities on cytopathic effects and replication of H1N1 and SARS-CoV-2 in MDCK cells and Vero E6 cells, respectively. In the mouse H1N1 influenza A virus induced acute lung injury (ALI) model, total of 4 daily injections of 1 x 10^10 particles of ASC-EVs administration resulted in significantly increased survival rate by 30 – 40%, recovery of body weight, and improved clinical disease score from 9 dpi. In the Syrian hamster SARS-CoV-2 induced ALI model, total of 4 daily injections of ASC-EVs at a dose of 3 x 10^10 or 1 x 10^10 particles resulted recovery of body weights from 5 dpi, in a dose-dependent manner, by 9.7% - 12.75%. Further, ASC-EV treatment resulted in significant downregulation of viral genes in lung tissue. To elucidate the molecular mechanisms of the observed anti-viral effects of ASC-EVs, the role of multiple miRNAs and proteins present in the ASC-EVs were assessed in vitro. We identified one specific protein that conveyed anti-viral efficacy against the two studied viruses including SARS-CoV-2. Loss and gain of function studies revealed that this protein may be involved in the anti-viral efficacy of the ASC-EVs.
Summary/Conclusion: Our findings support the concept that that ASC-EVs have anti-viral effects against virus induced ALI, which may have implications for the treatment of not only treatment COVID-19, but also future ALI-inducing virus diseases.

PF02.14 | Plant polyphenolics as potential antiviral candidates against SARS-CoV-2

Farrukh Aqil[1]; Raghuram Kandimalla[2]; Divyasha Saxena[3]; Margaret Wallen[4]; Disha Moholkar[2]; Jeyaprakash Jeyabalan[4]; Wendy Spencer[4]; Jon Gabbard[3]; Kenneth Palmer[3]; Ramesh C. Gupta[5]
[1]University of Louisville, Louisville, USA; [2]University of Louisville, Louisville, KY 40202, Louisville, USA; [3]Center for Predictive Medicine, University of Louisville, Louisville, KY 40202, USA, Louisville, USA; [4]3P biotechnologies Inc., Louisville, KY 40202, Louisville, USA; [5]3P biotechnologies and Department of Pharmecology & Toxicology and Brown Cancer Center, University of Louisville, Louisville, KY 40202, Louisville, USA

Introduction: The incidence of viral pandemics has been one of many side effects of increased international commerce and travel. The most recent outbreak, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), started in late 2019. The threat of this virus has not waned as mutations continue to produce new variants capable of evading immunity acquired through vaccines and infection. Limited efficacious antiviral drugs and continued evolution of vaccine-resistant strains underscores the need for development of effective antiviral therapeutics. Here, we develop an easy-to-use BSL2 system to screen potential antiviral drugs against SARS-CoV-2. We show significant antiviral effects of plant polyphenolics in vitro. Select polyphenolics were advanced for testing against the live virus.
Methods: Exosomes were isolated from bovine colostrum powder by rehydration and differential centrifugation. We first established the exosome-PEI matrix (EPM)-based system to express key SARS-CoV-2 proteins, a subunit of the spike glycoprotein (S1), nucleocapsid (N), and replicase (RdRp) using expression plasmids. HEK293T cells were transfected with all three plasmids simultaneously and treated with test polyphenolics for 48 h and analyzed by Western blot. To determine potential antiviral activity, Vero E6 cells (20,000 cells/well) were seeded in 96-well plate and co-treated with putative antiviral polyphenolics and SARS-CoV-2 (200 pfu/well, USA/WA1/2020 strain). After 72 h incubation, cells were imaged for cytopathic effect (CPE) and cell viability measured using MTT assay.
Results: Our data show the EPM system efficiently introduced all three SARS-CoV-2 proteins (S1, N and RdRp) in HEK293T cells. Systematic screening of numerous compounds showed varying ability to inhibit their expression. Two agents – pomegranate-derived punicalagins (PC) and bilberry-derived anthocyanidins (Anthos) - dose dependently inhibited expression of all the three proteins. Co-treatment with PC and Anthos reduced virus induced cell death in Vero E6 cells with an EC50 value of 18 μM and EC50 73 μM, respectively. The antiviral activity of PC was confirmed by cell imaging for CPE where the viral replication was

completely inhibited at a higher concentration (EC90; 77 μM). No cytotoxicity was observed in Vero E6 cells up to a concentration of 200 μM.

Summary/Conclusion: The EPM vector introduces plasmids for high expression of viral proteins and provides an alternative model for screening antiviral therapeutics. Two potential antiviral polyphenolics were identified that have strong antiviral effects in live virus challenge assays. Animal studies in virus-specific animal modelare warranted for the further validation of the model system and plant therapeutics.

Funding: Supported from 3P Biotechnologies & from the Duggan Endowment.

PF03: Technologies and methods: Engineering and loading EVs and other particles

Chair: Dhanu Gupta – Department of laboratory medicine, Karolinska Institutet

PF03.01 | A facile method for PEGylation of plant-derived nanovesicles using PEG precipitation

Hyeyeon Joo[1]; Van Quy Nguyen[2]; Wooram Um[1]; Yong Woo Cho[3]; Jae Hyung Park[1]

[1]Sungkyunkwan Universtiy, Suwon-si, Republic of Korea; [2]Sungkyunkwan University, Suwon-si, Republic of Korea; [3]ExoStemTech Inc., Seongdong-gu, Republic of Korea

Introduction: Extracellular vesicles (EVs) are extracellular membrane vesicles which are endogenously released from cells. Dependent on their origin, EVs contain various bioactive components such as RNAs, lipids and proteins, representing unique biological functions. Therefore, EVs have been identified as mediators in cell-to-cell communications. Especially, plant-derived nanovesicles (PVs) have been introduced as a novel strategy due to its safety, cost-effectiveness and clinical favorability since they are derived from natural sources. However, the poor stability and rapid clearance have restricted the in vivo clinical applications. Here, we developed a highly versatile method to control the in vivo bioavailability by modifying their surfaces with poly(ethylene glycol) (PEG). PEGylated PVs (P-PVs) improved their stability by reducing their aggregation through physicochemical interactions.

Methods: Extracellular nanovesicles were originated from sprouts of Brassica oleracea, known to have abundant antioxidants and anti-inflammatory phytochemicals. When excessive amount of PEG was introduced, the hydrophobic exclusion allowed PEG to preferentially precipitate PVs. Physicochemical properties were investigated by assessing size distribution, surface zeta potential and transmission electron microscopy (TEM). Finally, internal protein concentrations of PVs and P-PVs were quantitatively compared to explore the bioactivity loss of PVs.

Results: To optimize the proper PEG contents, three different concentrations (8, 10, and 12 wt%) were prepared. The results of size analysis and zeta potential exhibited that PEG has successfully attached to the PVs surface depending on the concentration levels. Additionally, we assessed antioxidant activity as a representative function of PVs and P-PVs and no remarkable differences were found in 8 and 10% samples, implying the internal content and its activities can be preserved in lower concentration than 12 wt%.

Summary/Conclusion: In summary, we introduced the PEG precipitation as a facile method for surface editing of PVs. Especially, PEGylation process by hydrophobic exclusion showed a highest yield under 10 wt% PEG. Size and zeta potential results were maintained for 24 hours, and TEM images confirmed the size stability in presence of 10 wt% PEG. Additionally, the protein concentration and antioxidant activity test exhibited that this method does not affect to the quantity and quality of internal contents. Overall, it is evident that PEGylation of PVs may be highly useful methodology for modulating PVs with improved stability.

Funding: This research was supported by the Korean Fund for Regenerative Medicine(KFRM) grant funded by the Korea government(the Ministry of Science and ICT, the Ministry of Health & Welfare). (21A0503L1-11)

PF03.02 | Biophysical studies of the interaction of TNAP with the lipid membrane during biomineralization

Bruno Z. Favarin[1]; Heitor G. Sebinelli[2]; Thierry Azaïs[3]; Nadine Nassif[3]; Ana Paula Ramos[4]; Massimo Bottini[5]; José Luis Millán[6]; Antonio josé da Costa-Filho[7]; Pietro Ciancaglini[8]

[1 1]Molecular Biophysics Laboratory, Department of Physics, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo [2]Dept. Chemistry, FFCLRP-USP, SP, Brazil [3] Sorbonne Université, CNRS, Collège de France, Laboratoire de Chimie de la Matière Condensée de Paris (LCMCP), F-75005 Paris, France, paris, France; [2]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) - Universidade de São Paulo (USP), Ribeirão Preto, Brazil; [3]Sorbonne Université, CNRS, Collège de France, Laboratoire de Chimie de la Matière Condensée de Paris (LCMCP), F-75005 Paris, France, Paris, France; [4]Faculdade de Filosofia,

Ciências e Letras de Ribeirão Preto (FFCLRP) da Univeridade de São Paulo (USP), Av. Bandeirantes, 3900, Brazil; [5]University of Rome Tor Vergata, Roma, Italy; [6]Sanford Burnham Prebys, La Jolla, CA, USA, La Jolla, USA; [7]Molecular Biophysics Laboratory, Department of Physics, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil; [8]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) da Univeridade de São Paulo (USP), Ribeirão Preto, Brazil

Introduction: Endochondral calcification is mediated by matrix vesicles (MVs), a special class of extracellular vesicles responsible for the initial precipitation of calcium phosphates. The MV membrane has higher levels of Tissue-Nonspecific Alkaline Phosphatase (TNAP) than the plasma membrane. TNAP is a the glycosylphosphatidylinositol (GPI)-anchored phosphomonohydrolase capable of generating inorganic phosphate (Pi) from ATP and inorganic pyrophosphate (PPi) hydrolysis, therefore initiating biomineralization. The lipid composition of membranes can modulate TNAP activity, but little is known about the mechanics of this process. Here we propose that the lipid composition influences the distance between the enzyme and the membrane resulting in different activity and mineralization characteristics. TNAP mutants were designed for use of EPR spin probes in Cys aminoacids to be able to map the proximity of the enzyme to the membrane. Two mutants, Pro307Cys and Ala420Cys, are particularly close in the structure to the GPI anchor motif
Methods: The mutant enzymes were reconstituted into dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and dimyristoyl-sn-glycero-3-phosphocholine (DMPC) liposomes. Catalytic activity and in vitro mineralization assays were performed. Minerals were analyzed by ATR-FTIR (Pi/CO band ratio).
Results: The TNAP mutants in DPPC were shown to be active: TNAP Pro307Cys ($1.6x10^3$ M-1.s-1) and TNAP Ala420Cys ($9.2x10^2$ M-1.s-1). There was a decrease in catalytic efficiency of approximately 1.7 times may be due to the presence of Cys in a position close to the site catalytic, thus making interaction with substrate difficult. A Attenuated Total Reflectance fourier transformed infrared spectroscopy (ATR-FTIR) results show an Pi/CO band ratio of 2.16 for TNAP Pro307Cys and a ratio 2.19 for TNAP Ala420Cys, similar values of mineral forming. In more fluid lipids (DMPC) the catalytic efficiency of DMPC-TNAP Pro307Cys was $1.7x10^3$ M-1.s-1 and DMPC-TNAP Ala420Cys was $1.1x10^3$ M-1.s-1. Regarding the ATR-FTIR Pi/CO ratio, it was found lower value for TNAP Pro307Cys (1.79) than to TNAP Ala420Cys (2.10).
Summary/Conclusion: The results show that greater catalytic efficiency factors are not always the main causes of mineral formation, and that the type of lipid (phase state) seems to cause greater effects in the biomineralization process. In order to have more information, further investigations are in process using ssNMR and Cryo-EM.
Funding: Supported by FAPESP: (2019/08568-2; 2021/02768-0); CNPq (304021/2017-2)

PF03.03 | Characterizing the impact of cell culture conditions in hollow fiber bioreactors on cells and EVs

Brianna Huszti[1]; Erika Duggan[2]; Thomas Maslanik[1]; John P. Nolan[2]
[1]Cellarcus Biosciences, La Jolla, USA; [2]Scintillion Institute, San Diego, USA

Introduction: Introduction: Translation of EV research from the bench to the clinic requires transition from small-scale flask-based EV production to large-scale EV production in bioreactors. Culture conditions vary between these systems which may affect the composition and utility of cells and EVs produced. We performed rigorous, quantitative analysis of cells and EVs from flasks and bioreactors to examine the impact of different cell culture conditions.
Methods: Methods: HEK239T cells cultures were maintained under standard culture conditions for either T-225 flasks or a hollow fiber bioreactor. Cells and EVs were harvested and analyzed using flow cytometry or a standardized, single vesicle flow cytometry (vFC) assay, respectively. Tetraspanin (TS) expression was measured using PE-conjugated monoclonal antibodies against CD9, CD63, and CD81 and phosphatidylserine was measured using PE-conjugated Annexin V.
Results: Results: HEK293T cells from both flasks and bioreactors express high levels of CD9 and CD81 and dimly express CD63. Expression of these markers is reduced in cells from bioreactors. EVs from both flask and bioreactor HEK293T culture supernatants express phosphatidylserine and CD81. CD9 expression was below the limit of detection of the vFC assay on EVs from flasks (LOD 25 antibodies/EV) but was detectable in a subpopulation of EVs from a bioreactor. CD63 expression was below the LOD in EVs from flasks or bioreactors.
Summary/Conclusion: Summary/conclusion: Analysis of TS expression in cells and EVs from flasks and bioreactors using flow cytometry and vFC reveals altered expression of cargo important to EV-physiology. These changes may impact function or suitability of EVs in downstream applications. Understanding changes elicited by differing culture conditions is important to assessing the suitability of an EV production method. Transitioning to large-scale EV production or making other alterations to cell culture conditions must be supported by rigorous characterization using standardized, suitable assays.

PF03.04 | Customizing the extracellular vesicles release and effect by strategizing surface functionalization of titanium

Miquel Antich-Rosselló[1]; Maria Antònia Forteza-Genestra[2]; Javier Calvo[3]; Antoni Gayà[3]; Marta Monjo[4]; Joana Maria Ramis[4]
[1]Institut d'investigació Sanitària de les Illes Balears, Palma De Mallorca, Spain; [2]Institut d'investigació Sanitària de les Illes Balears, Palma, Spain; [3]Banc de Sang i Teixits de les Illes Balears, Palma, Spain; [4]Universitat de les Illes Balears, Palma, Spain

Introduction: Metallic material functionalization with Extracellular Vesicles (EVs) is a desirable therapeutic approach to improve regenerative procedures. EVs are a heterogeneous population of communication nanovesicles released by cells that are being intensively investigated for their use in therapeutics.
Methods: Here platelet derived EVs for Ti surface coating were selected due to their demonstrated osteoinductive properties. Among the different functionalization strategies available, drop casting on machined Ti surfaces, drop casting on nanostructured TiO2 surfaces and polymeric entrapment with polydopamine were compared.
Results: Our results show that each functionalization strategy leads to differences in the size of EV populations attached to- and released from- the metallic implants, which, in turn, leads to variations in their osteogenic capability measured through alkaline phosphatase activity and calcium deposition.
Summary/Conclusion: In conclusion, the functionalization strategy used has an important effect on the resulting implant functionality, probably due to the heterogeneous EVs nature. Thus, the methodological approach to metallic material functionalization should be carefully chosen when working with extracellular vesicles in order to obtain the desired therapeutic application.
Funding: This research was funded by Instituto de Salud Carlos III, co-funded by the ESF European Social Fund and the ERDF European Regional Development Fund (contract to J.M.R; MS16/00124 and and project PI17/01605) and the Direcció General d'Investigació, Conselleria d'Investigació, Govern Balear (contract to M.A.R.; FPI/2046/2017) and the Institut d'Investigació Sanitària de les Illes Balears (contract to M.A.F.G; ITS2018-002-TALENT PLUS JUNIOR PROGRAM, JUNIOR18/01).

PF03.05 | Development of red blood cell based reference materials for EV studies

Kai Härkönen[1]; Juha Prittinen[2]; Ulla Impola[1]; Petra Ilvonen[3]; Saara Laitinen[1]
[1]Finnish Red Cross Blood Service, Helsinki, Finland; [2]University of Eastern Finland, Finnish Red Cross Blood Service, Helsinki, Finland; [3]Finnish Red Cross Blood Service, University of Helsinki, Helsinki, Finland

Introduction: There is a great need for reference materials for EV analyses i.e for calibration purposes and comparison of data from different laboratories. One of the special needs is in clinical flow cytometry in EV therapy development. Most artificial nanoparticles used for calibration are made from synthetic material such as polystyrene or latex, having a different refractive index than natural EVs. As red blood cells (RBC) possess a rich source of microparticles RBC concentrates offers an alternative to current reference materials.
Methods: RBCs are pelleted and washed from leukocyte reduced erythrocyte concentrates with gentle centrifugation steps. RBC degradation is performed either with sonication or extrusion with syringe-driven single-use polycarbonate extruders with pore sizes of 1000 nm to 200 nm. Ghosting of RBCs is done with repeated freeze-thaw treatment followed by washing of RBC membranes with centrifugation. CD235a intra- and extracellular fluorescent antibodies have been used for immunostainings of RBCs and RBC nanoparticles.
Results: RBC degradation with sonication seems to produce particles with more variable size and morphology than extrusion. Production of nanoparticles from ghosted RBCs may result in a purer end product, but it seems that cytoskeletal proteins, such as spectrin, may support forming of nanoparticles during degradation. However, the mass of non-encapsulated proteins should be removed from the sample afterwards to ensure high purity of reference material.
Summary/Conclusion: As only a small amount of RBC material from collected whole blood is required for abundant reference material production, artificial RBC-EVs may offer a new product for blood services. The use of RBCs will not only allow the production of EV-like particles with natural lipid composition but also offers the possibility to use RBC specific marker (CD235a) as an internal standard.
Funding: Business Finland ecosystem funded project EVE
Academy of Finland – GeneCellNano Flagship

PF03.06 | EV-inspired Nano Cell-Vesicle Technologies (nCVTs): fusion between cellular components and synthetic nanocarriers

Giorgia Pastorin
National University of Singapore, Singapore, Singapore

Introduction: Extracellular vesicles (EVs) are a class of cell-derived lipid-bilayer membrane vesicles secreted by almost all mammalian cells and involved in intercellular communication by shuttling various biological cargoes.
The imitation of this natural process that enables cells to communicate with both neighbouring and distant cells has inspired the development of new nanotechnological strategies to improve the delivery of drugs at their site of action. Scientists have recently exploited these EVs to either load them with drugs or to develop artificial EV mimetics that preserve biocompatibility and intrinsic targeting ability towards diseased cells.
Methods: We have recently conceived a nano-biohybrid system building on the idea of "combining the best of two worlds": these nano-Cell Vesicle Technology Systems (nCVTs) are obtained through the fusion of cell-derived components and conventional synthetic materials.
More specifically, 2e7 cells were first emptied of intracellular contents to produce cell ghosts (CGs) according to a modified protocol that preserved the structure and minimized membrane flipping. Next, 1 × 10e7 cell ghosts were added to a 2 mg lipid thin film (95 : 5 molar weight of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 0.05 mg of cholesterol), where the film was subsequently hydrated, encapsulating the cellular membranes within giant liposomes. The suspension was then extruded to produce nCVTs that are at the nanoscale and consist of the fusion of both lipid and CG membranes from the large multi-lamellar liposomes that originally encapsulated the CGs.
Results: Through this method it is possible to efficiently load small anticancer drugs, antisense oligonucleotides and/or proteins while retaining the intrinsic ability of cellular components to be recognized and uptaken by target cells. Hence, by selecting the starting cells as a source of nCVTs, it is possible to impart both homotypic properties (as demonstrated by preferential transfection of pre-adipocytes with pre-adipocyte-derived nCVTs) and heterotypic targeting (as shown between immune cells and cancer cells) to these bio-hybrid systems.
Summary/Conclusion: Our nCVTs represent an unreported chimeric drug delivery system with ideal properties in terms of nano-size (within 200 nm, which enable them to reach and accumulate at the diseased area), surface cues (key proteins and lipids that preserve the targeting properties inherited from their original parent cells) as well as ease of loading and functionalization (from the synthetic components), which pave the way towards new advances in the field of nanomedicine.
Funding: This work was supported by the National University of Singapore, NanoNash Program (R-148-000-296-114)

PF03.07 | Extracellular vesicles at the air liquid interface of bioprinted bovine mammary epithelial cells

Giulia Silvestrelli[1]; Markus Rimann[2]; Susanne E. Ulbrich[1]; Mara D. Saenz-de-Juano[1]
[1]*ETH Zurich, Zurich, Switzerland;* [2]*Zurich University of Applied Sciences (ZHAW), Wädenswil, Switzerland*

Introduction: Extracellular vesicles (EVs) in the bovine mammary gland are involved in physiology and pathology. To date, a 3D culture model to investigate their specific functions and properties in vitro is missing. We cultured bioprinted primary bovine mammary epithelial cells (pbMEC) grown at the air liquid interface (ALI) to closely resemble the real tissue and characterize the secreted EV.
Methods: A layer of Geltrex™ followed by a layer of pbMEC were jetted by a 3DDiscovery bioprinter on top of a Transwell®. Cells were then kept under ALI conditions. Apical fluid was collected and EVs isolated by differential centrifugation, size exclusion chromatography and ultracentrifugation. The EVs isolation method was validated by electron microscopy and western blot. We analysed the EVs by tunable resistive pulse sensing (TRPS), isolated the EVs RNA and evaluated the RNA fragments length. Finally, we performed an RT-qPCR analysis for the mammary epithelial cell-specific miRNAs bta-miR-200c-3p and let7a-5p.
Results: Despite the low initial volume of 20-30 μL of apical fluid, the number of particles measured by TRPS was 2.6x10^6 ± 1.6 x10^6. The RNA length of apical EVs was on the small RNAs range. Furthermore, we detected both miRNAs let7a-5p and bta-miR-200c-3p.
Summary/Conclusion: Printed pbMEC cultured under ALI conditions produced EVs. Despite the low input material, the amount of EV in the apical fluid was sufficient for the detection of mammary epithelial cell-specific miRNAs. Our results suggest that this model could be suitable for studying mammary glands EVs in vitro
Funding: ETH Research Grant ETH-53 16-1.

PF03.08 | Lactococcus lactis can surprisingly produce intracellular caveolin-enriched vesicles

Anthony Flourieusse[1]; Emma Schenckbecher[1]; Pauline Bourgeois[1]; Déborah Legrand[1]; Alain Rouleau[2]; Clarisse Labbé[1]; Thomas Bescond[1]; ANNIE Frelet-Barrand[3]

[1]CNRS, Besançon, France; [2]UBFC, Besançon, France; [3]CNRS, besancon, France

Introduction: Membrane proteins (MPs) perform a wide variety of functions vital to the survival of organisms. They are involved in numerous pathologies and are thus important drug targets. In spite of their functional and biotechnological importance, their study remains difficult due to their hydrophobicity and low abundance in the cells. Their overexpression in heterologous systems is mandatory for their detailed structural and functional characterization. However, this strategy leads to numerous obstacles such as their toxicity to hosts and the quality of the MP produced in these systems, especially for structural studies.
Methods: An original approach to produce MP-enriched vesicles was tested using the ability of a small membrane protein, caveolin 1β, to generate membrane vesicles within the cytoplasm when heterologously overexpressed in various hosts including insect cells and bacteria. Such structures have already been observed in Gram negative bacteria such as Escherichia coli and were named heterologous caveolae (h-caveolae) [1]. The overexpression of caveolin 1β to produce h-caveolae has been tested in Gram positive bacteria, Lactococcus lactis, since these bacteria appeared to emerge as a good alternative to E. coli for MP overexpression [2,3] although they display a very different lipid composition [4,5].
Results: Biochemical and biophysical studies have been carried out to realize a deeper characterization of such nanovesicles.
Summary/Conclusion: Surprisingly, h-caveolae formation was noticed within L. lactis bacteria and displayed size comparable to E. coli h-caveolae. These lactoccocal caveolae could be further used for co-expression of MPs of pharmaceutical interest.
Funding: This project has been founded by the ANR Caveotank (2017-2021).

PF03.09 | Modified Crispr/Cas9 technology for microRNA elimination from extracellular vesicles

Maxim Karagyaur[1]; Nataliya Basalova[2]; Maxim Vigovskiy[3]; Mariya Skryabina[4]; Daniyar Dyikanov[4]; Maria Illarionova[4]; Anna Ivaschenko[4]; Anastasia Efimenko[3]

[1]Institute for Regenerative Medicine, Lomonosov Moscow State University, Moscow, Russia; Faculty of Medicine, Lomonosov Moscow State University, Moscow, Russia, Moscow, Russia; [2]Institute for Regenerative Medicine, Medical Research and Education Center, Lomonosov Moscow State University, Moscow, Russia, Moscow, Russia; [3]Institute for Regenerative medicine, Medical Research and Education Center, Lomonosov Moscow State University, Moscow, Russia; Faculty of Medicine, Lomonosov Moscow State University, Moscow, Russia, Moscow, Russia; [4]Faculty of Medicine, Lomonosov Moscow State University, Moscow, Russia, Moscow, Russia

Introduction: Modulation of the extracellular vesicles (EV) composition allows studying the role of individual components in the EV biological effects. One of the most interesting class of components are miRNA (miR). Today there are a number of approaches (ExoFect, saponin..) that help to modify miR amount. However, all of them raise questions about the preservation of the EV native structure and functions. Therefore, we have offered an approach based on CRISPR/Cas9 technology to remove miR from EV.
Methods: To edit miR genes (21 or 29c), a modification of the CRISPR/Cas9 technology named SpCas9-D10A in a lentiviral vector with a pair of targeting vectors was used. The transduced cells (MSC) were sorted and cultured, and the interesting DNA regions from this cells clone were analyzed by Sanger sequencing. A conditioned medium with subsequently EV isolation by ultrafiltration was obtained from the necessary clones. The biological properties of EV were analyzed on in vitro model of TGFb-induced fibroblasts to myofibroblasts transdifferentiation.
Results: The sequencing results showed a high level of genes editing. However, the most common outcome of editing was not excision, but the loss of a portion of the genes arm. Despite this, using real-time PCR, we have shown that both transduced cells or appropriate EV had not mature forms of the studied miR in comparison with control samples. Moreover, EV from transduced cells retain their shape and size, as well as the expression of markers. Removal of miR21, and to a greater extent miR29c, dramatically reduces the ability of EV to inhibit fibroblasts to myofibroblasts transdifferentiation, that correlate with our previous in vitro data.
Summary/Conclusion: Thus, genome editing using modified CRISPR/Cas9 technology might be used to modulate the amount of miR within EV. It is important that not only complete removal of the gene miR, but also partial disruption of its structure leads to impaired expression of mature miR and EV biological function.
Funding: The study was supported by RSF#30007.

PF03.10 | NPP1 and TNAP act synergistically and complementarily on ATP hydrolysis during matrix vesicle-driven biomineralization

Pietro Ciancaglini[1]; Luiz Henrique Andrilli[1]; Bruno Z. Favarin[2]; Heitor G. Sebinelli[3]; Maytê Bolean[1]; Ana Paula Ramos[4]; Massimo Bottini[5]; José Luis Millán[6]

[1]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) da Univeridade de São Paulo (USP), Ribeirão Preto, Brazil; [2]Faculdade de FIlosofia, Ciencias e Letras de Ribeirao Preto (FFCLRP) da Universidade de Sao Paulo (USP), RIbeirao Preto, Brazil; [3]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) - Universidade de São Paulo (USP), Ribeirão Preto, Brazil; [4]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) da Univeridade de São Paulo (USP), Av. Bandeirantes, 3900, Brazil; [5]University of Rome Tor Vergata, Roma, Italy; [6]Sanford Burnham Prebys, La Jolla, CA, USA, La Jolla, USA

Introduction: Endochondral ossification is mediated by matrix vesicles (MVs)- a special class of extracellular vesicles responsible for the initial precipitation of calcium phosphates (CaP). These vesicles are enriched in tissue-nonspecific alkaline phosphatase (TNAP), that through its phosphomonohydrolytic activity produces inorganic phosphate (Pi), from adenosine-5'-triphosphate (ATP) and inorganic pyrophosphate (PPi) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (NPP1), an ATP phosphodiesterase, producing adenosine-5'-monophosphate (AMP) and PPi. In this study, we investigated both the kinetics of ATP hydrolysis using proteoliposomes containing TNAP and NPP1 as MV-biomimetic models, and their combined effects on in vitro biomineralization.

Methods: Liposomes composed by 1,2-dipalmitoylphosphatidylcholine (DPPC) and carrying TNAP and/or NPP1 were prepared using different lipid/enzyme molar ratios. Non-stationary state kinetics assays of ATP hydrolysis were performed in the absence/presence of Suramin, a NPP1 inhibitor. In vitro mineralization turbidimetry assay at 340 nm was used to evaluate the potential of the proteoliposomes in the propagation of CaP precipitation. ATR-FTIR and SEM/EDS were used to investigate the chemical composition of the precipitated minerals.

Results: NPP1-containing proteoliposomes exhibited both phosphomonohydrolase and pyrophosphohydrolase activities as revealed by the presence of ADP and AMP, respectively, as by-products in all the assays. The presence of 0.5 mM of Suramin reduced by 65% and 75% the amount of ADP and AMP, respectively, formed after 48 hours of reaction. The presence of TNAP in NPP1-containing proteoliposomes decreased Suramin inhibition. All the proteoliposomes harboring TNAP and NPP1 produced amorphous CaP with a Ca/Pi molar ratio of 0.875 as revealed by SEM/EDS.

Summary/Conclusion: The formation of PPi in the precipitated minerals in the presence of NPP1-containing proteoliposomes was confirmed by ATR-FTIR as revealed by a decrease in the intensity of the band related to the Pi stretching (∼1000 cm-1). Taken together, the results prove that TNAP:NPP1-DPPC proteoliposomes support the propagation of CaP, mimicking the enzymatic processes of MVs. Different proportion of the enzymes in the presence and absence of a NPP1 inhibitor can be used to elucidate the complex balance of Pi/PPi homeostasis in biomineralization.

Funding: FAPESP (2019/08568-2), CNPq (130366/2020-0 and 304021/2017-2), Brazil-France USP-COFECUB Uc Sv 184/20 grant

PF03.11 | Selective enrichment of extracellular vesicles by bio-layer interferometry for improved early biomarker detection

Rebekka Van Hoof[1]; Alexandra Van Driessche[1]; An Jacobs[1]; Pascale berckmans[1]; Michal Szymonik[1]; Patrick Wagner[2]; Jef Hooyberghs[3]; Inge Nelissen[1]

[1]Flemish Institute for Technological Research (VITO), Health Unit, Boeretang 200, 2400 Mol, Belgium, Mol, Belgium; [2]University of Leuven, Laboratory for Soft Matter and Biophysics, Celestijnenlaan 200D, 3001 Leuven, Belgium, Leuven, Belgium; [3]Flemish Institute for Technological Research (VITO), Data Science Hub, Boeretang 200, 2400 Mol, Belgium, Mol, Belgium

Introduction: The sensitivity of biomarker detection in minimally invasive liquid biopsies, where only a blood samples is taken, can potentially be improved by selectively enriching and analyzing extracellular vesicles (EVs). Whereas kits for the analysis and enrichment of EV populations bearing protein markers such as CD9, CD63, CD81 and EpCAM exist, the capture of disease related EV populations and subsequent analysis of additional biomarkers have not been widely studied. Moreover, not many point-of-care applications using EVs for diagnostics exist. In this study, we aimed to demonstrate the use of bio-layer interferometry for this purpose. This optical analytical technique allows for real-time monitoring of the functionalization of a sensor tip with antibodies and the specific binding of EVs displaying the targeted epitope.

Methods: Conditioned media were harvested from 3D hollow-fiber bioreactor culture of the human monocytic mono-mac-6 cell line, and EVs were isolated using size exclusion chromatography. The EV fractions were analyzed for their concentration, purity and protein markers using fluorescent nanoparticle tracking analysis, high-sensitivity flow cytometry and western blotting. We functionalized streptavidin-coated sensor tips with biotinylated antibodies against the well-known EV surface marker CD63, and

monitored EV binding on the tip in real-time. Next, the captured EV population was released and used for genotyping analysis by digital PCR.
Results: We observed that the CD63+ EV binding signal was specific in comparison to an isotype IgG control, and could be further increased by adding a blocking step after the functionalization. Furthermore, we demonstrated successful release of the tip-bound EVs with sufficient recovery to allow for subsequent digital PCR analysis of the β-actin housekeeping gene.
Summary/Conclusion: Our results show that bio-layer interferometry is a suitable technique to analyze the capture of subpopulations of EVs displaying a specific epitope while also allowing downstream biomarker characterization. Further research may demonstrate the potential for point-of-care applications in diagnostics.
Funding: This work was financed by the Research Foundation Flanders (FWO; Grant agreement 1S69320N), the European Union's H2020 Safe-N-Medtech project (Grant agreement N° 814607), and the Flemish Institute for Technological Research (VITO nv).

PF03.12 | Vesicle-like nanoparticles derived from Pomegranate exert anti-inflammatory properties and promote wound healing in vitro: Does industrial processing affect these bioactive properties?

Christian M. Sánchez-López[1]; Claudia Honrubia-Usina[2]; Carla Soler[2]; Pedro Pérez-Bermúdez[3]; ANTONIO MARCILLA[4]
[1]Universitat de Valencia, BURJASSOT, Spain; [2]Universitat de València, BURJASSOT, Spain; [3]Universitat de València, BURJASSOT (VALENCIA), Spain; [4]Universitat de Valencia, Burjassot, Spain

Introduction: Fruits enriched in polyphenols, such as pomegranates, have gained attention due to their therapeutic properties and promising biological activities. Here, we report the presence of vesicle-like nanoparticles in pomegranate juice (PgEVs), confirm their biomedical potential and test whether pasteurization and lyophilization treatments affect PgEVs yield and biological functions.
Methods: Juice was obtained manually and stored at -80°C. Pasteurization was carried out at 62°C for 30 min, and lyophilization was carried out for 24 h. PgEVs were isolated by differential centrifugation followed by SEC, and characterized using NTA and TEM, and LC-MS/MS (EV-Track ID EV220000). Wound-healing assays were performed in CACO-2 human intestinal epithelial cell line (ATCC®, HTB-37TM), and anti-inflammatory assays were performed on THP-1-XBlue™-CD14 human macrophages (InvivoGen), using the Quanti-blueTM reagent (Invivogen). PgEVs citotoxicity was assessed by MTT assays. All assays were performed on FBS-free culture media.
Results: Vesicle-like nanoparticles were isolated and characterized. PgEVs isolated from non-industrial treated juice showed significant higher particle concentrations than Pasteurized and Lyophilized PgEVs, as confirmed by NTA and TEM. Pasteurization affected PgEVs integrity, while Lyophilized PgEVs maintained similar morphologies than non-treated PgEVs. Proteomic analyses identified 130 proteins in PgEVs, including suggested Plant-EV markers such as TET-8, Vsp proteins and Patellins. PgEVs showed no citotoxicity and powerful anti-inflammatory properties in LPS-stimulated macrophages. In addition, PgEVs showed significant wound-healing properties. Both Pasteurized and Lyophilized treated PgEVs maintained those properties, but the required higher concentration to achieve those effects.
Summary/Conclusion: Vesicle-like nanoparticles derived from Pomegranate have anti-inflammatory properties and promote wound healing in vitro, demonstrating their important role in the biological effects related to this fruit juice. Industrial processing of the juice could affect the yield and integrity of the isolated PgEVs, as well as their functional properties. The bioactive properties can be maintained increasing the dosage. Investigations are underway to identify molecules and effectors responsible for those actions.
Funding: Supported by the Agencia Estatal de Investigación, Ministerio de Ciencia e Innovación, Spain (Grant PID2019-105713GB-I00/AEI /10.13039/501100011033), and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (Grant PROMETEO/2020/071). C.M.S-L is the recipient of a predoctoral fellowship (ref. PRE2020-092458) funded by AEI /10.13039/501100011033. Part of "Red Traslacional para la Aplicación Clínica de Vesículas Extracelulares, Tentacles", (RED2018-102411-T, Agencia Estatal de Investigación, Spain). Patent Nr P202130397.

PF03.13 | An extracellular vesicle-based vaccine platform displaying native viral envelope proteins to tackle viral emerging diseases

Robert Z. Mamoun[1]; Katarzyna Polak[1]; Noémie Greze[1]; Maëlle Lachat[1]; Delphine Merle[1]; Steve Chiumento[1]; Christelle Bertrand-Gaday[2]; Bernadette Trentin[1]
[1]Ciloa SAS, Montpellier, France; [2]INRAe, Montpellier, France

Introduction: Extracellular vesicles (EVs) secreted by antigen presenting cells (APCs) carry accessory proteins like ICAM and major histocompatibility complex (MHC) molecules loaded with antigenic peptides. Such EVs can trigger humoral and cellular immune responses against pathogens and can be considered as natural vaccines. On the basis of these EV properties, combined to a technology to engineer EVs in vivo with native membrane proteins, we developed several candidate vaccines against different enveloped viruses, of which, retroviruses, arboviruses and coronaviruses.
Methods: EVs were engineered to present the major membrane antigens of HIV, Chikungunya and SARS-CoV-2 viruses. In order to benefit from the adjuvant properties of EVs, we used an immunization protocol that combines DNA prime injections with an exosome boost. The DNAs encode viral membrane proteins that, when injected, are targeted in situ to the EVs, in order that the viral antigens are presented in an autologous immune environment. The exosomes of the boost, harbor the viral membrane antigens and are produced using cell cultures.
Results: Using immunochemical and biochemical tools, we showed that the viral membrane proteins presented by these EVs are in fully native and mature conformation; thus, the engineered EVs mimic the shape of the different original viruses.
Mice injected with Chikungunya and Coronavirus candidate vaccines and rabbits injected with HIV candidate vaccine developed strong humoral and cell mediated immunity with high levels of neutralizing antibodies.
Summary/Conclusion: Engineered EVs will be the basis for a vaccine platform that will help fighting future viral pandemics: a simple exchange of the viral membrane protein antigen for that of the emerging virus will lead to an adapted vaccine.
Funding: French government FUI grants

PF03.14 | ATEI™(Antibody-Targeted Exosome Immuno-oncology) Technology: Fc Binding Domain-Engineered Extracellular Vesicles for Universal Active Targeted Delivery

Hyungtaek Jeon; Kyung Youn Hong; Byong Seung Cho
ExoCoBio Inc., Seoul, Republic of Korea

Introduction: The development of a variety of drug delivery system has been active to deliver drugs to specific targets to reduce side effects of drugs and maximize efficacy to deliver the necessary amounts of drugs. EVs that load therapeutic cargos and deliver them to target cells can be a perfect drug delivery platform. Targeted anticancer drugs have been developed to adjust specifically on cancer cells, but treatment effects can be maximized if they can act more selectively on cancer tissues to be treated when administered in human body. We have developed an innovative ATEI™ platform technology of EVs engineered with a Fc binding domain which can interact to any kinds of antibodies and were able to confirm specific delivery to target tumor cells through co-administration with antibodies. In addition, it was confirmed that it plays an effective role as and anti cancer agent by loading drugs into EVs equipped with Fc domains or fusing various proteins including cytokines to directly target tumor cells or active immune cells to control the tumor microenvironment. Although the delivery of EV-mediated drugs is still in its infancy, it has low toxicity, low immunogenicity, and high engineering ability, which accelerate the development of targeted immuno-cancer drugs using EVs.
Methods: Cells were prepared by transfecting HEK293 cells with a plasmid DNA including and Fc-binding domain. Stable cell lines were prepared through monoclonal isolation. After that, EVs were isolated and purified by our methods and then separated again through UC. A certain amount of the durgs was loaded into the EVs through incubation with the target antibody. After that, the results were confirmed through in vitro and in vivo experiments.
Results: A novel scaffold protein possesses two functions simultaneously. 1) Function capable of binding to Fc binding region such as antibody(Universal Targeting). 2) To enable presentation by engineering an protein of interest to the N-terminal(Protein Delivery). In addition, it is possible to load chemical anticancer drugs into the EVs(Drug loading). By combining these three core technologies, it was shown that up to 44 times more efficient chemical or protein drug delivery become possible.
Summary/Conclusion: Our ATEI™ technology is the world's first to utilize dual function based on EV delivery system. This platform enables the delivery of specific proteins, and when combined with antibodies, targeting to specific cells or tissues is possible. This novel engineered EVs are new concept of universal targeting because it can bind to proteins and antibodies having all FC-binding domains. In addition to efficient delivery using and antibody, additional protein drugs can be engineered to develop novel immuno-cancer durgs or immune-enhancing agents.

PF03.15 | Development of a novel EV-loading cargo protein methodology for therapeutic applications

Etienne Lourdin - De Filippis; Noémie Greze; Maëlle Lachat; Katarzyna Polak; Delphine Merle; Robert Z. Mamoun; Bernadette Trentin
Ciloa SAS, Montpellier, France

Introduction: Extracellular Vesicles (EVs) can potentially deliver a variety of cargos to any type of organs. To achieve that, there is a need for EVs customized with chosen proteins on their surface, for specific organ targeting, and/or with a chosen therapeutic cargo. However, developing reliable and robust technologies to customize EVs for their use as therapeutic vectors in vivo is still a challenge.
Methods: Our aim was to evaluate an existing technology (Patent WO2011/036416) that addresses naturally any protein cargo into the EV lumen. Originally, this technology targets a protein under the EV membrane. We adapted this technology for the reversible release of the protein into the EV lumen.
To reach this goal, we use two proteins that interact together in a reversible way. The first one is a carrier protein (CP, patent in process), the second is the protein of interest. The CP was inserted in between two peptides i) a Src myristylated peptide for membrane anchoring and ii) a Pilot Peptide (CilPP) that interacts with ESCRT machinery and sorts the protein into EVs. Next, the cargo protein of interest, was fused to a CP interacting peptide (CPIP, patent in process), that allows its encapsulation inside the EVs.
Results: NanoLuc (nLuc), Antares2 (a fusion of CyOFP1 and nLuc), and Oct4 proteins were used as cargos. Their targeting inside the EVs was assessed by Western blots, luminescence, BRET assays and by protection from Proteinase K digestion. The reversibility of the interaction between CP and CPIP to release the cargo was also tested.
Summary/Conclusion: In conclusion, we demonstrated the robustness of a technology that addresses efficiently any protein cargo into EVs, either by direct interaction with EV membrane or by a potentially reversible interaction with a CP protein addressed under the EV membrane.
The next step of this work will be to combine the cargo loading with specific cell targeting in order to develop a powerful tool for the EV-mediated delivery-based therapeutics.

PF04: Fundamental biology: Cell-EV interaction, uptake, fusion, and cargo delivery

Chair: Pascale Zimmermann – K. U. Leuven

PF04.01 | Characterizing the interactions between cancer derived EVs and neutrophil subsets and their impact on NETosis and triple negative breast cancer metastasis

Emilie Solymoss[1]; Molly L. Shen[2]; Anna Shen[3]; Sébastien Tabariès[4]; Matthew G. Annis[4]; Peter M. Siegel[4]
[1]Goodman Cancer Research Centre of McGill University, Montréal, Canada; [2]Department of Biomedical Engineering, Faculty of Medicine and Health Sciences, McGill University, Montreal, Canada; [3]Goodman Cancer Research Centre of McGill University, Montreal, Canada; [4]Rosalind and Morris Goodman Cancer Research Centre, McGill University, Montreal, Canada

Introduction: Individually, cancer derived EVs as well as neutrophils and their release of NETs have been shown to aid in cancer progression and metastasis. Our study aimed to characterize EV and neutrophil interactions by quantifying neutrophil uptake of EVs and the subsequent effects EVs had on NETosis across two neutrophil subpopulations.
Methods: Five cell lines were used in our study: non-transformed mouse mammary epithelial cells (NMuMG), non-metastatic (67NR) and metastatic 4T1 TNBC cell lines (parental (4T1-P), liver (2776), and lung (526) metastatic 4T1). EVs were isolated from conditioned media (CM) by ultracentrifugation and the integrity and size was confirmed by NTA. Immature low-density (iLDNs) and high-density neutrophils (HDNs) were isolated from tumor-bearing Balb/c mice by density centrifugation. An optimized concentration of isolated EVs were co-cultured with either iLDNs or HDNs prior to fixation and immunofluorescent staining for NETs. NETosis was quantified based on the resulting microscopy images.
Results: In agreement with previous findings, iLDNs showed increased NETosis compared to HDNs. Indeed, iLDNs cultured with EV-depleted CM from metastatic cell lines (4T1-P, 2776, 526) underwent more NETosis than HDNs under the same conditions. For both iLDNs and HDNs, the highest NETosis incidence was when the neutrophils were primed with EVs derived from the 2776 and 526 metastatic cell lines. In comparison, CM and EVs from non-tumorigenic NMuMG cells did not induce NETosis beyond background levels.
Summary/Conclusion: Our findings suggest metastatic cancer derived EVs can promote NETosis to a greater extent than EVs from non-metastatic cells and that iLDNs are more prone to NETosis in response to metastatic cancer derived factors. Ongoing experiments aim to quantify neutrophil uptake of EVs derived from cell lines expressing fluorescent membrane-localizing proteins.

PF04.02 | Effects of X-ray irradiation on extracellular vesicles isolated from head and neck cancer cells

Inga S. Juvkam[1]; Olga Zlygosteva[2]; Nina Edin[2]; Eirik Malinen[2]; Hilde Kanli Galtung[1]; Tine Merete Søland[3]
[1]*Institute of Oral Biology, University of Oslo, Oslo, Norway;* [2]*Department of Physics, University of Oslo, Oslo, Norway;* [3]*University of Oslo, Oslo, Norway*

Introduction: Extracellular vesicles (EVs) are important in cell-to-cell communication and have been found to play a role in modulating non-targeted effects in tumour and host cells not directly exposed to ionizing radiation. We aimed to investigate and compare the proteome of an oral squamous cell carcinoma (OSCC) cell line and their released EVs after exposure to photon radiation. Furthermore, we aimed to investigate the effect of these EVs after uptake in non-irradiated cancer and normal cells.
Methods: Human OSCC cells were irradiated with X-ray doses of 0, 2 or 5 Gy. 24 hours after irradiation, EVs were isolated from the irradiated OSCC cells by centrifugation, ultrafiltration and size-exclusion chromatography and characterized by nanoparticle tracking analysis, flow cytometry and transmission electron microscopy. Irradiated OSCC cells and their associated EVs underwent liquid chromatography-mass spectrometry analysis for protein identification. Uptake studies of irradiated OSCC EVs by non-irradiated OSCC cells and oral fibroblasts were performed by staining EVs with PKH67 and obtaining confocal images after incubation with the recipient cells. The influence of EV uptake on proliferation and migration of the recipient cells were studied.
Results: Proteins involved in mediating apoptosis (CTL2, TR10A, ADA17) were overexpressed in EVs released by irradiated OSCC cells while proteins involved in DNA repair (PARP1, APTX, HAT1, among others) were upregulated in irradiated OSCC cells. Uptake of irradiated OSCC EVs did not alter the proliferation or migration of non-irradiated OSCC cells or oral fibroblasts.
Summary/Conclusion: Two different protein profiles, both potentially of importance for cytoprotection of photon radiation, were identified in irradiated OSCC cells and their associated EVs. Our results do not exclude that non-targeted radiation effects might occur through other signalling channels than EVs released from irradiated cells.

PF04.03 | Exosomal miR-92a-1-5p selectively secreted by tumor cells may serve as diagnostic biomarker and contributes to bone-metastatic prostate cancer

Lijuan Yu
Krefting Research Centre, Gothenburg, Sweden

Introduction: Prostate cancer (PCa) bone metastasis is the major reason for prostate cancer-related death. In PCa bone metastasis, the imbalance in bone homeostasis is a critical step and osteolysis occur before any invasion. Our previous work has demonstrated that exosomes secreted from osteoblastic PCa promote osteoclastogenesis and osteolysis. In this study, we are expecting to identify key osteolytic molecules involved in this process, which will facilitate the development of novel biomarkers or therapeutic targets to improve patient outcomes.
Methods: MiR-92a-1-5p was identified from small RNA sequencing from PCa exosomes and normal exosomes. Stably overexpressing miR-92a-1-5p MDA PCa 2b cells were constructed by lentivirus and exosomes were isolated by ultracentrifugation. Patient serum collection was approved by the ethics committee at Air Force Medical University. Serum ALP and serum exosomal miR-92a-1-5p expression were detected. Osteoclast differentiation induced by Exo-miR-92a-1-5p was examined and tumor bone metastasis was performed in a nude mouse model.
Results: We identified a key osteolytic exosomal molecule, exosomal miR-92a-1-5p, that was selectively sorted into prostate cancer exosomes naturally or artificially overexpressed, promoted osteoclast differentiation in vitro and increased osteolysis in vivo by targeting MAPK1. MAPK1 downregulation and the subsequent inhibition of FoxO1 promoted osteoclast differentiation. Furthermore, bone metastasis was accelerated following 4-week bone marrow education with Exo-miR-92a-1-5p. More importantly, exosomal miR-92a-1-5p was negatively associated with the bone metabolic biomarker alkaline phosphatase and may sever as a diagnostic biomarker for bone-metastatic PCa with AUC=0.7431.
Summary/Conclusion: Our findings not only identify a role of exosomal miR-92a-1-5p in prostate cancer bone metastasis, but also suggest exosomal miR-92a-1-5p as a potential early diagnostic biomarker for bone-metastatic PCa.
Funding: This work was supported by the China Postdoctoral Science Foundation to L.Y. [under Grant No. F121NF0020].

PF04.04 | Extracellular vesicles from different cell sources yield specific transcriptional responses primarily at low doses

Daniel W Hagey[1]; Beklem Bostancioglu[1]; Jeremy Bost[2]; André Görgens[1]; Samir El Andaloussi[3]

[1]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, Stockholm, Sweden; [2]Department of laboratory medicine, Karolinska Institutet, Stockholm, Sweden; [3]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, Huddinge, Sweden

Introduction: Extracellular vesicles (EV) have been established to play important roles in cell-cell communication and shown promise as therapeutic agents. Although the majority of the evidence for these functions has come from studies applying EVs directly to cells in vitro, there has yet to be a comprehensive comparison of the effects of EVs from different cell sources or at different doses.
Methods: In this work, we derived EVs from twelve different cell lines (Bone marrow, umbilical cord, and Wharton's Jelly mesenchymal stem cells, BJ-5ta fibroblasts, HUVEC endothelial, Panc-1 epithelial, HEK293T adherent, HEK293 freestyle, AE602 umbilical cord, CAP amnion, THP1 monocyte and Jurkat T-cell lines) and applied them to human fibroblasts at doses between 20 and 200 000 per cell. We analyzed their effects on the target cell transcriptome by RNA-sequencing and confirmed these using functional assays.
Results: Global analysis revealed EV dosage to have a more significant effect than cell source, with high doses resulting in downregulated exocytosis and upregulated lysosomal activity regardless of EV cell source. In contrast, low doses of EVs produced the most cell source-specific responses, with the greatest number and most distinct sets of genes differentially expressed. Classifying EV source cells as mesenchymal-, HEK-, endothelial-, amnion- or immune-derived showed cellular transcriptional responses and EV-mRNA to cluster based on the EV source cell type group. Moreover, fibroblast transcriptional responses were reflective of the EV source cell's niche, such that mesenchymal- and amnion-derived EVs activated wound healing and proliferation phenotypes, respectively. Finally, by mapping the abundance of EV-specific transcripts found in fibroblasts, we could infer their dosage-dependent uptake by target cells. This suggested that immune cell-derived EVs were uniquely potent in this regard.
Summary/Conclusion: We find that high doses of EVs induce a generalized lysosomal response, but are required to detect EV-borne transcripts in target cells. In contrast, low doses of produce EV cell source-specific responses that reflect the activity of their source cell niches. This study provides important insights into the cell biology of the EV uptake response that should be considered in the design of future EV research.
Funding: The Swedish Cancer Society

PF04.05 | β-adrenergic stimulation accelerates uptake of plasma extracellular vesicle in porcine coronary artery endothelial cells

Trude Aspelin[1]; Michael Frisk[2]; Espen Stang[3]; Anne-Marie Siebke Trøseid[4]; Reidun Øvstebø[1]
[1]The Blood Cell Research Group, Department of Medical Biochemistry, Oslo University Hospital, Ullevaal, Norway, Oslo, Norway; [2]Institute for Experimental Medical Research, Oslo University Hospital and University of Oslo, Oslo, Norway, Oslo, Norway; [3]Department of Pathology, Oslo University Hospital Rikshospitalet, Oslo, Norway, Oslo, Norway; [4]The Blood Cell Research Group, Department of Biochemistry, Oslo University Hospital Ullevaal, Oslo, Norway, Oslo, Norway

Introduction: In coronary pig blood, we have previously demonstrated release of norepinephrine (βAR agonist) and concurrent reduction in extracellular vesicles (EV) during 3 min stimulation of sympathetic nerves to the heart - a situation relevant to myocardial ischemia and stress. We have detected β2AR receptors on plasma EV and they may thus be involved in acute coronary EV uptake. The aim of this study was to examine acute EV uptake in porcine coronary endothelial cells (PCAEC) induced by βAR stimulation.
Methods: EV were isolated from pooled citrate plasma (n = 3 pigs) using size exclusion chromatography (SEC; 0.5 mL fractions). EV in fraction 8+9 were concentrated on 100kDa-filter, labelled with MemGlow488 (MG-EV), re-isolated on SEC, concentrated on 100kDa-filter and stored at -80 until analysis. PCAEC were cultured on chambered coverglass to about 50 % confluence. Nuclei were stained with DAPI. Then medium was replaced with 0.2 mL serum-free medium before βAR agonist (isoprenaline, 10 μM) or vehicle, and 3.5 x 10< sup> 9 MG-EV were added. Cellular EV uptake was detected by measuring the change in PCAEC fluorescence using confocal microscope. Recordings (ideally 8 cells/chamber) were performed before adding MG-EV, immediately after, and in 5 min intervals for 20 min. Experiments were repeated 3 times for each treatment. EV were characterized by western blot, nanoparticle tracking analysis and electron microscopy.
Results: Vehicle-treated cells were able to take up EV and obtained maximum fluorescence (maxFL) 14.8 ± 1.0 min after EV administration (n = 22 cells). Addition of isoprenaline accelerated this process and reduced time to maxFL to 8.5 ± 1.4 min (n = 17 cells, $P < 0.001$).
Summary/Conclusion: EV are important for intercellular communication by transferring biomolecules regulating physiological processes in recipient cells. Here, we show that plasma EV are constitutively taken up by porcine coronary artery endothelial cells, and β-adrenergic stimulation (isoprenaline) accelerates the process possibly via demonstrated β2AR on EV.
Funding: Fondsstiftelsen, Oslo University Hospital

PF04.06 | Induction of the NF-κB signaling pathway by plasma-derived exosomes from HNSCC patients

Diana Huber[1]; Linda Hofmann[1]; Florian von Strachwitz[1]; Sonja Ludwig[2]; Ramin Lotfi[3]; Cornelia Brunner[1]; Thomas K. Hoffmann[1]; Marie-Nicole Theodoraki[1]
[1]Department of Otorhinolaryngology, Head and Neck Surgery, Ulm University Medical Center, Ulm, Germany, Ulm, Germany; [2]Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Mannheim, Mannheim, Germany, Mannheim, Germany; [3]Institute of Clinical Transfusion Medicine and Immunogenetics Ulm, German Red Cross Blood Transfusion Service, Baden Wuerttemberg- Hessian Ulm, Ulm, Germany, Ulm, Germany

Introduction: As one of the most immunosuppressive cancers, head and neck squamous cell carcinomas (HNSCC) show an increased NF-κB activation with downstream production of Treg and MDSC attracting immune suppressive factors. Exosomes are small extracellular vesicles that mediate cell-to-cell communication. Plasma-derived exosomes from HNSCC patients contain immune modulatory molecules that can alter the function of immune cells and contribute to the immunosuppressive tumor microenvironment (TME). We investigate if plasma-derived exosomes of HNSCC patients activate NF-κB signaling and which upstream signaling pathways are involved.
Methods: Exosomes were isolated from plasma of HNSCC patients by size-exclusion chromatography. Plasma-derived exosomes were incubated with primary macrophage cultures to investigate the effects on NF-κB signaling, upstream and downstream signaling pathways. NF-κB activation was determined by western blot analysis of IκB and pIκB and by NF-κB ELISA using nuclear extracts. Upstream TLR3 dependent signaling pathways were investigated by immunofluorescence of IRF3 and RIPk1 as well as cytoplasmic helicase dependent pathways. Downstream signaling was evaluated by CCL5, CXCL11 and CCL22 ELISA and PCR analysis of immune suppressive factors. T cell attraction was investigated by migration assays.
Results: The visible exosome dependent NF-κB activation in macrophages was reversable by addition of NF-κB inhibitors Bay, CAPE but mostly by Curcumin. Activated NF-κB signaling resulted in production of downstream immune suppressive chemokines and cytokines. Evaluation of upstream signaling showed mainly a TLR3 dependent activation of NF-κB.
Summary/Conclusion: The results show that plasma-derived exosomes from HNSCC patients interact with macrophages and are able to alter the NF-κB signaling pathway, leading to NF-κB activation. The possible reversion of NF-κB activation by several inhibitors may be useful for future clinical therapeutic strategies on modulation of the tumor microenvironment.
Funding: Deutsche Krebshilfe

PF04.07 | Manipulating the uptake of extracellular vesicles in prostate cancer

Ardalan Mansouri[1]; Pedro Matos Filipe[2]; Natasja Dits[3]; Andrea Sacchetti[1]; Judith Farrés[4]; Guido W. W. Jenster[3]; Martin E. E. van Royen[5]
[1]Department of Urology, Erasmus MC, Rotterdam, The Netherlands, Rotterdam, Netherlands; [2]Anaxomics Biotech, Barcelona, Spain; [3]Department of Urology, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, Netherlands; [4]Information Technology Department, Anaxomics Biotech, Barcelona, Spain, Barcelona, Spain; [5]Department of Pathology, Erasmus MC, Rotterdam, The Netherlands, Rotterdam, Netherlands

Introduction: Prostate cancer is a common cancer with the chance of metastasis among men. Cell-cell communication via secreting extracellular vesicles (EVs) has a role in tumor metastasis (1, 2). Accordingly, developing EV-uptake inhibitors as research tools to study the EV-uptake may lead to developing more efficient anti-metastasis therapies. In the current study, we used a drug repurposing approach to identify compounds that inhibit EV-internalization.
Methods: PNT2C2 normal prostate epithelial cells were treated with combined more than 2000 compounds from the Prestwick and the LOPAC1280 libraries for 16 h. Subsequently, EVs derived from the PCa cell line DU145, isolated via several centrifugation and ultracentrifugation steps and labeled with PKH26, were added to the cells for 3 h. At the end, EV-uptake was measured using the Opera Phenix HCS system. For validation, serial dilution of compounds and two incubation times of 16 h and 30 min were considered. Flowcytometry (FCM) was additionally used for data validation. Ingenuity Pathway Analysis (IPA) was performed to explore the mechanism of action of the inhibitory compounds.
Results: From more than 300 effective compounds, 15 inhibitors and 10 inducers were considered for the validation steps. Four EV-uptake inhibitors and four inducers were validated in independent experiments. The inhibitors exhibited a ~3-fold decrease in EV-uptake as compared to the controls, while inducers caused less than a ~1.5 folds increase in EV-uptake. Interestingly, two inhibitors had an almost immediate effect on EV-uptake within 30 min of incubation.
IPA analysis of the protein targets of the inhibitors revealed that EV-uptake inhibiting compounds mostly affect the pathways related to regulation of P glycoproteins and ATPases**.
**The name of the compounds and molecules cannot be mentioned at the moment, because of patent/publication concerns.

Summary/Conclusion: Compound screening led to discovery of at least two novel EV-uptake inhibitors, which will be further evaluated as tools to manipulate the EV-uptake to investigate the role of EV cell-cell communication during tumor growth and metastasis.
Funding: Supported by proEVLifeCycle project funded by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 860303.

PF04.09 | Role of N-glycosylations in mesenchymal stromal cell-derived extracellular vesicles' uptake and functionality

Marta Clos Sansalvador[1]; Marta Monguió-Tortajada[2]; Charles Williams[3]; Miriam Moron-Font[4]; Antoni Bayes-Genis[2]; Santiago Roura[2]; Marcella Franquesa[4]; Francesc E. Borràs[4]
[1]1. IVECAT, REMAR-IGTP Group & Nephrology Service, "Germans Trias i Pujol" Health Science Research Institute & University Hospital, Can Ruti Campus, Badalona, Spain 2. Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain, Badalona, Spain; [2]ICREC Research Program, Health Science Research Institute Germans Trias i Pujol, Can Ruti Campus, Badalona, Spain, Badalona, Spain; [3]6. Glycotechnology Laboratory, CIC biomaGUNE, San Sebastián, Spain 7. Exosomes Laboratory, Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA). Derio, Bizkaia, Spain, San Sebastián, Spain; [4]1. IVECAT, REMAR-IGTP Group & Nephrology Service, "Germans Trias i Pujol" Health Science Research Institute & University Hospital, Can Ruti Campus, Badalona, Spain, Badalona, Spain

Introduction: Extracellular vesicles derived from mesenchymal stromal cells (MSC-EV) are increasingly considered a powerful cell-free alternative therapy for the treatment of various inflammatory disorders. MSC-EV are safe cargo transporters of molecular mediators and messengers that have demonstrated to function in a similar way to their producing cells in terms of immunomodulation and tissue damage repairing. However, specific mechanisms behind these processes are still not fully understood and results are often controversial.
Methods: In this study, we focused on the role of EVs' N-glycosylations in mediating EV uptake and MSC-EV functionalities, such as cell recruitment and angiogenesis promotion. Immortalized Wharton's Jelly MSC-derived EVs (iWJ-MSC-EV) were isolated by size exclusion chromatography (SEC), characterized by bead-based flow cytometry, treated with PNGase-F enzyme to remove N-glycosylations, and functionally analyzed.
Results: Our results showed that iWJ-MSC-EV recruited both pro-angiogenic (endothelial cells) and pro-regenerative cells (allogeneic MSC), and induced tube formation capacity by endothelial cells. Conversely, removal of N-glycosylation through PNGase-F treatment abolished these effects. Lectin microarrays of both types of EV confirmed differences in the glycosylation pattern of PNGase-F-treated EV in some N-acetylglucosamine binding lectins, mannose-binding lectins and fucose binding lectins. We also observed iWJ-MSC-EVs' N-glycosylations are fundamental for EV-capture by resting endothelial cells. However, TNFα-activated endothelial cells increased capture of PNGase-F-treated EV compared to control endothelial cells, suggesting an alternative capture pathway not affected by the enzyme treatment. Studies to reveal these receptor/ligand interactions are underway.
Summary/Conclusion: Overall, these results further stress the importance of glycosylations in EV biology and function.

PF04.10 | TERT mediated interactions between Neuroblastoma cells and non-cancerous neuronal cells via extracellular vesicles

Tal Manko[1]; Ishai Luz[1]; Meshi Zorsky[2]; Gad Vatine[2]; Ester Priel[1]; Tomer Cooks[1]
[1]The Shraga Segal Department of Microbiology and Immunology, Ben Gurion University of the Negev, beer sheva, Israel; [2]The Department of Physiology and Cell Biology, Ben Gurion University of the Negev, beer sheva, Israel

Introduction: Extracellular vesicles (EVs) play an important role in cell-to-cell communication, particularly in cancerous cells, allowing them to interact with their microenvironment. Notably, 85%-90% of cancers use telomerase to achieve immortalization. Telomerase reverse transcriptase (TERT) is the catalytic unit in telomerase involved in multiple molecular and cellular mechanisms (attributed to splicing variants) such as apoptosis prevention, gene regulation, heat shock, Wnt signaling and telomere elongation. Despite extensive neuroblastoma (NB) research, there are still many gaps in our knowledge regarding tumorigenesis and its molecular driving forces. Neurons are non-replicated cells, thus, the role of TERT is associated with different functions. However, as the neuron cell becomes cancerous, TERT levels increase and tumor tissues with positive-telomerase activity correlate with poor prognosis. Due to the important roles of both EVs and TERT in cancer, we hypothesized that TERT plays a role in EVs with secretion, cargo, and effects on non-cancerous cells.
Methods: NB cell lines from mouse spinal cord representing different onsets were co-cultured with mouse motor neuron like cells for rtPCR of splice variants of TERT and neuron biomarkers. EVs from all cells were extracted according to the MISEV

2018 guidelines. After treatments with AGS499, a synthetic compound that increase TERT expression and activity in neuron cells, EVs were measured using Nano tracking analysis. NB cells were co-cultured with IPSCs derived endothelial cells for TEER measurement done by Epithelial Volt/Ohm (TEER) Meter 3.
Results: We have shown that treatment with AGS499, affected the quantitative secretion of EVs in both cancerous NB cells in different onsets and in non-cancerous motor-neuron cells. In addition, we showed that different TERT levels in the cells were positively correlated with EVs secretion levels. Finally, we showed that NB cells co-cultured with IPSCs derived endothelial cells, cause changes in TEER levels representing tight junctions in the BBB.
Summary/Conclusion: Our findings indicate that NB cells use TERT-driven EVs, potentially promoting tumorigenesis.

PF04.11 | Transfection reagent artifact accounts for some reports of extracellular vesicle function

Russell McConnell; Madeleine Youniss; Bhargavee Gnanasambandam; Palak Shah; Jonathan Finn; Sriram Sathyanarayanan
Codiak BioSciences, Cambridge, USA

Introduction: Extracellular vesicles (EV) are important mediators of cell communication and physiology. EVs are frequently investigated by transiently transfecting cells with plasmid DNA to produce EVs modified with protein(s) or nucleic acid(s) of interest. DNA-transfection reagent complexes (DTC) are approximately the same size as EVs, raising the possibility that some purification procedures may fail to separate these two species and activity arising from transfection may be improperly attributed to EVs.
Methods: EV producing cells were transiently transfected with plasmid DNA encoding an EV-targeted Cre recombinase. Differential and density gradient ultracentrifugation were used to purify EVs from cell culture supernatant or DTC from cell-free culture media. Cre localization to EVs was confirmed by Western blot and plasmid DNA detected by PCR. Cells stably expressing a fluorescent Cre reporter were used to assess functional enzyme delivery in recipient cells.
Results: Reporter cells treated with ultracentrifuge pellet material (UCP) showed robust and reproducible signal, however fractionating UCP with an iodixanol density gradient revealed that reporter activity was not associated with EV-enriched fractions. UCP isolated from identical transfection conditions, but lacking cells (and therefore EVs), showed similar Cre activity levels and distribution in iodixanol gradients, suggesting that Cre activity was due to contaminating DTC and not EV-mediated delivery of Cre protein. Moreover, steps commonly taken to remove plasmid DNA from EV samples, such as media exchanges and treatment with nucleases, are ineffective at avoiding this artifact.
Summary/Conclusion: Due to the pernicious nature of DTC in these cellular assays, some reports of EV function are likely artifacts produced by contaminating DTC and not EV-mediated cargo delivery.

PF05: EVs role in physiology and pathology

Chair: Michela Pozzobon, Department of Women's and Children's Health, University of Padova, Padua, Italy

PF05.01 | A protein corona around extracellular vesicles is essential for their function in aiding angiogenesis and wound healing

Martin Wolf[1]; Rodolphe W. Poupardin[1]; Constantin Blöchl[2]; André Cronemberger Andrade[1]; Fausto Gueths Gomes[1]; Patrica Ebner Peking[1]; Balazs Vari[1]; Essi Eminger[1]; Heide-Marie Binder[1]; Anna M Raninger[1]; Nicole Maeding[1]; Gabriele Brachtl[1]; Andreas Spittler[3]; Thomas Heuser[4]; Astrid Obermayer[2]; Christian G Huber[2]; Katharina Schllmoser[5]; Hans-Dieter Volk[6]; Dirk Strunk[1]
[1]*Cell Therapy Institute, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI - TReCS), Paracelsus Medical University (PMU), Salzburg, Austria, Salzburg, Austria;* [2]*Dept. of Biosciences, Paris Lodron University Salzburg, Salzburg, Austria;* [3]*Anna Spiegel Center of Translational Research, Medical University, Vienna, Vienna, Austria;* [4]*Vienna Biocenter Core Facilities, Medical University, Vienna, Vienna, Austria;* [5]*Department of Transfusion Medicine and SCI-TReCS, PMU, Salzburg, Austria, Salzburg, Austria;* [6]*BCRT & Institute of Medical Immunology, Charite - Univeritätsmedizin Berlin, Germany, Berlin, Germany*

Introduction: Evidence is accumulating indicating that extracellular vesicles (EVs) can carry a protein corona comparable to the corona around synthetic nanoparticles in protein rich environment. We could recently show that EVs derived from therapeutic grade placental stroma cells (PLX) contribute to their efficacy in aiding re-vascularization of ischemic tissue. Here we investigated whether a protein corona around these PLX-EVs contributes to their function.
Methods: EVs were enriched from PLX-conditioned media via tangential flow filtration (TFF), followed by further purification via size exclusion chromatography (SEC) or ultracentrifugation. Identity and purity of the different EV preparations was

analysed by immunoblotting, super resolution microscopy and transmission electron microscopy (TEM). EV functionality was determined during in vitro angiogenesis, modulation of immune cell proliferation, and in vivo skin wound healing in a humanized mouse model. Proteomics were used to identify active components in EV fractions. Finally, we re-established a functional protein corona with defined factors around naked EVs.

Results: EV identity according to MISEV2018 criteria was confirmed via immunoblots. With super-resolution microscopy we found considerable heterogeneity of tetraspanin (CD9, CD63 and CD81) expression at single EV level. TFF-purified PLX-EVs displayed proangiogenic potential, inhibited T cell proliferation in vitro, and enhanced vessel density and full thickness skin wound healing in a mouse model. When further purified via ultracentrifugation or SEC, EVs lost these functions. This effect was accompanied by a loss of their protein corona as visualized by negative contrast TEM. Re-establishing the protein corona with defined factors restored their function.

Summary/Conclusion: TFF-purified PLX-EVs carry a functional protein corona. Corona removal resulted in a loss of function. The guided corona restoration with defined factors restored their function.

Funding: This work was supported by funding from the European Union's Horizon 2020 research and innovation program (grant agreement no. 733006 to Dirk Strunk), by Land Salzburg IWB/EFRE 2014 - 2020 P1812596 and WISS 2025 20102-F1900731-KZP EV-TT (to Dirk Strunk).

PF05.02 | Acute effects of interesterified fats on numbers and thrombogenic activity of circulating extracellular vesicles

Amal Alanzeei[1]; Shin Soyama[1]; Aseel Alkoblan[2]; Pippa Gibson[2]; Sarah Berry[2]; Wendy L Hall[2]; Parveen Yaqoob[1]
[1]University of Reading, Reading, United Kingdom; [2]King's College London, London, United Kingdom

Introduction: Interesterified (IE) fats are used by the food industry, particularly in spreads, to confer suitable functionality to certain foods without the adverse health effects of trans fats. However, the effects of these fats on cardiovascular disease (CVD) risk markers remain to be elucidated. Extracellular vesicles (EVs) are nano-sized membrane derived vesicles released by activated or dying cells, reported to have pathophysiological effects in CVD, and are modified by dietary fat intake, suggesting their potential as a novel biomarker.

Methods: Samples of 35 healthy subjects were used for EV analysis for this randomized controlled, double-blind, crossover study conducted at King's College London (KCL). Subjects received four test meals in random order containing 50 g test fat (spreadable butter, IE spread, functionally-equivalent non-IE spread, control rapeseed oil), 15 g protein and 85 g carbohydrate. This research was approved by the Research Ethics Committee at KCL. Blood was sampled at baseline and 3 postprandial time points (2, 4 and 8h) and EV fractions obtained from 500 μL of platelet-free plasma by size exclusion chromatography using the IZON qEVOriginal column. EV subtypes and numbers were analysed using flow cytometry and Nanoparticle Tracking Analysis (NTA) respectively. The thrombogenic activity of EVs was assessed using tissue factor-based thrombin generation and fibrinolysis assays.

Results: There was a pronounced postprandial increase in plasma triglyceride concentrations and the number and thrombogenic activity of EVs regardless of treatment. There was no influence of the IE spread relative to non-IE spread on EV number, thrombin generation or fibrinolysis, but there were some differences in the mean size of EVs following consumption of different types of fat.

Summary/Conclusion: There was a pronounced postprandial increase in the number and thrombogenic activity of circulating EVs following a high-fat meal, but this is not altered by replacing the fat source with commercial IE fats.

Funding: This project was supported by BBSRC DRINC grants BB/N021185/1 and BB/N020987/1

PF05.03 | Blood-derived extracellular vesicles change their oxidative properties after myocardial infarction

Vytautas Žėkas[1]; Reet Kurg[2]; Kristiina Kurg[2]; Daiva Bironaitė[3]; Mantas Radzevičius[4]
[1]Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine, Institute of the Biomedical Sciences, Faculty of Medicine, Vilnius University, Vilnius, Lithuania; [2]Institute of Technology, Tartu University, Tartu, Estonia; [3]Dept. of Regenerative Medicine, State Research Institute Centre for Regenerative Medicine, Vilnius, Lithuania; [4]Dept. of Physiology, Biochemistry, Microbiology and Laboratory Medicine, Institute of Biomedical Sciences, Faculty of Medicine, Vilnius University, Vilnius, Lithuania

Introduction: We investigated the amount and composition of extracellular vesicles (EVs) derived from male 40-60-year-old healthy controls and same age and sex post-myocardial infarction (post-MI) patients' blood samples and showed their oxidative-related properties. Our study aimed to determine the EV yield and composition differences between both groups as well as to find out if there were differences between EV mediated oxidative stress reactions.

Methods: We included 15 post-MI patients and 25 healthy controls in the study. Local Vilnius University Bioethics Committee approved the study protocol. Procedures were in compliance with the Helsinki Declaration. Informed consent was obtained. EVs were isolated by ultracentrifugation and their concentration and properties were analyzed using nanotracking analysis (NTA). To characterise EVs TSG101 was detected using western blot and flotillin-1 was detected using fluorescent flow cytometry (FFC) in the EVs samples. CD9 concentration was detected in each EV sample by the FFC method. Thioredoxin, Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX), extracellular signal-regulated kinases 1/2 (ERK1/2) and protein kinase B (Akt B) was detected by western blot. H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) fluorescence detected by FFC was used as a marker to measure EV-induced oxidative stress in the human vein endothelium cells (HUVEC) culture.
Results: We measured higher EVs concentration in healthy controls than in the post-MI group (7.07±3.1 E+10 ml vs 3.1±1.9 E+10 ml, $P < 0.001$) and a higher level of CD9-positive exosomes (MFI 275±39.5 vs 252±13, $P < 0.001$). Thioredoxin, extracellular signal kinases 1/2 (ERK 1/2) and protein kinase B (Akt B) were detected in all EV samples but at higher levels in post-MI EVs samples. Post-MI patient EVs gave a lower oxidative stress response in the culture of the HUVEC cells than the EVs of the healthy controls ($P < 0.001$).
Summary/Conclusion: We conclude that post-MI patient blood sample EVs have stronger anti- than pro-oxidative properties and these could help fight against post-MI consequences.

PF05.04 | Exploring the small RNA component of extracellular vesicle populations released from human saphenous vein vascular smooth muscle cells and investigating their effect on cell proliferation

Antoniya Pashova[1]; Lorraine M. Work[1]; Stuart A. Nicklin[2]
[1]*University of Glasgow/SNBTS, GLASGOW, United Kingdom;* [2]*Institute of Cardiovascular & Medical Sciences, University of Glasgow, Glasgow, UK, Glasgow, United Kingdom*

Introduction: Vascular smooth muscle cell (VSMC) proliferation is one of the main drivers of neointimal formation that leads to vein graft occlusion after coronary artery bypass grafting with autologous saphenous vein (SV). Here, the small RNA content of EVs secreted by VSMCs under control and pathophysiological stimuli was investigated, as well as the effects of EVs on proliferation of recipient VSMC.
Methods: SV SMCs were cultured in control media (quiescence) or with media +20ng/mL platelet-derived growth factor (PDGF-BB) as a proliferative stimulus. RNAseq differential expression analysis of the small RNA component of EVs-derived from vehicle control cells (vEVs) or PDGF-stimulated cells (pEVs) was carried out and validated by qPCR. Bioinformatics was employed to identify potential miRNA gene targets. Gene set enrichment analysis was carried out through the ToppGene bioinformatics tool. Recipient quiesced or PDGF-stimulated SV SMCs were treated with vEVs or pEVs and the effect on cell proliferation was measured using bromodeoxyuridine (BrdU) assay.
Results: EVs were successfully isolated from conditioned culture medium and characterised. RNAseq analysis identified 6 differentially expressed miRNAs between the two groups (miR-24, -224, -1, let-7A2, -21, -409), all of which were upregulated in pEVs compared to vEVs. Three miRNAs were validated by qPCR (miR-24, -224, and -409). Gene set enrichment analysis suggested that all three validated miRNAs might be involved in the regulation of biological processes relevant to the process of neointimal formation including cell growth/proliferation/migration/wound healing/apoptosis. No significant difference in proliferation of recipient quiesced and PDGF-stimulated SMCs was observed when treated with vEVs versus pEVs.
Summary/Conclusion: The expression of miR-24, -224, and -409 is upregulated in pEVs suggesting a potential role of this EV population in the regulation of cellular processes underlying the development of neointimal formation.
Funding: Funded by the British Heart Foundation (FS/16/55/32731) and the College of Medical, Veterinary and Life Sciences, University of Glasgow

PF05.05 | Extracellular matrix attenuation at the ocular drainage system by SMAD7 miRNA loaded Extracellular Vesicles, an approach for Glaucoma disease

Elie Beit-Yannai; Saray Tabak; Valeria Feinshtein; Sofia Schreiber-Avissar
Department of Clinical Biochemistry and Pharmacology, Beer-Sheva, Israel

Introduction: Failure to regulate elevated IOP is the major risk factor for the development of primary open-angle glaucoma (POAG), established by the disruption of trabecular meshwork (TM) function, which leads to increased resistance to the aqueous humor (AH) outflow. Extracellular vesicles (EVs) hold immense promise for utilization as drug delivery agents due to their nature as biological nanoparticles that facilitate intercellular molecular transport, participating in the communication between the non-pigmented ciliary epithelium (NPCE) and the TM tissue in the ocular drainage system. So far, small interfering RNAs (siRNAs)

have been successfully loaded into EVs for a variety of delivery applications, but the potential use of NPCE derived EVs for siRNA delivery to TM cells regulating the Wnt-TGFβ2 signaling has scarcely been explored.
Methods: NPCE derived EVs were isolated by a series of ultra-centrifugations, their size and concentration were determined by Tunable Resistive Pulse Sensing (TRPS) technology. To verify whether engineered EVs are more efficacious on TM cells modulation, anti-fibrotic (SMAD7) siRNA was loaded into EVs by electroporation. EV's structural integrity was evaluated using cryo-TEM analysis, and the efficiency of the loading process was estimated by fluorescent principle, including TECAN device and confocal microscopy. Engineered EVs were added to pre-cultured TM cells for 24hr. qRT-PCR was used to verify the transfer of selected siRNA to TM cells and Western blot analysis was used to evaluate qualitative effects on Wnt-TGFβ2 proteins expression.
Results: EVs were successfully loaded with exogenous siRNA, reaching 23% positive EVs to the fluorescent siRNA (siGLO) marker, avoiding aggregates formation and vesicle rapture. The therapeutic potential of EVs-mediated siRNA delivery was demonstrated by the mRNA (53%) knockdown of SMAD7 in TM cells. Examination of Wnt-TGFβ2 revealed a significant elevation in the levels of β-Catenin, pGSK3β, N-Cadherin, K-Cadherin, and TGFβ2 proteins in TM cells, treated with NPCE EVs loaded with SMAD7 siRNA.
Summary/Conclusion: NPCE derived EVs can overcome the barriers for efficient siRNA molecule delivery to TM cells, which may be beneficial as a medical intervention for monitoring the Wnt-TGFβ2 signaling pathway as a therapeutic target to lower IOP.
Funding: The ISRAEL SCIENCE FOUNDATION (grant No. 1545/20)

PF05.06 | Helium conditioning increases cardiac fibroblast migration which effect is not propagated via soluble factors or extracellular vesicles

Csenger Kovácsházi[1]; Marek Jelemenský[2]; Kristína Ferenczyová[3]; Monika Hofbauerová[4]; Bernadett Kiss[5]; Éva Pállinger[6]; Ágnes Kittel[7]; Viktor nabil Sayour[5]; Anikó Görbe[8]; Csilla Pelyhe[5]; Szabolcs Hambalkó[5]; Lucia Kindernay[2]; Miroslav Barančík[2]; Péter Ferdinandy[9]; Monika Barteková[10]; Zoltán Giricz[9]
[1]Semmelweis University, Department of Pharmacology and Pharmacotherapy, Budapest, Hungary, Budapest, Hungary; [2]Institute for Heart Research, Centre of Experimental Medicine, Slovak Academy of Sciences, 84104 Bratislava, Slovakia;, Bratislava, Slovakia; [3]Institute for Heart Research, Centre of Experimental Medicine, Slovak Academy of Sciences, 84104 Bratislava, Slovakia;, bra, Slovakia; [4]Institute of Physics, Slovak Academy of Sciences, Dúbravska cesta 9, SK-84511 Bratislava, Slovakia; Centre of excellence for advanced material application, Slovak Academy of Sciences, Dúbravská cesta 9, SK-84511 Bratislava, Slovakia;, Bratislava, Slovakia; [5]Semmelweis University, Department of Pharmacology and Pharmacotherapy, Budapest, Hungary;, Budapest, Hungary; [6]Semmelweis University, Department of Genetics, Cell- and Immunobiology, Budapest, Hungary, Budapest, Hungary; [7]Institute of Experimental Medicine, Eötvös Loránd Research Network, Budapest, Hungary, Budapest, Hungary; [8]Semmelweis University, Department of Pharmacology and Pharmacotherapy, Budapest, Hungary; Pharmahungary ltd., Szeged, Hungary;, Budapest, Hungary; [9]Semmelweis University, Department of Pharmacology and Pharmacotherapy, Budapest, Hungary; Pharmahungary ltd., Szeged, Hungary, Budapest, Hungary; [10]Institute for Heart Research, Centre of Experimental Medicine, Slovak Academy of Sciences, 84104 Bratislava, Slovakia; Institute of Physiology, Faculty of Medicine, Comenius University in Bratislava, 81372 Bratislava, Slovakia;, Bratislava, Slovakia

Introduction: Helium inhalation induces cardioprotection against ischemia/reperfusion injury, of which cellular mechanism is not fully elucidated. Extracellular vesicles (EVs) mediates cardioprotective mechanisms, but their function in helium-conditioning (HeC) is not elucidated yet. Fibroblasts are key regulators of post-ischemic remodelling, therefore they may take part in HeC-induced cardioprotection, as well. Therefore, we aimed to test, how HeC affects cardiac fibroblasts and if their HeC-induced EVs or other secreted factors mediates remodelling of the cardiac tissue.
Methods: Neonatal rat cardiac fibroblasts (NRCF) were exposed to glucose deprivation and HeC rendered by four cycles of 95% helium + 5% CO2 for one hour, followed by one hour of normal culturing conditions. 40 hours later, NRCF migration was analyzed and Western Blot and quantitative PCR were used to analyze the expression of fibroblast to myofibroblast transformation markers. From serum-free cell supernatant, medium-sized extracellular vesicles (mEVs) were isolated with differential centrifugation and characterised with WB, transmission electron microscopy and nanoparticle tracking analysis. The supernatant of HeC-treated NRCF was transferred to naïve NRCF or immortalized human umbilical vein endothelial cells (HUVEC/TERT2) and migration and in vitro angiogenesis assay was performed.
Results: HeC accelerated the migration of NRCF. Meanwhile, HeC did not increase the expression of myofibroblast markers. HeC tended to decrease mEV secretion of NRCFs, but supernatant of HeC-NRCF neither accelerate the migration of naïve NRCF, nor affect the angiogenic potential of HUVEC/TERT2.
Summary/Conclusion: Since HeC increased the migration of NRCF but HeC-NRCF mEVs did not affect the function of remote cells, HeC may exert its cardioprotective effect via NRCFs, but does not affect cardiac remodelling remotely via NRCF mEVs.

Funding: Project was supported by 2020-4.1.1.-TKP2020; VEKOP-2.3.3-15-2016-00016; STIA-KFI-2019; NKFIA; NVKP-16-1-2016-0017; K_21-139105; VEKOP-2.3.3-15-2016-00016; STIA-KFI-2019. VEGA no. 2/0104/20; APVV-18-0548; M.J. and K.F. were supported by National Scholarship Programme of the Slovak Republic from Slovak Academic Information Agency; Z.G. is supported by ÚNKP-18-4-E-17; ÚNKP-19-4-SE-94 and by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. V.N.S was supported by EFOP-3.6.3-VEKOP-16-2017-00009 and by the Gedeon Richter Excellence PhD Scholarship. C.K. was supported by NTP-NFTÖ-21-B-0300.

PF05.07 | Podocyte-specific Extracellular vesicles yield novel insight into intercellular signaling in the Glomerulus

Annika Pape[1]; Kilian Teicher[1]; Inka Homeyer[1]; Milagros Wong[1]; Anja Obser[1]; Kian Deheshwar[2]; Nicola Wanner[1]; Maja Lindenmeyer[3]; Franz Lennard. Ricklefs[4]; Catherine Meyer-Schwesinger[2]; Victor Puelles[1]; Tobias Huber[1]; Fabian Braun[1]
[1]III. Department of Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, Hamburg, Germany; [2]Institute of Cellular and Integrative Physiology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, Hamburg, Germany; [3]III. Department of Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, III. Department of Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, Germany; [4]Department of Neurosurgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Introduction: Extracellular vesicles (EVs) have been identified to play an essential role in basic pathological processes such as malignant, metabolic and autoimmune diseases. Nevertheless, knowledge about their role in kidney health and disease remains scarce. A new group of EVs, shed upon cell stress with the ability to induce a proliferative effect in neighboring cells, was recently identified in cell culture models as well as experimental glomerulonephritis. This study aimed to characterize these medium sized EVs (mEVs) shed by glomerular epithelial cells, podocytes, and the signaling propagated by them.
Methods: Using differential centrifugation (1.500g for 20 minutes, 16.000g for 60 minutes at 4°C in standard plastic 50ml conical tubes) and filtration (5μm) we established a protocol to separate mEVs from cell culture supernatants, kidney tissue and urine samples. With Western Blot, immunofluorescence microscopy employing antibody stainings and pKH membrane dyes as well as image flow cytometry we investigated the release dynamics of podocyte specific mEVs in different models of murine podocyte damage in vitro and in vivo. Furthermore, cross culture experiments and life microscopy were used to determine the effect of podocyte specific mEVs on parietal epithelial cells. Besides this, proteomic analysis of EVs was performed to investigate the content in podocyte specific EVs shed upon different stressors.
Results: Podocyte-specific mEVs were detected in baseline podocyte culture supernatant, murine kidney tissue as well as the urine of healthy human volunteers. Quantification revealed a drastic increase of vesicle release upon podocyte damage both in vitro and in vivo. Interestingly, podocyte-specific EVs exerted different effects on the proliferative and migratory behavior of primary parietal epithelial cells depending on the initial stressor. Proteomics revealed first candidate proteins differentially abundant in EVs released upon podocyte stress, that could prove responsible for these different effects.
Summary/Conclusion: Our study represents the first investigation of podocyte-specific medium sized extracellular vesicles, their release dynamics, protein containments and functional implications in health and disease. As these vesicles can be separated without advanced equipment such as ultracentrifuges, they could also be a valuable source for biomarker research in various nephropathies.

PF05.08 | Potential of podocyte-derived urinary extracellular vesicles in the display of podocyte injury in membranous nephropathy

Karen Lahme[1]; Kian Deheshwar[1]; Gilliana Gloede[1]; Stephanie Zielinski[1]; Oliver Kretz[2]; Shuya Liu[2]; Fabian Braun[3]; Tobias B. Huber[2]; Lukas Heintz[1]; Desiree Loreth[1]; Wiebke Sachs[1]; Catherine Meyer-Schwesinger[1]
[1]Institute of Cellular and Integrative Physiology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, Hamburg, Germany; [2]III. Department of Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Hamburg, Germany; [3]III. Department of Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, Hamburg, Germany

Introduction: Membranous nephropathy (MN) is an autoimmune glomerulonephritis induced by circulating autoantibodies directed against podocyte foot process proteins. MN is characterized by the accumulation of aggregated proteins in injured podocytes. The upregulation of the ubiquitin-proteasome system (UPS) occurs during disease progression and correlates with podocyte injury in rodent models. Here we tested whether the abundance of podocyte-derived urinary extracellular vesicles (EVs) changes and whether the content mirrors podocyte injury.

Methods: EVs were isolated from mouse and human urine by differential ultracentrifugation. For the human urine, EVs were additionally isolated by ultrafiltration and size exclusion chromatography. EVs were quantified by nanoparticle tracking analysis, characterized by electron microscopy and analyzed by immunoblotting. The proteostatic content of EVs and glomeruli was analyzed in 3 murine models of podocyte injury (experimental MN, adriamycin nephritis, and type 2 diabetes). Pulldown of podocyte-derived EVs was established from human urine via immunoprecipitation and EVs from nephrotic patients were investigated.
Results: Both human and murine urinary EVs contain UPS components. The amount of podocyte-derived EVs increases differentially in murine podocyte injury models: The abundance increases in a dose- and time-dependent manner in experimental MN, but not in adriamycin nephritis and diabetic nephropathy. The proteostatic content changes in a disease-dependent manner, mirroring the disease-associated proteostatic situation of podocytes. Human podocyte-specific EVs differ in size from total human urinary EVs and, depending on the nephrotic syndrome, the amount and content of podocyte-derived EVs differs between patients.
Summary/Conclusion: Analyses of podocyte-derived urinary EVs have the potential to give insight in the proteostatic status of podocytes, possibly reflecting origin (and prognosis) of the underlying injury.

PF06: Fundamental biology: EV biogenesis (from prokaryotes to eukaryotes), component loading, and release

Chair: Vincent Hyenne – INSERM / CNRS

Chair: Irma Schabussova, Medical University of Vienna

PF06.01 | Aberrant Serine Metabolism in Cancer Cells Promotes Secretion of Extracellular Vesicles

Tomofumi Yamamoto[1]; Jun Nakayama[2]; Kagenori Ito[2]; Yusuke Yamamoto[2]; Takahiro Ochiya[3]
[1]Department of Molecular and Cellular Medicine, Institute of Medical Science, Tokyo Medical University, Tokyo, Japan., Chuo-Ku, Japan; [2]Labolatory of Integrative Oncology, National Cancer Center Research Institute, Tokyo, Japan., Chuo-Ku, Japan; [3]Department of Molecular and Cellular Medicine, Institute of Medical science, Tokyo Medical University, Shimjuku-ku, Japan

Introduction: Cancer cells secrete more extracellular vesicles than normal cells and create their tumor microenvironment. Although we reported several EV secretion mechanisms so far, the mechanism of cancer EV secretion still remains elusive (Urabe et, al., Sci. Adv., 2020). To reveal the universal mechanisms of EV secretion in cancer, we sought to determine key molecules, combined a microRNA (miRNA) library screen with high-throughput EV quantification.
Methods: The amount of EVs was measured by ExoScreen, which is an ultra-sensitive detection method of EVs by measuring surface protein of EVs, such as CD9 and CD63 (Yoshioka et al., Nat Commun., 2015). EVs were isolated by ultracentrifugation. We performed this screen in 2 types of cancer cells (colon and lung). We found an miRNA and its target genes, which regulated EV secretion in both cell lines. Finally, we validated the effect of this miRNA and its target genes in other types of cancer (breast, ovarian, multiple myeloma, head and neck, pancreas, and melanoma).
Results: Here we identified miR-891b and its direct target gene, phospho-aminotransferase 1 (PSAT1), which significantly promote EV secretion and cancer metastasis. Loss of function experiments with PSAT1 pathways showed that serine metabolism is critical for aberrant EV secretion in cancer. Of note, the miR-891b/PAST1 axis regulated EV secretion in multiple types of cancer, suggesting a common mechanisms of cancer EV secretion. Interestingly, we found overexpression of PSAT1 in metastatic breast cancer cells (lymph-node metastasis, lung metastasis, bone metastasis), and enhanced PSAT1 expression remarkably induced bone metastasis in a breast cancer xenograft model by activating osteoclastogenesis via EVs.
Summary/Conclusion: These data suggested a miR-891b/PAST1 axis is a universal mechanisms of cancer EV secretion and enhanced bone metastasis in breast cancer cells.

PF06.02 | Bleeding risk in patients with thrombocytopenia: low platelet concentration prevents aggregation and release of procoagulant extracellular vesicles

Naomi C. Buntsma[1]; Aleksandra Gasecka[2]; Sytske Talsma[3]; Jagoda Rytel[4]; Krzysztof Mądry[4]; Grzegorz Basak[4]; Edwin van der Pol[5]; Rienk Nieuwland[6]
[1]Department of Neurology, Laboratory of Experimental Clinical Chemistry & Biomedical Engineering and Physics, Amsterdam UMC, the Netherlands, Amsterdam, Netherlands; [2]1st Chair and Department of Cardiology, Medical University of Warsaw, Warsaw, Poland & Laboratory of Experimental Clinical Chemistry, Amsterdam UMC, Amsterdam, the Netherlands, Amsterdam,

Netherlands; [3]Laboratory of Experimental Clinical Chemistry, Amsterdam UMC, Amsterdam, the Netherlands, Amsterdam, Netherlands; [4]Department of Hematology, Oncology and Internal Medicine, Medical University of Warsaw, Warsaw, Poland, Warsaw, Poland; [5]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Amsterdam, Netherlands; [6]Department of Clinical Chemistry; Vesicle Observation Center; Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands

Introduction: Aggregation of platelets and the release of procoagulant extracellular vesicles (EVs) both play a pivotal role in hemostasis. However, why hemostatic processes only start to fail when platelet concentrations are below 5E7/mL, increasing the bleeding risk, is yet unknown. We previously demonstrated that platelet-platelet interaction is essential for both platelet aggregation and the subsequent release of platelet-derived EVs (PEVs). Here, hemostatic mechanisms are investigated in a patient group with low platelet concentrations, thereby aiming to explain the increased risk of bleeding.
Methods: Blood was collected from idiopathic thrombocytopenia purpura (ITP) patients (n=10) and healthy controls (n=3). Aggregation of platelets was induced by thrombin-receptor activating peptide (TRAP) or saline (control), and measured for 6 minutes in citrate-anticoagulated blood (Multiplate). Subsequently, PEV concentrations were measured by flow cytometry (Apogee A60-Micro, scatter triggering, EV detection limit 160 nm).
Results: TRAP induced platelet aggregation and a 2.2-fold increase in PEV concentration in blood from healthy controls. In contrast, neither platelet aggregation nor an increase in PEV concentration was observed when TRAP was added to blood from ITP patients.
Summary/Conclusion: In ITP patients, platelet activation does neither result in platelet aggregation nor in the release of PEVs. This finding supports our hypothesis that PEVs are mostly released when platelet activation is followed by platelet-platelet interaction. In addition, these findings suggest that below a "critical platelet concentration" multiple hemostatic mechanisms become impaired, being both platelet aggregation and the release of PEVs, which together contribute to an increased risk of bleeding.
Funding: N. Buntsma acknowledges the Dutch Heart Foundation, research grant CINTICS 2018B031.
A. Gasecka acknowledges funding from the Polish National Science Centre, research grant PRELUDIUM 2018/31/N/NZ7/02260.

PF06.03 | Cigarette smoke extract exposure alters fatty acid profiles and levels of oxidatively modified proteins in extracellular vesicles released by airway epithelial cells

Sandra Buratta[1]; Anna Sansone[2]; Elisabetta Chiaradia[3]; Lorena Urbanelli[1]; Roberto Maria Pellegrino[1]; Alessia Tognoloni[3]; Raffaella Latella[4]; Giada Cerrotti[1]; Brunella Tancini[4]; Carla ferreri[2]; Carla Emiliani[1]
[1]Università di Perugia, Perugia, Italy; [2]Consiglio Nazionale delle Ricerche Research Area of Bologna, Bologna, Italy; [3]Department of Veterinary Medicine, University of Perugia, Perugia, Italy; [4]Department of Chemistry, Biology and Biotechnology, University of Perugia, Perugia, Italy

Introduction: Here, we evaluated levels of oxidized proteins and fatty acid composition of Extracellular Vesicles (EVs) released by BEAS-2B cells exposed to cigarette smoke extract (CSE). No studies have investigated such molecular composition, even if it has been demonstrated that CSE induces the release of EVs with modified protein and RNA profiles from airway epithelial cells [Benedikter et al., 2019].
Methods: Viability and intracellular ROS levels in BEAS-2B cells treated with CSE were measured by MTT and DCFH-DA, respectively. EVs, isolated from cell culture media by a differential centrifugation protocol, were characterized by Scanning Electron Microscopy and Immunoblotting [Kowal et al., 2016] Levels of carbonylated protein, which are an early marker for protein oxidation, were measured by Oxyblot. Fatty acid composition and the identification of positional/geometrical isomers of fatty acids were identified by GC-MS [Sansone et al., 2020].
Results: CSE-EVs presented elevated levels of carbonylated proteins, compared to EVs from controls. CSE exposure induced only few changes in fatty acid profiles of cells. Extensive remodeling of phospholipid acyl chains was observed in CSE-EVs, i.e. high levels of palmitic (16:0) and arachidic (20:0) acids and decreased levels of sapienic (6 cis-16:1 n-10) and oleic (9 cis-18:1 n-9) acids. These changes accounted for the higher content of saturated fatty acid (SFA) and lower levels of monounsaturated (MUFA) in CSE-EVs, compared to controls.
Summary/Conclusion: CSE-EVs are characterized by 1) high content of carbonylated proteins, thus suggesting that CSE-treated cells can remove oxidized proteins via EVs in order to reduce their harmful effects; 2) high SFA/MUFA ratio, a membrane parameter important for EV stability, EV uptake and, in part, for EV-mediated pro-inflammatory responses. This study could be a starting point for the utilization of circulating EVs as biomarkers for the diagnosis of CSE-induced lung damage and/or cigarette smoke-related diseases.

PF06.04 | Comparison of bacterial cell- and extracellular vesicle-associated small RNAs and their potential to interact with human transcripts

Kylie I. Krohmaly[1]; Marcos Perez-Losada[2]; Andrea L. Hahn[3]; Robert Freishtat[4]
[1]Center for Genetic Medicine Research, Children's National Hospital, washington, USA; [2]Computational Biology Institute, Department of Biostatistics and Bioinformatics, Milken Institute School of Public Health, The George Washington University, Washington, USA; [3]Children's National Hospital, Washington, USA; [4]Center for Genetic Medicine Research, Children's National Hospital, Washington, USA

Introduction: Characterizing the influence of the microbiome on both acute and chronic airway conditions is critical to improving their management. Small RNAs (sRNAs), commonly packaged in extracellular vesicles (EVs), can regulate interspecies gene expression and potentially facilitate microbiome-host communication. We aim to identify and compare bacterial sRNAs in the EV and cytoplasm, and to determine potential microbe-host interactions.
Methods: We selected four common respiratory microbes, Haemophilus influenzae (Hi), Moraxella catarrhalis (Mc), M. nonliquefaciens (Mn), and Streptococcus pneumoniae (Sp) (ATCC). We isolated EVs from cultured bacterial supernatant via precipitation and SEC. Cell pellet and EV RNA was extracted and sRNA-sequencing performed on NovaSeq (Illumina). sRNA sequences were aligned and annotated via Partek, sRNA Detect, ARNold, and BLAST.
Results: The mean number of reads per sample was about 15 million. Each species was slightly different, but the majority of sRNAs were unannotated (41-43%), followed by tRNAs (6-52%). The remaining sRNAs were coding RNA (6-35%), rRNA (0-4%), and too short to align (6-31%). We calculated EV:Cytoplasm abundance ratios for the sRNAs. Any sRNA two times more abundant in either the EV or the cytoplasm was considered enriched. Hi and Sp had more EV-enriched sRNAs compared to Mc and Mn (26%, 20%, 2.6%, and 0% respectively), while Mc and Mn had more cytoplasm-enriched sRNAs compared to Hi and Sp (51%, 31%, 6.6%, and 5.2% respectively). Alignment of bacterial sRNA sequences to the human genome to determine potential human-microbe interactions was low (4-18% of sRNAs). Realignment incorporating RNA secondary structure with base pairing is underway.
Summary/Conclusion: We found that Hi and Sp preferentially packaged sRNAs in EVs, while Mc and Mn preferentially retained sRNAs in the cytoplasm. By incorporating sRNAs secondary structure, we will be able to identify potential microbe-host interactions in the human airway.

PF06.05 | Contractile activity of human primary myotubes in culture affects the microRNA and protein cargo of secreted extracellular vesicles

Kari Bente Foss Foss Haug[1]; Vigdis Aas[2]; Berit S. Brusletto[1]; Trude Aspelin[3]; Anne-Marie Siebke Trøseid[4]; Saba Qureshi[5]; Desima SO Eid[5]; Ole Kristoffer Olstad[1]; Tuula Nyman[6]; Reidun Øvstebø[3]
[1]Department of Medical Biochemistry, Oslo University Hospital, Ullevål, Norway, Oslo, Norway; [2]Department of Life Science and Health, Oslo Metropolitan University (OsloMet), Norway, Oslo, Norway; [3]The Blood Cell Research Group, Department of Medical Biochemistry, Oslo University Hospital, Ullevaal, Norway, Oslo, Norway; [4]The Blood Cell Research Group, Department of Biochemistry, Oslo University Hospital Ullevaal, Oslo, Norway, Oslo, Norway; [5]Department of Life Sciences and Health, Oslo Metropolitan University (OsloMet), Norway, Oslo, Norway; [6]Department of Immunology, University of Oslo and Oslo University Hospital, Norway, Oslo, Norway

Introduction: Physical activity is important for prevention and treatment of life style diseases. During exercise, contracting skeletal muscles release bioactive factors including extracellular vesicles (EVs) that can communicate with other cells and mediate beneficial effects. In the present study, we have examined how electrical pulse stimulation (EPS) of primary human myotubes, a model of exercise, affects the microRNA- and protein-cargo of secreted EVs.
Methods: Muscle biopsies from obese patients with type 2 diabetes (n=6) were used to isolate, in vitro cultivate, and differentiate satellite cells into mature myotubes. Low frequency EPS was applied to myotubes for 24 h, and EVs were collected from serum free media the next 24 h. EVs were isolated, separated and concentrated into microvesicles (MV) and exosomes by combined centrifugation (17 000g) and filtration (0,22 um and 100 kDa filters). EV size and concentration were revealed by nanoparticle tracking analysis, tetraspanins analysed by flow cytometry, Hsc70/Hsp70 and calnexin by Western blotting and morphology visualized by transmission electron microscopy. EV content was studied by high resolution proteomics (LS-MS/MS) and transcriptomics (Affymetrix microarray) combined with bioinformatics using Ingenuity Pathway Analysis (IPA).
Results: Size, concentration and CD63- and CD81-levels of MV and exosomes were unaffected by EPS. The protein content of MV and exosomes was clearly different, and they both showed significantly changed protein levels after EPS. Whereas the microRNA content of MV was basically unchanged by EPS, the exosomes appeared with a significantly altered microRNA pattern. IPA revealed that pathways involved in cell growth and protein synthesis were the most affected by EPS.

Summary/Conclusion: EPS clearly changed the microRNA- and protein patterns of EVs secreted from primary myotubes, indicating that physical training of skeletal muscles may convey beneficial effects to other cells and organs, reflected through the cargo load of secreted EVs.
Funding: The Norwegian Diabetes Association

PF06.06 | Donor cell-specific aspects of the small extracellular vesicle production

Maria Harmati[1]; Timea Boroczky[2]; Matyas Bukva[2]; Gabriella Dobra[3]; Edina Gyukity-Sebestyen[1]; Krisztina Buzas[4]
[1]Laboratory of Microscopic Image Analysis and Machine Learning, Institute of Biochemistry, Biological Research Centre, Szeged, Hungary, Szeged, Hungary; [2]Laboratory of Microscopic Image Analysis and Machine Learning, Institute of Biochemistry, Biological Research Centre, Szeged, Hungary; Doctoral School of Interdisciplinary Medicine, University of Szeged, Szeged, Hungary; Department of Immunology, Albert Szent-Györgyi Medical School, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary, Szeged, Hungary; [3]Laboratory of Microscopic Image Analysis and Machine Learning, Institute of Biochemistry, Biological Research Centre, Szeged, Hungary; Doctoral School of Interdisciplinary Medicine, University of Szeged, Szeged, Hungary, Szeged, Hungary; [4]Laboratory of Microscopic Image Analysis and Machine Learning, Institute of Biochemistry, Biological Research Centre, Szeged, Hungary; Department of Immunology, Albert Szent-Györgyi Medical School, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary, Szeged, Hungary

Introduction: It is widely demonstrated that extracellular vesicle (EV) release depends on the donor cells and the microenvironmental stimuli. Today, there are several quantitative methods to measure EV production, but comparative studies are still limited. Here, we investigated different mammalian cell cultures – as in vitro models of various healthy cells and tumors – to compare their capacity for EV release and vesicular protein production.
Methods: We set up a heterogeneous cell culture panel (n=17) paying attention to the diverse origin, morphology and disease state (normal-tumor cells). Small EVs (sEVs) were isolated by differential filtration and ultracentrifugation, then subjected to nanoparticle tracking analysis and BCA protein assay. Primary mouse mesenchymal stem cell (MSC) isolation was performed in accordance with the national and European animal ethics guidelines (clearance no.: XVI./78/2018).
Results: The average number of produced sEVs per cell per unit of time was higher and more variable in the group of tumor cells compared to the normal cells. In contrast, the protein content of the tumor cell-derived sEVs was lower. For instance, THP-1 human monocytic leukemia cells produced the most sEVs and the primary MSCs released the highest amount of vesicular protein. In general, the vesicular protein production correlated with the population doubling time and the cell size. Beside the cell physiology, culture conditions also influenced the sEV production, as it was significantly higher in the first 24 h after passage.
Summary/Conclusion: These data further evidence that the ability for EV production depends on several internal and external parameters providing a unique and continuously adapting communication route for all cells. At the same time, exploring the quantitative differences in sEV production between cells may provide valuable information for describing the pathophysiology of different tumors or assess the efficacy of sEV-based therapies.
Funding: This study was supported by the following research grants: MEC_R 141434, GINOP-2.2.1-15-2017-00052, ÚNKP-20-3, University of Szeged, Szent-Györgyi Albert Research Fund (2021).

PF06.08 | Extracellular vesicles from Vibrio cholerae contain AT-rich DNA and shorter mRNAs that do not correlate with their protein products

Petter Langlete[1]; Anders Krabberoed[2]; Hanne C. Winther-Larsen[3]
[1]Norwegain Institute of Public Health, Oslo, Norway; [2]University of Oslo, Oslo, Norway, Oslo, Norway; [3]University of Oslo, Oslo, Norway

Introduction: Bacterial membrane vesicles (MVs) are found to be secreted from most bacterial species at all stages of growth and are associated with a range of phenotypes such as biofilm formation, bacterial communication, phage and antibiotic resistance. These 50 – 250 nm sized spheres contain DNA, RNA, lipopolysaccharides (LPS) and proteins, thereby resembling their mother cell but in a non-replicative form. Membrane vesicles are found to elicit a protective immune response in a range of host and are currently used for epidemic control of meningitis in humans. Our research group are exploring the possibility to use BEV as vaccines in fish for aquaculture. In so doing we need to ensure their safety for use in food for human consumption. An understanding of the packaging of their cargo content is therefore of essence.
Methods: We have fully performed proteomics, DNA and RNA sequencing of isolated BEV and compared that to the contents of the mother cell from which the BEV has been arrived. We have performed these studies using the bacteria Vibrio cholera as a model.

Results: We found BEV with both singe and double membranes in our isolated BEV fraction. From the DNA sequencing we found that specific part of the DNA was specifically packaged into the BEVs, in particular those containing the regulatory ToxR binding protein sequence AAAAAANATNAAA. Subtracting rRNA from the total RNA content, the mRNA content of the BEV seen to be depending on size and purine content. There are also a positive bias for certain noncoding functional RNAs. The proteins of the BEV have no correlation with protein size, there are a positive selection of the outer membrane proteins OmpK, OmpT, OmpU, OmpV and the Pencillin binding protein activator YcfM, In addition there is an enrichment of proteins from Chromosome II compared to Chromosome I (Chr II more associated with virulence factors).
Summary/Conclusion: Our findings suggest a specific packaged of genetic material into BEVs is pending on size and sequence. We also have data that propose that this genetic material does not seem to be translated in the vesicles themselves, which is a central question in EV biology and function. In accordance with data from other researchers there is a specific packaging of protein cargo.
Funding: Norwegian Research Council.
University of Oslo.

PF06.09 | IL-8/CXCR1 axis induces biogenesis of small extracellular vesicles from A2780 ovarian cancer cells with a pro-tumorigenic profile

Albano Caceres-Verschae[1]; Belen Gaete-Ramirez[2]; Cristóbal Cerda-Troncoso[3]; Jorge Cancino[4]; Patricia V. Burgos[5]; Lara J. Monteiro[6]; Manuel Varas-Godoy[7]
[1]PhD Program in Biomedicine, Faculty of Medicine, Universidad de los Andes, Av. La Plaza 2501 Las Condes, Santiago , Chile., LAS CONDES, SANTIAGO, Chile; [2]Cancer Cell Biology Laboratory, Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Lota 2465, Providencia, Santiago, Chile., Santiago, Chile; [3]Organelle Phagy Lab, Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Lota 2465, Providencia, Santiago, Chile., Santiago, Chile; [4]Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Lota 2465 Providencia, Santiago, Chile, Santiago, Chile; [5]Organelle Phagy Lab, Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Lota 2465, Providencia,Santiago, Chile, Santiago, Chile; [6]Biology of reproduction Laboratory, Center for Biomedical Research and Innovation (CIIB), Universidad de los Andes, Avenida La Plaza 2501 Las Condes, Santiago, Chile, Las Condes, Chile; [7]Cancer Cell Biology Laboratory, Centro de Biología Celular y Biomedicina (CEBICEM), Facultad de Medicina y Ciencia, Universidad San Sebastián, Santiago, Chile., Las Condes, Santiago, Chile

Introduction: Small extracellular vesicles (sEVs) play an important role in tumor progression, and sEV of cancer stem-like cells (CSCs) can contribute to metastasis. In several tumors CSCs express CXCR1, but its contribution in the biogenesis of metastatic sEV remains unclear. We hypothesize that IL-8/CXCR1 activation could modify the production sEVs with a pro-tumorigenic activity.
Methods: Ovarian cancer cells A2780 which overexpress CXCR1 were stimulated with IL-8, and proteins involved in sEVs biogenesis (Alix, HRS, TSG101 and CD9) were evaluated by western blot. sEV were characterized measuring proteins enriched in sEVs by western blot, and their size and concentration were determined by Nanotracking Analysis. The effect of sEVs in recipient cells was performed in endothelial (HUVEC) and low invasive ovarian cancer (OVCAR) cells evaluating tube and sphere formation assay, respectively. The protein content of sEVs was evaluated by Mass-Spectrometry.
Results: We observed that activation of IL-8/CXCR1 in A2780 cells increased the level of proteins related to sEVs biogenesis accompanied with an increase in the sEVs release. Also, transfer of sEVs released in response to IL-8/CXCR1 activation induced tube formation in HUVEC and sphere formation in OVCAR3 cells. Proteins involved in angiogenesis and cell proliferation (FDZ2, PI3KC2A, among others) were enriched in this sEV and could be responsible to induce pro-tumorigenic properties.
Summary/Conclusion: Our findings suggest that the IL-8/CXCR1 axis could modulate the biogenesis and release of sEVs with pro-tumorigenic activity, becoming this axis a new target to abrogate metastasis mediated by sEVs.
Funding: FONDECYT 1190928, ANID/BASAL/FB210008

PF06.10 | Mutated β-catenin regulates extracellular vesicles machinery in hepatocellular carcinoma

Camille DANTZER[1]; Aude BRUNEL[2]; Jean-Frédéric BLANC[1]; Mireille VERDIER SAGE[2]; Barbara BESSETTE[2]; Fabrice LALLOUE[2]; Violaine MOREAU[1]; Clotilde BILLOTTET[1]
[1]UMR1312 Inserm, Bordeaux, France; [2]UMR1308 Inserm, Limoges, France

Introduction: Hepatocellular carcinoma (HCC) is the most common primary liver cancer; it represents the 6th most frequent tumor in the world and the 4th leading cause of cancer-related death. Due to the lack of efficient therapies and the difficulty to detect the pathology during early stages, HCC has a poor prognosis. Today, the immunotherapy represents the first-line treatment for advanced HCC with beneficial effect on overall survival. Despite this therapeutic advance, clinical data suggest that immunotherapy could be less effective in patients with β-catenin-mutated HCC. These tumors are characterized by an environment devoid of immune infiltrates, leading to resistant-immunotherapy tumors. However, the role of ß-catenin promoting this immune escape and leading to tumor cells trigger immunosuppressive cascades is not yet fully understood. Our project focuses on the role of ß-catenin signaling in tumor/immune cells communication through extracellular vesicles (EVs).
Methods: Using transcriptomic analysis, we showed an alteration of gene expression involved in EVs machinery in HepG2 cells upon knock-down of mutated ß-catenin. We also revealed by nanoparticle tracking analysis and flow cytometry, a modulation of EVs secretion when β-catenin is mutated.
Results: We further identified two target genes of the EVs machinery, RAB27A and SDC4, whose expression is ß-catenin-dependent. Thus, these results suggest that ß-catenin mutations inhibit EVs formation and/or secretion in liver tumor cells. As EVs and their contents are main factors for intercellular communications, we now hypothesized that the decrease of EVs production could lead to defective recruitment of leukocytes, making these tumors poor in immune infiltrates and resistant to immunotherapy.
Summary/Conclusion: Our results provide a new knowledge on the impact of ß-catenin mutations on EVs biogenesis. This may allow the development of a new tool from liquid biopsies to stratify HCC patients for immunotherapy response.

PF06.11 | Norovirus interactions with Enterobacter cloacae result in lipidomic and metabolomic changes in bacterial extracellular vesicles

Chanel Mosby-Haundrup; Melissa Jones
University of Florida, Gainesville, USA

Introduction: Human noroviruses (HNoVs) are the leading cause of gastroenteritis. An incomplete understanding of HNoV pathogenesis has hindered development of effective antivirals. Due to difficulty in culturing HNoV in vitro, murine norovirus (MNV) is used as a surrogate virus. It was recently shown that the interaction of norovirus and Enterobacter cloacae (E. cloacae) results in gene expression changes and increased production of bacterial extracellular vesicles (bEVs). Proteomic analysis of E. cloacae bEVs saw significant differences in protein content between bEVs produced in the presence and absence of MNV. Based on these findings, our hypothesis is that the presence of MNV will result in changes to the bEV lipidomic and metabolomic profiles.
Methods: E. cloacae was incubated with MNV and grown to produce bEVs. Low speed spins pelleted bacterial cells and the supernatant underwent sequential ultracentrifugation steps and 0.2μm filtration to recover bEVs. The bEVs were sent off for global lipidomics and metabolomics.
Results: Significant differences were found in the lipidomic and metabolomic profiles of the bEVs in the presence of MNV compared to controls. An increased abundance of lipids in the phosphatidylethanolamine (PE) class are of particular interest as increased PE lipids have been seen in hypervesiculation mutants and are thus consistent with previously published data demonstrating that MNV increases vesicle production.
Summary/Conclusion: The changes in the bEVs lipidomic and metabolomic profiles support changes previously seen in the protein cargo of the bEVs. The data also suggests that the presence of MNV alters the biogenesis pathway for E. cloacae bEVs whereby bEV formation shifts from explosive cell lysis to membrane blebbing. These findings will help to further the understanding of how norovirus interacts with commensal bacteria and lead to a better understanding of norovirus pathogenesis as well as how commensal bacteria respond to external stressors through bEV production.
Funding: R21 NIH grant# R21AI140012
Pilot Grant funded by the Southeastern Center for Integrative Metabolomics

PF06.12 | Response of blood cells to a Listeriolysin O and its mutant LLO Y406A

Apolonija Bedina-Zavec[1]; Rebeka Podgrajšek[2]; Ana Špilak[2]; Maja Jamnik[2]; Matic Kisovec[1]; Gašper Šolnič[2]; Nejc Petrišič[2]; Veronika Kralj Igič[3]; Gregor Anderluh[2]; Marjetka Podobnik[1]
[1]National Institute of Chemistry, Department of Molecular Biology and Nanobiotechnology, Ljubljana, Slovenia, Ljubljana, Slovenia; [2]Department of Molecular Biology and Nanobiotechnology, National Institute of Chemistry, SI-1000 Ljubljana, Slovenia, Ljubljana, Slovenia; [3]Biomedical Research Group, Faculty of Health Sciences, University of Ljubljana, SI-1000 Ljubljana, Slovenia, Ljubljana, Slovenia

Introduction: Listeriolysin O (LLO) is a toxin of the intracellular pathogen Listeria monocytogenes, that forms pores in cholesterol-rich lipid membranes of host cells. Its action depends on pH. The pH optimum of LLO is pH 5.5, a condition found in late endosomes, while it can bind to the membrane,form pores and damage cells even at neutral pH. Our group generated the LLO mutant Y406A whose activity at room temperature is even more dependent on pH than the activity of the wild type (LLO wt). We compared the effect of LLO wt and LLO Y406A on various cancer blood cells with the effect on healthy cells isolated from blood.
Methods: The cell lines K562 and Raji were used. PBMCs were obtained from the blood of healthy donors (with informed consent and according to the study protocol approved by the National Medical Ethics Committee (0120-21/2020/4)). Cells were incubated with LLO or its mutant Y406A for 30 min at 37°C. Cytotoxicity was measured by quantification of cellular membrane integrity (propidium iodide and trypan blue staining) and by cell viability assay (Presto Blue). ROS (reactive oxygen species)-generation activity and the amount of ATP in cells were also measured. EVs were isolated by differential centrifugation. The concentration of larger EVs in the samples was measured by flow cytometry using Annexin-FITC. The concentration of smaller EVs in the samples was measured by DLS.
Results: The results showed that the toxicity of LLO Y406A to cancer and healthy blood cells was very low at physiological pH (7.4). Treatment with LLO Y406A resulted in reduced cell viability by 20% at concentrations 300 to 7000 times higher than treatment with LLO wt. In addition, LLO wt and LLO Y406A were found to be more toxic to cancer blood cells than to healthy blood cells under physiological conditions. Concentration of LLO wt needed to kill 20 % of cells is 2 to 20 times higher for healthy cells than for cancer cells. Concentration of LLO Y406A needed to kill 20 % of cells is 2 times higher for healthy cells than for cancer cells. As pH decreases, the activity of both proteins continues to increase. At pH 6.5, concentration of LLO wt needed to kill 20 % of cells is 4 times higher for healthy cells than for cancer cells; and concentration of LLO Y406A needed to kill 20 % of cells is 3 to 15 times higher for healthy cells than for cancer cells. Increased extracellular vesiculation in response to LLO wt and LLO Y406A was detected at protein concentrations 10 to 100 times lower than initial cell death. LLO wt and LLO Y406A did not cause an increase in ROS, but LLO wt caused an increase in ATP levels.
Summary/Conclusion: The differences in the toxicity of LLO wt and LLO Y406A to cancer and healthy cells, as well as the increased activity of proteins at pH 6.5, suggest that both LLO wt and LLO Y406A are of potential interest for stimuli responsive applications and cancer treatment.
Funding: Slovenian research agency, P1-0391.

PF06.13 | Syndecans and tetraspanins: partners in the sorting of signaling proteins to small extracellular vesicles?

Rania Ghossoub[1]; Raphael Leblanc[2]; Stéphane Audebert[3]; Eric Rubinstein[4]; Guido David[5]; Pascale Zimmermann[6]
[1]Cancer Research of Marseille - Aix-Marseille University, Marseille, France; [2]CRCM, Marseille, France; [3]Centre de Recherche en Cancérologie de Marseille (CRCM), Aix-Marseille Université, Marseille, France; [4]Centre d'Immunologie et des Maladies Infectieuses, Inserm, CNRS, Sorbonne Université, CIMI-Paris, 75013 Paris, France, paris, France; [5]Centre de Recherche en Cancérologie de Marseille, Marseille, France; [6]K. U. Leuven, Leuven, Belgium

Introduction: Exosomes, small extracellular vesicles of endosomal origin, are master regulators of cell-to-cell signaling in physiology and disease. Exosomes are highly enriched in tetraspanins and syndecans, the latter occurring mainly in proteolytically-cleaved form. While both protein families are membrane scaffolds appreciated for their role in exosome formation, composition and activity, we currently ignore whether tetraspanins and syndecans work together to control exosome composition. Syndecans can regulate the compartmentalization of tetraspanins (and associated cargos) into exosome [1].
Methods: Small extracellular vesicles from MCF7 breast cancer cells were isolated by serial ultracentifugation and analyzed by proteomics and nanoparticle tracking analysis. The expression of proteins of interest were modulated using loss- and gain- of function experiments and the
effects were further analyzed by immunoblots, biochemical analysis and confocal microscopy.
Results: We showed that Tetraspanin-6 associates with syndecan-4 and acts as a negative regulator of exosome release by supporting the lysosomal degradation of syndecan-4 and syntenin [2]. Strikingly, our recent data show that syndecans control the balance between cellular and exosomal ADAM10, a sheddase regulating a plethora of signaling receptors implicated in neurodegenerative diseases, inflammation and cancer.
Summary/Conclusion: Our work highlights various mechanisms by which syndecans control the biology of exosomes. These findings clarify our understanding of the molecular determinants governing exosome formation, composition and heterogeneity. This would help to rationalize their use as source of biomarkers and therapeutic tools.

[1] M. F. Baietti et al., « Syndecan-syntenin-ALIX regulates the biogenesis of exosomes », Nat Cell Biol, vol. 14, no 7, p. 677-685, juill. 2012, doi: 10.1038/ncb2502.
[2] R. Ghossoub et al., « Tetraspanin-6 negatively regulates exosome production », Proc Natl Acad Sci U S A, vol. 117, no 11, p. 5913-5922, mars 2020, doi: 10.1073/pnas.1922447117.

PF06.14 | TP53 mutations correlate with the non-coding RNA content of small extracellular vesicles in melanoma

Maureen Labbé[1]; Estelle Menoret[1]; Franck Letourneur[2]; Benjamin Saint-Pierre[2]; Laurence De Beaurepaire[3]; Joelle Veziers[4]; Brigitte Drenos[5]; Marc G Denis[6]; Nicolas Boisgerault[1]; Jean-François Fonteneau[1]; Christophe Blanquart[1]; Delphine Fradin[1]
[1]CRCI2NA, INSERM, Nantes Université, Nantes, France, Nantes, France; [2]Université de Paris, Institut Cochin, INSERM, CNRS., 75014 Paris, France, Paris, France; [3]IECM, ONIRIS, INRAE, USC1383, Nantes, France, Nantes, France; [4]SC3M, SFR Sante F. Bonamy, FED 4203, UMS Inserm 016, CNRS 3556, Nantes F-44042, Nantes, France; [5]Dermatology Department, Director of the Unit of Cell and Gene Therapy CHU Nantes, CIC 1413, CRCI[2]NA, University Nantes, Nantes, France; [6]Department of Biochemistry, Nantes University Hospital, Nantes, France, Nantes, France

Introduction: Secretion of RNAs in extracellular vesicles (EV) is a recently recognized form of cell-to-cell communication in which RNAs can change gene expression of recipient cell. Some RNAs are preferentially enriched into EVs, but mechanisms controlling this specific sorting remain poorly understood. Here, we investigated the impact of mutations on NRAS, BRAF, and TP53 genes on the non-coding RNA content of melanoma small EV (sEV).
Methods: Melanoma cell lines established from local patients with different mutational status were cultured in EV-depleted media for 24h. sEV were isolated from culture supernatant using differential ultracentrifugation and characterized for size and concentration by transmission electronic microscopy and Tunable Resistive Pulse Sensing experiments. The expression of sEV marker proteins were assessed by western blot. Profiling of non-coding RNAs (ncRNAs) were performed by next-generation sequencing for microRNAs (miRNAs), and by micro-array for long non-coding RNAs (lncRNAs). Enriched RNA motifs in sEV and sequences recognized by RNA binding proteins were identified by bioinformatic analyses. Sam68 protein quantification was performed by western blot.
Results: We showed that TP53-mutated cells release lncRNAs and miRNAs that are distinct from those secreted by TP53 wild-type melanoma cells, whereas BRAF and NRAS mutations did not modulate the RNA cargo of sEV. Furthermore, we found that long and small ncRNAs enriched in TP53-mutated sEV share a common sequence motif, highly similar to the RNA binding motif of Sam68, a protein interacting with hnRNP proteins. Finally, we found that Sam68 protein is enriched in TP53-mutated melanoma cell lines, leading probably to this selective sorting.
Summary/Conclusion: Our results support the existence of cellular mechanisms for selective export of different RNA classes into small EVs depending on the TP53 mutational status of the cell. sEV-derived nucleic acids could provide clinically relevant information on cancer progression or other pathophysiological features.

PF07: Technologies and methods: EV quantitation

Chair: Zoltán Varga – Biological Nanochemistry Research Group, Institute of Materials and Environmental Chemistry, Research Centre for Natural Sciences

Chair: Dario Brambilla, National Research Council of Italy – Institute on Chemical Sciences and Technology

PF07.01 | Comparing digital detection platforms in high sensitivity immune-phenotyping of Extracellular Vesicles

Roberto Frigerio[1]; Angelo Musicò[2]; Gabriella Andriolo[3]; Lucio Barile[3]; Alessandro Gori[4]; Marina Cretich[4]
[1]Consiglio Nazionale delle Ricerche, Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC), Milan, Italy, Milano, Italy; [2]Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC), Consiglio Nazionale delle Ricerche, Milano, Italy; [3]Istituto Cardiocentro Ticino, Ente Ospedaliero Cantonale, Lugano, Switzerland; [4]Consiglio Nazionale delle Ricerche, Istituto di Scienze e Tecnologie Chimiche "Giulio Natta" (SCITEC), Milan, Italy, Milano, Italy

Introduction: Extracellular Vesicles (EVs) based diagnostic practices must comply with the putative clinical value of low to very low abundant EV sub-types within complex media. In this regard, the enormous diagnostic potential of EVs could be unleashed

by the extraordinary sensitivity offered by digital biosensing platforms, namely capable of reaching the single molecule detection level. As far as immune-phenotyping of EVs coupled to digital readout, two platforms are finding their place in the spotlight: the Single Particle Interferometric Reflectance Imaging Sensing (SP-IRIS) and the Single Molecule Array technology (SiMoA).
Methods: EVs from human Cardiac progenitor Cells (CPCs) isolated by Tangential Flow Filtration were characterized by Western Blotting (WB), Nanoparticle Tracking Analysis (NTA) and Cryo-Electron Microscopy. The sample was used to provide a side by side multiparametric comparison of the two platforms by studying the abundance of CD9/CD63/CD81. Monoclonal antibodies anti-CD9, anti-CD63 and anti-CD81 were selected for this work and, for a fair comparison of data, applied to both SP-IRIS and SiMoA platforms using custom functionalization protocols of chips and beads respectively.
Results: Calibration curves were generated with different assay schemes. After assessing that the tetraspanin monoclonal antibodies used in this work are not cross-reactive to mouse, to promptly compare the two digital platforms, we mimicked a liquid biopsy by spiking the human CPCs - EVs into mouse serum. The lowest detectable concentration of EVs were determined leading to a limit of detection (LOD) of 10^8 EV/mL for the SP-IRIS platform and 10^7 EV/mL for the SiMoA instrument showing that both platforms sensitivity is affected by sample matrix effect (1 order of magnitude higher LODs compared to spiking in PBS).
Summary/Conclusion: Despite the higher sensitivity demonstrated by SiMoA platform compared to SP-IRIS in this assay, careful consideration of multiple features such as reading time, multiplexing, dedicated software and additional information provided by SP-IRIS (size, biomarker co-localization) have to be considered.
Funding: This work was partially funded from the European Union's Horizon 2020 research and innovation program under grant agreements No. 951768 (project MARVEL)

PF07.02 | Development of an aptamer-based fluorescence polarisation assay for quantification and characterisation of extracellular vesicles

Tarlan Esalmi Arshaghi[1]; Jerry Clifford[2]; Stephanie J Davies[2]; Frank Barry[3]
[1]Regenerative Medicine Institute , National University of Ireland Galway, Galway, Ireland, GALWAY, Ireland; [2]Valitacell Ltd., Dublin, Ireland, Dublin, Ireland; [3]Regenerative Medicine Institute , National University of Ireland Galway, Galway, Ireland., GALWAY, Ireland

Introduction: Extracellular vesicles (EVs) have attracted wide interest in recent years due to their potential applications in regenerative medicine, as biomarkers for disease, and because of their role in cell-cell interactions. However, EV isolation, quantification, and characterisation remain challenging in terms of purity and specificity as well as time- and cost-effectiveness. This work aims to develop a novel and high-throughput EV quantification tool based on the interaction between a fluorescently labeled probe and a specific EV surface component, using fluorescence polarisation (FP) for detection. The method analyses the change in the polarisation of emitted light between unbound and bound probes, with the observed polarisation in a mixture of labeled probe and target being proportional to the fraction of bound probe. This property of FP allows its use to quantify the amount of EVs in a sample.
Methods: Two distinct strategies have been investigated, with probes targeting (i) specific EV surface markers (Tetraspanins e.g., CD63) for EV sub-population quantification or (ii) the EV phospholipid bilayer membrane for total EV quantification. Commercially available fluorescently labeled CD63 binding aptamers and proteins, and lipophilic dyes have been evaluated. EVs derived from HEK and MSC cultures have been purchased (HansaBioMed) or isolated through PEG-precipitation and ultracentrifugation, and particles quantified through Nano Tracking Analysis (NTA) or Imaging Flow Cytometry. Each probe candidate was incubated with EVs, and FP was measured over time using a Spark Cyto plate reader.
Results: Tetraspanin specific (Anti-CD63) aptamer probes and lipophilic dyes demonstrated increased fluorescence polarisation in response to increasing EV concentration.
Summary/Conclusion: This initial proof of concept supports the use of FP as a high throughput EV detection and quantification method, with the ability to provide both total and CD63+ve particle numbers.
Funding: Marie Curie Career FIT
Enterprise Ireland

PF07.03 | Identification of HER2 positive extracellular vesicles from breast cancer cells by nano-flow cytometry

Karin Ekström[1]; Nasibeh Karimi[2]; Cecilia Lässer[3]; Roger Olofsson Bagge[1]
[1]Sahlgrenska Center for Cancer Research and Wallenberg Centre for Molecular and Translational Medicine, Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; [2]Krefting Research Centre, Institute of Medicine at Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, Gothenburg, Sweden; [3]Krefting

Research Centre, Institute of Medicine at the Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden., Gothenburg, Sweden

Introduction: Breast cancer is the most common diagnosed cancer and the leading cause of cancer-related deaths among females world-wide. Human epidermal growth factor receptor 2 (HER2) is overexpressed in approximately 20 % of breast cancers, and treatment with anti-HER2 therapy improve clinical outcome. HER2 overexpression is evaluated in tumour tissue for the use as both a predictive and prognostic factor. Recent data suggests that serum from patients with HER2 positive breast cancer contain HER2 positive extracellular vesicles (EVs). The aim of this study was to establish a non-washing protocol to detect HER2 on individual EVs isolated from a HER2 positive breast cancer cell line using nano-flow cytometry (nano-FCM).
Methods: The HER2 overexpressing breast cancer cell line BT-474 was cultured and EVs were isolated from the conditioned media by ultracentrifugation. EV quantification and characterization was performed by Qubit protein quantification, nanoparticle tracking analysis, nano-FCM (using S16M-Exo size standard) and western blot (EV and non-EV markers). BT-474 EVs were labelled with HER2 antibody (VioBright FITC, 10X-300X dilution of antibody) and then diluted to reduce background fluorescence prior to nano-FCM analysis. As negative control, EVs from HER2 negative cells were used.
Results: BT-474 EVs were positive for the common EV markers CD9 and Flotillin, while the ER marker Grp-94 was absent in the isolated EVs. Most EVs were in the size range of 40-140 nm, as estimated by nano-FCM. Nano-FCM analyses of HER2 labelled BT-474 EVs showed that HER2 can be detected on the membrane of 10-40 % of the EVs. The HER2 positivity was also verified by Western blot analysis.
Summary/Conclusion: HER2 can be detected on individual EVs from the BT-474 breast cancer cell line using nano-FCM. Potentially, nano-FCM can be used to detect HER2 positive EVs isolated from breast cancer patients.
Funding: Assar Gabrielsson's foundation. Knut and Alice Wallenberg Foundation, Wallenberg Centre for Molecular and Translational Medicine, University of Gothenburg, Sweden

PF07.04 | Influence of purification and anticoagulant on analysis of plasma-derived EVs by imaging flow cytometry

Tobias Tertel[1]; Monika Wolny[2]; Oumaima Stambouli[1]; Ingvild Birschmann[2]; Bernd Giebel[1]
[1]*Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, Essen, Germany;* [2]*Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabeteszentrum Nordrhein-Westfalen, Universitätsklinik der Ruhr-Universität Bochum, Bad Oeynhausen, Deutschland, Bad Oeynhausen, Germany*

Introduction: Extracellular vesicles (EVs) are secreted by virtually all cells. Depending on their origin they appear as promising biomarkers in health and disease, especially if they can be recovered in plasma samples. However, there are many parameters that affect the detectability of EVs in given samples. Amongst others the anticoagulants being used as well as required processing steps in obtaining and preparing plasma for the EV analysis and especially the EV detection method of choice influence the diagnostic usability of EVs essentially.
Methods: Recently, we have qualified imaging flow cytometry (IFCM) as a valid method for the phenotypic characterization of antibody labelled EVs at the single object level. In contrast to many other novel single EV characterization methods, IFCM does not require any major pre- and post EV labelling processes. Antibodies can be simply added to EV containing biofluids and analyzed following a typically 60 minutes lasting incubation period. Using a panel of 20 different antibodies and applying our optimized IFCM analysis strategy, we now have investigated impacts of conventionally applied processing steps, i.e. the centrifugation step regularly applied for the cell and debris clearance of applied plasma samples, as well as that of the eight most frequently used anticoagulants on the detectability and the phenotypic appearance of EVs within the plasma of healthy donors.
Results: Our results demonstrate that the speed of the clearance centrifugation step (2,000, 2,500, 3,800 x g centrifugation, or following Ficoll gradient centrifugation) do not recognizably affect the composition of detected EV populations. In contrast, the choice of the anticoagulant significantly impacted the composition of the different EV populations. While we hardly observed any impact on myeloid cell derived EVs, e.g. CD16+ or CD71+ EVs, the abundance of CD9+, CD41+ and CD61+ EVs, assumedly platelet-derived EVs, was significantly affected. Most CD41+ or CD61+ EVs were recovered in serum or, when Heparin was used as anticoagulant, the lowest amount was detected in EDTA containing plasma. In contrast, HLA-ABC+ and HLA-DR+ EVs had lowest abundances in serum while with all other anticoagulants no differences in quantity are detected.
Summary/Conclusion: Thus, our analyses demonstrate significant impacts of anticoagulants on the detectability of certain EV types, especially on PL-derived EVs.

PF07.05 | MIFlowCyt-EV reporting of single vesicle flow cytometry methods and results

Thomas Maslanik[1]; Erika Duggan[2]; John P. Nolan[2]
[1]*Cellarcus Biosciences, La Jolla, USA;* [2]*Scintillion Institute, San Diego, USA*

Introduction: Introduction. Rigor and reproducibility are major issues for science in general, and the EV field in particular. It is increasingly appreciated that the key to data interpretation lies in the details of the methods used to produce the data. Single vesicle analysis using flow cytometry is a case in point, with literally hundreds of different ways to prepare and measure a sample using a flow cytometer, and the details of those methods and validation of their performance are required for understanding the data. The recent MIFlowCyt-EV guidelines address this need by providing a checklist of issues that must be addressed to enable data interpretation and sharing. Here we illustrate the reporting an EV FC method and results in a MIFlowCyt-compliant manner.
Methods: Methods. We measured individual EVs in culture media and plasma by single vesicle flow cytometry using a commercial flow cytometer and assay kit. Preanalytical steps included pelleting of cells, dilution, and/or concentration by ultrafiltration. The flow cytometers were qualified and calibrated using calibration beads to enable data reporting in standardized units. EV concentration, size, and surface cargo was measured by single vesicle flow cytometry (vFC) which incorporates validated reagents reagents, essential controls, and standardized protocols and data analysis.
Results: Results. We report size, concentration, and tetraspanin number per EV in standard units on EVs from blood and cell culture. Calculation of these parameters is described within the context of the sample preparation and assay protocols. Example calibration and control data that might be included in supplementary methods as suggested by the MIFlowCyt-EV ISEV position paper is reported. Data analysis protocols with an example of how to report a gating strategy and resulting data are illustrated. A walkthrough of the data repository and best practices for use are described.
Summary/Conclusion: Conclusion. Single EV analysis using flow cytometry offers great potential for understanding the diverse origins and functions of EVs, but only if performed in a way that allow assay specificity and sensitivity to be transparently documented. The MIFlowCyt-EV guidelines provide a framework that enables EV FC measurements to be reported in the context of essential experimental details, calibrations, and controls. Many of the concepts and procedures developed for EV FC are extensible to other single EV counting, sizing, and cargo analysis methods.

PF07.07 | Quantitative analysis of molecular cargo transfer from cells to EVs

John P. Nolan; Erika Duggan
Scintillion Institute, San Diego, USA

Introduction: Introduction. EVs carry molecular cargo from their cell of origin, but the mechanisms of cargo selection and loading into EVs are not well understood. We used quantitative single cell and vesicle flow cytometry to measure membrane protein expression on cells and EVs.
Methods: Methods. PC3 cells were cultured, media collected, and EVs concentrated using ultrafiltration (100K MWCO). Cell surface markers were measured by flow cytometry (FC). EV concentration, size, and cargo were measured by single vesicle flow cytometry (vFC). Instruments were calibrated and intensity reported in units of antibodies per cell or EV.
Results: Results. PC3 cells express surface markers at high (>250K median copies/cell: CD71, CD29, CD44, CD54), medium (50K-250K copies: CD9, CD63, CD49f) and low (< 50K copies: CD81, EPCAM, EGFR, STEAP-1) abundances. Cell permeabilization reduced staining for CD9 and CD81 (due to disruption of the plasma membrane), and increased staining for CD63 and STEAP1 (due to accessibility of internal antigen). EVs expressed detectable (>~10 PE MESF) CD9, CD63, CD81 and CD29, with a fraction (~50%) also staining with AnnV. Expression was proportional to EV surface area, with surface densities ranging from a background of ~10 molecules/um2 to >1000 molecules/um2 for high abundance targets. Several high abundance markers (CD71, CD44, CD54) were not detectable on EVs, suggesting differential packaging of cell surface cargo into released EVs. CD63 was expressed at low abundance overall, but a subset of smaller EVs (< 100 nm) expressed CD63 at high surface density (~1000 um2).
Summary/Conclusion: Conclusions. We find that the abundance and surface density of cargo on vesicles can be higher or lower than on the cell of origin. Some abundant cell surface molecules (CD71, ICAM, CD44) were undetectable on EVs, while others (CD9, CD81, CD29) were present at surface density similar to cells. CD63 was present at high density on smaller EVs, consistent with enrichment of CD63 on small exosomes formed inside the cell.

PF07.08 | Shape prediction of single nanoparticles by on-chip trajectory tracking and deep learning analysis

Takanori Ichiki
The University of Tokyo, Bunkyo, Japan

Introduction: We have developed a model for multiparametric analysis of nanoparticles in liquids that combines single-particle measurement of scattered light-imaged nanoparticles with deep learning analysis. We aimed to obtain more information from the measurement data of on-chip Nanoparticle Tracking Analysis (on-chip NTA), which is a single particle measurement technology for nanoparticles in liquids, which has enabled us to evaluate particle size and surface charge. In this study, we developed a deep learning model to predict the shape of differently shaped nanoparticles using the Brownian motion trajectories obtained from NTA measurements.
Methods: Development of the shape estimation model consisted of three stages: NTA measurements for raw data acquisition, creation of a data set for deep learning, and deep learning. First, the Brownian motion trajectories of spherical gold nanoparticles (AuNP, 80 nm diameter) and gold nanorods (AuNR, 45 nm in diameter × 180 nm in length) were measured by NTA. Gold nanoparticles in a microfluidic channel were irradiated with a laser (405 nm), and the scattered light was imaged (0.01 fps) by dark-field laser observation. Then, the center of gravity of the scattered light from each nanoparticle was tracked, and the time-series 2D coordinate data for each particle was acquired. The time series trajectory data were given a correct answer label indicating whether the trajectory was related to spherical or rod-shaped particles and was used as the deep learning data set. The data set was randomly divided into training data, validation data, and test data. We designed and built deep learning models using implemented Fully Connected Neural Network (FCNN), Long Short-Term Memory (LSTM), One-Dimension Convolution Neural Network (1DCNN), and Bidirectional Long Short-Term Memory.
Results: The accuracy of the shape prediction model, using the time series trajectory data of Brownian motion of spherical and rod-shaped gold nanoparticles with almost the same mass and self-diffusion coefficient, reached 70.8% when the shape prediction model developed by the combination of 1DCNN and Bi-LSTM was used.
Summary/Conclusion: In conclusion, the methodological potential for realizing multiparametric analysis of nanoparticles in liquids has been demonstrated. By the deep learning analysis of the trajectory of Brownian motion of nanoparticles in liquid, we obtained the information of the particle shape.

PF07.09 | Single-step approach to prevent swarm detection in extracellular vesicle flow cytometry

Naomi C. Buntsma[1]; Mona Shahsavari[2]; Britta A. Bettin[3]; Chi Hau[4]; Aleksandra Gasecka[5]; Rienk Nieuwland[6]; Edwin van der Pol[3]
[1]Department of Neurology, Laboratory of Experimental Clinical Chemistry & Biomedical Engineering and Physics, Amsterdam UMC, the Netherlands, Amsterdam, Netherlands; [2]Biomedial Engineering and Physics & Laboratory of Experimental Clinical Chemistry, Amsterdam UMC, Amsterdam, Netherlands; [3]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Amsterdam, Netherlands; [4]Department of Clinical Chemistry; Vesicle Observation Center, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands; [5]1st Chair and Department of Cardiology, Medical University of Warsaw, Warsaw, Poland & Laboratory of Experimental Clinical Chemistry, Amsterdam UMC, Amsterdam, the Netherlands, Amsterdam, Netherlands; [6]Department of Clinical Chemistry; Vesicle Observation Center; Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands

Introduction: Flow cytometry (FCM) is commonly used to characterize single extracellular vesicles (EVs) in biological fluids. However, EV FCM can be hampered by swarm detection. Swarm detection occurs when multiple particles are simultaneously illuminated. Therefore, without appropriate dilution, a single event signal might be caused by multiple particles, leading to meaningless concentration estimates. To find the minimum dilution (to maximize EV counts) without swarm detection, it is currently recommended to run a dilution series. However, a dilution series is laborious and unpractical. Here, we aim to find and validate a single-step approach, specifically for our assay and FCM, to minimize sample dilution and prevent swarm detection.
Methods: Five human blood plasma samples from a published study (AFFECT EV) were selected, of which the total concentration of particles exceeding a side scatter cross section of 10 nmˆ2 ranges from 1E9 to 1E11/mL. To systematically investigate the relation between the count rate and swarm detection, dilution series of each sample were prepared and measured by FCM (Apogee A60-Micro) using side scatter triggering.
Results: To avoid swarm detection while optimizing EV counts, samples require a dilution ranging from 35-fold to over 650-fold, resulting in count rates ranging from 4,000 events/s to 10,000 events/s. For all samples, swarm detection is absent for count rates < 4,000 events/s.

Summary/Conclusion: For our assay and FCM, swarm detection does not occur below count rates of 4,000 events/s. Based on this maximum count rate, the minimum sample dilution can be calculated by measuring the total particle concentration of an (over)diluted sample. This single-step approach assures that samples in clinical studies can be measured without swarm detection while maximizing EV counts. It needs yet to be validated whether this approach can be generalized to other flow cytometers.
Funding: N. Buntsma acknowledges the Dutch Heart Foundation, research grant CINTICS 2018B031.
M. Shahsavari acknowledges funding from the Dutch Research Council (NWO), research grant P18-26: Medical Photonics (MEDPHOT)
B.A. Betting acknowledges funding from the EMPIR program, METVESII (project 18HLT01).
A. Gasecka acknowledges funding from the Polish National Science Centre, research grant PRELUDIUM 2018/31/N/NZ7/02260.

PF07.10 | Traceable refractive index measurements of liquids to standardize concentration measurements of extracellular vesicles

Martine Kuiper[1]; Richard Koops[2]; Edwin van der Pol[3]; Rienk Nieuwland[4]
[1]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Delft, Netherlands; [2]VSL, National Metrology Institute, Delft, Netherlands, Delft, Netherlands; [3]Department of Clinical Chemistry, Vesicle Observation Center, Department of Biomedical Engineering and Physics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, Amsterdam, Netherlands; [4]Department of Clinical Chemistry; Vesicle Observation Center; Amsterdam University Medical Center, University of Amsterdam, Amsterdam, Netherlands

Introduction: The concentration of extracellular vesicles (EVs) in body fluids is a promising biomarker. When EV concentration measurements by flow cytometry are combined with Mie theory to relate light scattering signals (of EVs) to diameter, EV concentrations can be reported within a given size range. A required input variable of Mie theory is the refractive index (RI) of the liquid surrounding the EVs. This RI, however, has never been traceably measured. Here, we determine the RI of Dulbecco's phosphate buffered saline (DPBS) using a metrologically traceable set-up and show that the previously assumed RI substantially differs from the actual RI of DPBS.
Methods: We developed a metrological goniometer to measure the RI of liquids with an expanded uncertainty of 1.3E-6. We measured the RI of fresh DPBS (Corning) at a wavelength of 405 nm, which is commonly used in flow cytometry. The measurement was performed at controlled environmental conditions with a temperature of (19.90 ± 0.03) °C.
Results: We found that the RI of DPBS is 1.344599, which is 5E-4 lower than assumed previously. In daily practice, when the lower size gate of a flow cytometer is set at 400 nm, this difference in RI results in a gate uncertainty of 11 nm, corresponding to a 9% uncertainty in measured EV concentrations.
Summary/Conclusion: We determined the actual RI of DPBS, which has practical consequences for standardizing EV concentration measurements by flow cytometry, because the RI of DPBS directly affects the accuracy of the size calibration.
Funding: This work is part of the project 18HLT01 METVES II and received funding from the EMPIR programme co-financed by the Participating States and from the European Union's Horizon 2020 research and innovation programme.

PF07.11 | Uncertainty in EV concentration determination by nanoparticle tracking analysis (NTA)

Jeff Bodycomb[1]; Julie Chen Nguyen[2]
[1]HORIBA Scientific, Piscataway, USA; [2]HORIBA Scientific, Irvine, USA

Introduction: EV concentration is routinely reported and many reports show an expected variation of results when similar animals are analyzed. This variation has multiple components, for example variation due to differences between animals and uncertainty of the final measurement (e.g., NTA). Understanding measurement uncertainty improves interpretation of experimental data. In addition, understanding how to control uncertainty can guide experimental design by clarifying the trade-off between effort and uncertainty.
Methods: Lyophilized exosomes were obtained from Abcam (Lyophilized MCF7 Exosome Standards, lot GR3401014-1). These are derived from MCF7 a human cancer cell line and characterized by Abcam. Exosomes were reconstituted following manufacturer instructions, and diluted in Dulbeccos phosphate buffered saline (dPBS). NTA was performed with a HORIBA ViewSizer 3000 multi-laser NTA instrument. Multiple lasers were used to ensure analysis of all EV sizes in the sample. Laser power settings were 210 mW blue, 12 mW green, 8 mW red and camera gain was 30 dB.

Results: A single aliquot of diluted exosomes was measured six times in succession. The overall particle concentration was 2.63e7 p/mL with a standard deviation of 1.13e6 p/mL. This reflects a coefficient of variation of 4.31%. In these measurements, an average of 1640 particles were observed in each measurement.
Concentration for each size range was obtained and the concentration uncertainty of each bin estimated from the repeats. Plotting the uncertainty as a function of number of analyzed particles shows an approximate square root dependence.
Summary/Conclusion: Repeated measurements can be used to estimate measurement uncertainty for stable dilutions of EV's. The observed coefficient of variation, 4.3% is close to but somewhat higher than the expected from the number of particles counted and Poisson statistics. The predicted value is 1/sqrt(1640) or 2.5%. Repeatability for the concentration in each size class roughly followed Poisson statistics. This indicates that repeatability improves in proportion to the square root of measurement time.

PF07.12 | Towards unbiased and specific staining of EVs allowing for detection in complex particle isolations

Dimitri Aubert[1]; Alice Law[1]; rebecca J. Lees[1]; Rob Tempest[1]; Ben Peacock[2]
[1]NanoFCM, Nottingham, United Kingdom; [2]NanoFCM Co., Ltd., Nottingham, United Kingdom

Introduction: Extracellular vesicles (EVs) are often co-isolated with other non-EV particles of similar density or size, especially when derived from biofluids. Common characterization of size and/or concentration may provide total particle information without specificity towards EVs. Many popular advanced techniques for EV characterization utilize fluorescent labelling to identify subpopulations of EVs.
Methods: Nano-flow cytometry (nFCM) performed on a NanoAnalyzer has the benefit of elucidating both the total particle population as well as the total EV and/or specific EV subpopulations by detecting scattered and fluorescent light. Whilst antibody staining is an option for identifying specific targets, the use of EV compatible dyes allowing fast determination of EV concentrations within complex particle isolations will greatly facilitate research. Dyes used in this study include CFSE, Cell Mask, Memglow, PKH, and many more.
Results: However, currently there are caveats to be aware of for some commercially available dyes. nFCM allowed for in depth comparison of the efficacy of several dyes identifying capabilities such as, high intensity fluorescence, self-quenching and low background, even distribution and correlation with size. Several limiting factors were also identified including, desorption and leeching, micelle formation, and poor specificity.
Summary/Conclusion: Direct comparison of these commonly available dyes will help to inform researchers as to the most applicable dyes for specific scenarios, forming a roadmap for future EV research.

PF08: Reproduction & Metabolism

Chair: Nicole Noren Hooten – National Institute on Aging, U.S. National Institutes of Health

Chair: Alireza Fazeli – Unit of Reproductive and Developmental Medicine, Department of Oncology and Metabolism, The Medical School, University of Sheffield, Sheffield, UK

PF08.01 | Early pregnancy adipocyte-derived small extracellular vesicles microRNAs potentially impact infant adiposity through adipogenesis related signaling pathways

Laura Cechinel[1]; Evan Nadler[2]; Sudha Rao[3]; Pooja Kunte[4]; Krishna Sukla[4]; Chittaranjan Yajnik[4]; Robert J. Freishtat[5]
[1]Children's National Medical Center, Washington, USA; [2]Children's National Hospital and The George Washington University, Washington, USA; [3]Genotypic Technology Pvt Ltd., Bangalore, India; [4]King Edward Memorial Hospital, Pune, India; [5]Children's National Hospital, Washington, USA

Introduction: Introduction: Maternal nutritional status and other in utero exposures are associated with infant adiposity, childhood obesity, and adult cardiometabolic disease. We hypothesize that maternal adipocyte-derived small extracellular vesicles (ad-sEVs) carry miRNAs during early gestation that are associated with high infant adiposity via enhanced fetal adipogenesis.
Methods: Methods: Ad-sEVs were isolated from maternal blood at 8 weeks gestation from a representative subset (n=28) of a maternal-infant cohort (IndiaGDM cohort, n>330; the study was approved by the ethics committee). Newborns were classified high (n=12) or low adiposity (n=16) according to the sum of skinfolds. EV RNA libraries were prepped and sequenced on Illumina NextSeq for about 10 million reads per sample. Data were analyzed in Partek™Flow™. Sequences (16–40nt) were aligned

and differentially expressed miRNAs analyzed using DESeq2. Ingenuity Pathway Analysis predicted mRNAs targeted by these miRNAs.

Results: Results: We identified 8 differentially expressed (FC≥|1.5|; P≤0.05; no FDR) ad-sEV miRNAs between mothers of babies with high vs. low adiposity. These included 1 upregulated (i.e., miR-144-3p – an upregulator of adipogenesis) and 7 downregulated miRNAs (e.g., miR-215-5p and miR-486-5p - associated with childhood obesity). These miRNAs are predicted to target 922 mRNAs. Among them, adipogenesis signaling pathway members STAT5B and FGFR3 are predicted to be upregulated. In addition, STAT3 and PTEN signaling are involved in adipogenesis regulation. Among the differentially regulated pathways, STAT3 signaling was one of the most upregulated ($P < 0.001$; $z=+3.2$) and PTEN signaling is the second most downregulated ($P< 0.001$; $z=-1.6$).

Summary/Conclusion: Conclusions: Maternal ad-sEV miRNAs potentially enhance fetal adipogenesis through several signaling pathways, including STAT3 and PTEN. The findings provide new insights toward understanding the relationship between early fetal exposures, childhood obesity, and adult cardiometabolic disease

PF08.02 | Extracellular vesicles are stable carriers of adiponectin with insulin-sensitive properties

Alexia Blandin[1]; Jérémy Amosse[2]; Grégory Hilairet[3]; Maëva Durcin[3]; Xavier Prieur[4]; Luisa Vergori[5]; Cédric Dray[6]; Jean-philippe Pradère[7]; Séverine Dubois[8]; Jérôme Boursier[8]; Bertrand Cariou[9]; Soazig Le Lay[1]

[1]Inserm U1087 Institut du Thorax Nantes & SFR ICAT Univ Angers, Angers, France; [2]IRSET Laboratory, Inserm, UMR 1085, Rennes, France, Rennes, France; [3]Univ Angers SFR ICAT, Angers, France; [4]Université de Nantes, CNRS, INSERM, l'institut du thorax, Nantes, France; [5]Univ Angers, SFR ICAT, Angers, France; [6]Restore UMR1301 Inserm, 5070 CNRS, Université Paul Sabatier,, Toulouse, France; [7]Restore UMR1301 Inserm, 5070 CNRS, Université Paul Sabatier, France, France; [8]CHU Angers, Angers, France; [9]Université de Nantes, CHU Nantes, CNRS, INSERM, l'institut du thorax, Nantes, France

Introduction: Adipose tissue (AT) plays a critical role in the metabolic crosstalk between organs, relayed by adipokines which participate to obesity-associated complications. Recent studies evidenced AT-derived EV (AT-EV) as important mediators of obesity-associated metabolic dysfunctions. We performed AT-EV adipokine profiling to identify EV-associated factors that could relay EV metabolic effects.

Methods: AT-EV were purified from conditioned media of visceral AT from lean or obese (ob/ob) mice by differential centrifugation. We isolated large (13K) and small (100K) AT-EV that were further characterized by a combination of biochemical, microscopical and physical methods.

Results: We evidenced the ability of AT to secrete both large (lEV) and small (sEV) EV subpopulations, a secretion that is enhanced in obese mice compared to lean animals. Adiponectin was the most enriched adipokine in AT-EV under its oligomeric active forms. Adiponectin mainly distributed at the EV external surface for both AT-EV or plasma EV, as a result of unspecific adsorption of soluble adiponectin. EV-associated adiponectin maintained its insulin-sensitizing properties on target cells and its injection in high fat diet-fed mice prevented the animals from the development of insulin-resistance.

Summary/Conclusion: Altogether, our results highlight EV as new original carriers of metabolic forms of adiponectin, that are likely to participate to its beneficial effects.

Funding: This work was supported by a research grant from Genavie and INSERM

PF08.03 | Extracellular vesicles released by steatotic hepatocytes alter adipocyte metabolism

Maria Azparren[1]; Justyna Mleczko[1]; Felix Royo[1]; Juan M. Falcon-Perez[2]

[1]CIC bioGUNE, Derio, Spain; [2]Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Exosomes Laboratory, 48160 Derio, Spain., Derio, Spain

Introduction: The composition of extracellular vesicles (EVs) is altered in many pathological conditions, and their molecular content provides essential information on features of parent cells and mechanisms of crosstalk between cells and organs. Metabolic Syndrome (MetS) is a cluster of clinical manifestations including obesity, insulin resistance, dyslipidemia and hypertension that increases the risk of cardiovascular disease and type 2 diabetes mellitus. We investigated the crosstalk between liver and adipocytes by characterising EVs secreted by primary hepatocytes isolated from Zucker rat model, and studied the effect they have on 3T3-L1 adipocytes.

Methods: By using differential ultracentrifugation, density gradient, NTA, cryo-TEM, Western-blotting, Raman spectroscopy, proteomics and lipidomicss we perform a deep molecular characterisation of EVs secreted by lean and obese primary hepatocytes. In addition by using radio labelling we analyse the bio distribution of these vesicles, and by using metabolomics we study the effects that these hepatocytes-secreted vesicles produce on adipocytes metabolism.

Results: We found that steatotic hepatocytes secrete EVs with significantly reduced exosomal markers in comparison with their lean counterpart. Moreover, proteomic analysis revealed that those EVs reflect the metabolic state of the parent cell in that the majority of proteins upregulated relate to fat metabolism, fatty acid synthesis, glycolysis, and pentose phosphate pathway. In addition, hepatocytes-secreted EVs influenced lipolysis and insulin sensitivity in recipient 3T3-L1 adipocytes. Untargeted metabolomic analysis detected alterations in different adipocyte metabolic pathways in cells treated with hepatic EVs.
Summary/Conclusion: Our work showed that steatosis has a significant impact in the amount and composition of EVs secreted by hepatocytes. Moreover, our data point to the involvement of hepatic-EVs in the development of pathologies associated with Metabolic Syndrome.

PF08.04 | Infrared spectra signatures of extracellular vesicles derived from endothelial cells cultured in long-term hyperglycemic conditions

Carina Rząca[1]; Andrzej Wróbel[1]; Paweł Moskal[2]; Ewa Ł Stępień[1]
[1]Department of Medical Physics, M. Smoluchowski Institute of Physics, Faculty of Physics, Astronomy and Applied Computer Science, Jagiellonian University, Kraków, Poland, Kraków, Poland; [2]Department of Experimental Particle Physics and Applications, Marian Smoluchowski Institute of Physics, Faculty of Physics, Astronomy and Applied Computer Science, Kraków, Poland

Introduction: Long-term hyperglycemic conditions cause changes in endothelial cell properties: they became stiffer and less motile. These changes promote endothelial dysfunction and lead to microvessels damage. Extracellular vesicles (EVs) are a heterogeneous group of small vesicular structures released by different types of cells, including microvascular endothelial cells. Stress and metabolic conditions are reflected in the molecular content of endothelial cell-derived EVs. This study aims to investigate modifications in endothelial EVs molecular composition under hyperglycemic conditions using the Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) method.
Methods: EV samples were isolated by ultracentrifugation of conditioned media collected from a telomerase-immortalized human microvascular endothelium cell line (TIME) cultured in normoglycemic (NG) and hyperglycemic conditions (HG). For FTIR spectroscopy, a sample of EVs washed with distilled water was dried at RT directly on the diamond crystal. Then, 127 scans of the sample were collected in a range of 800–4000 cm−1and processed at a nominal resolution of 4 cm−1. The secondary structure of proteins was defined by decomposing the second derivative of the Amide I band (1600 – 1700 cm−1).
Results: FTIR results showed that NG and HG vesicles have different content of protein and lipid components. Statistically significant differences were found in protein phosphorylation, acyl chain length, and lipid to protein ratio. We have shown that HG and NG vesicles also differ in protein secondary structure: side chain, inter β-sheet, β-sheet, random coil, α-helix, and β-turn.
Summary/Conclusion: We propose that EVs are a waste fraction to transport or remove metabolic components from endothelial cells under HG conditions. We have shown that ATR-FTIR analysis of EV samples is a fast and simple method to show a metabolic effect of long-term hyperglycemia.
Funding: The work was supported by the National Science Centre (Poland) OPUS 17 to prof. E. Stępień (No. 2019/33/B/NZ3/01004).

PF08.06 | Profiling variations in human primary skeletal muscle myoblasts to identify putative biomarkers of ageing

Maria F. Fernandez Rhodes; Andrew J. Capel; Neil R.W. Martin; Mark P. P. Lewis; Owen G. Davies
Loughborough University, Loughborough, United Kingdom

Introduction: Sarcopenia (loss of muscle mass and function) affects 5-50% of population over the age of 60 and is currently a global public health concern. Skeletal muscle (SM) has been described as a secretory organ, involved in autocrine and paracrine activities. SM extracellular vesicles (SM-EVs) are emerging as key players mediating effects such as myogenesis, controlling the cell cycle and Wnt signalling pathways implicated in maintaining Pax3 and Pax7 expression in myogenic precursor cells. Age-related changes in cell phenotype (e.g., senescence) are known to have an impact on EV release and composition. However, the impact of sarcopenia has not been well described. In this study, we aimed to provide the first comprehensive profile of variations in young and old human primary skeletal muscle cells (HPMCs) to begin to predict their contribution to native SM function and begin to identify potential biomarkers of ageing.
Methods: HPMCs were recovered from skeletal muscle biopsies isolated from young (18-25 years old) and older (>60) participants. Cells were expanded on Matrigel coated flasks and subsequently sorted using MACS separation for the myoblast marker CD56. CD56+ cells were differentiated in EV-depleted myogenic medium for a period of 5 days.

Results: Conditioned medium was collected and SM-EVs isolated using an optimised ultrafiltration and size exclusion chromatography (SEC) protocol. Differences in particle morphology (TEM), concentration (NTA), zeta-potential (Zetasizer) and marker profiles (WB, ExoELISA, nanoFCM) were evaluated. Raman spectroscopy was applied as a label-free method to identify variations in the biochemical signatures of EVs isolated from young and aged muscle biopsies. Finally, protein profiles were compared using mass spectrometry.
Summary/Conclusion: Variations detected between younger and older individuals related to their EV composition have the potential to inform strategies for the identification of biomarkers in current and future models of skeletal muscle ageing and related diseases.

PF08.08 | The effect of high-fat diet on extracellular vesicles of the mouse testis

Ellis K. FOK; Samuel Tin Yu LAW; Kathleen Hoi Kei CHOY; Cyan Sze Yan CHAN; Tingting ZHENG
School of Biomedical Sciences, The Chinese University of Hong Kong, New Territories, Hong Kong

Introduction: Environmental stimuli such as stress and diet alter the sperm epigenome, particularly the small RNAs carried by the sperm that are inherited to the next generation and mediates paternal epigenetic inheritance. Previous studies have shown that the extracellular vesicles secreted by the epididymis epithelia convey the small RNAs to sperms. However, although the sperm that is capable of fertilising an oocyte in assisted reproduction is produced in the testis, the effect of these stimuli on testicular EVs (tEVs) and subsequently the sperm epigenome remain unexplored.
Methods: Mice were fed with a high-fat diet (HFD) or chow diet (SD) for 24-26 weeks. The tEVs were isolated from the testes using a recently developed method that involved a one-step tissue dissociation followed by an affinity column-based isolation of the membraneous vesicles. The physical properties of tEVs were compared by the transmission electron microscope. The small RNA cargoes of tEVs were investigated by small RNA sequencing. The uptake of tEVs by sperm was monitored by fluorescence microscopy and the small RNAs profile in sperm were validated by realtime-PCR.
Results: Our results showed that tEVs obtained from HFD- and SD-fed mice displayed similar hallmark cup-shape morphologies. Interestingly, tEVs isolated from HFD-fed mice were significantly larger (HFD 320.5±99.8 nm vs SD 251.9±81.0nm, $p < 0.001$). The tEVs carried negligible levels of tRNA that mediates the paternal epigenetic inheritance of metabolic traits. The tEVs isolated from HFD-fed mice had lower levels of miRNAs and snoRNAs. We further identified eight miRNA cargoes that showed differential abundance in tEVs isolated from HFD- and SD-fed mice. Notably, while the ejaculated sperm took up the tEVs from HFD- and SD-fed mice with comparable efficiency, the increased miR34b and miR34c in HFD tEVs did not cause an increase of these two miRNAs in the ejaculated sperm.
Summary/Conclusion: HFD alters the small RNA profiles of tEVs. However, the differential abundance of miRNA cargoes in tEVs did not correlate with the levels of miRNAs carried by the ejaculated sperm.
Funding: This work was supported in part by grants from the Research Grant Council of Hong Kong (TRS T13-602/21-N), Food and Health Bureau of Hong Kong (HMRF 06170476), and the Lo Kwee Seong Start-Up Fund.

PF08.09 | Type 2 diabetes is associated to low-grade intravascular hemolysis and smaller extracellular vesicle shedding from red blood cells

Sylvain Le Jeune[1]; Sihem Sadoudi[2]; Dominique Charue[2]; Salwa Abid[2]; Jean-Michel Guigner[3]; Dominique Helley[4]; Hélène Bihan[5]; Camille Baudry[5]; Hélène Lelong[6]; Tritan Mirault[7]; Robin Dhote[1]; Jean-Jacques Mourad[1]; Chantal Boulanger[2]; Olivier P. BLANC-BRUDE[8]
[1]Service de Médecine Interne et d'Hypertension Artérielle, AP-HP, Hôpital Avicenne, Bobigny, France, Paris, France; [2]Paris Center for Cardiovascular Research, Institut National de la Santé et de la Recherche Médicale U970, Université de Paris, France, Paris, France; [3]Institut de Minéralogie, de Physique des Matériaux et de Cosmochimie, IRD - CNRS UMR 7590 - MNHN, Sorbonne Université, Paris, France, P, France; [4]Service d'Hématologie Biologique, AP-HP, Hôpital Européen Georges Pompidou, Paris, France, Paris, France; [5]Service de Diabétologie, Endocrinologie et Maladies Métaboliques, AP-HP, Hôpital Avicenne, Bobigny, France, Bobigny, France; [6]Unité HTA, Prévention et thérapeutiques cardiovasculaires, Hôtel Dieu, AP-HP, Paris, France, Paris, France; [7]Service de Médecine Vasculaire et HTA, Hôpital Européen Georges Pompidou, AH-HP, Paris, France, Paris, France; [8]Paris Center for Cardiovascular Research, Institut National de la Santé et de la Recherche Médicale U970, Université de Paris Cité, France, Paris, France

Introduction: Type 2 diabetes (T2D) involves hyperglycemia, glycated hemoglobin (Hb), rigid red blood cells (RBC) and altered hemorheology. In T2D, circulating extracellular vesicles (EV) levels are elevated. And may come from RBC. In hemolytic anemia, intravascular hemolysis is associated to RBC remodeling, membrane phospholipid shuffling and phosphatidylserine (PS)

expression. These EV can carry Hb, heme, damage the endothelium and alter blood vessels. In T2D, reports diverge and it remains unclear whether RBC-derived EV vary in quantity or in characteristics. We studied intravascular hemolysis and EV shedding in T2D.

Methods: We collected blood from 109 T2D patients and 65 controls, purified RBC, saved plasma. We studied RBC contents and membrane fragments. In plasma, we characterized heme-related absorbance (Hb, Heme) by spectrophotometry (Soret band) and circulating EV by cryogenic transmission electron microscopy, nanoparticle tracking (NTA) and flow cytometry (CD235a). We studied the association of these biomarkers with micro- and macro-vascular complications of T2D. We stimulated RBC vesiculation in vitro by calcium influx or shear stress. We characterized RBC EV phenotypes and compared their activity using tests of thrombin activation (adapted CAT assay), and endothelial oxidant stress stimulation.

Results: Heme-related absorbance was increased in T2D plasma compared to controls (+57%) and more in obese diabetic (+27%). However, CD235a+RBC EV observed by FACS were not increased. T2D plasma EV, or those shed by T2D RBC (both types of stress), were all smaller (-27%) and rich in heme-related absorbance. In T2D, the raise in absorbance (+30%) was associated specifically to peripheral nerve injury, amongst all other vascular complication. CD235a+ RBC EV were not associated to any complication. In vitro, T2D RBC shed more EV then control RBC under the same stress. T2D RBC EV stimulated oxidant stress in endothelial cultures et thrombin activation in plasma, more intensely than control EV. Annexine-A5, a PS inhibitor, and hemopexin, a heme inhibitor, blocked these effects.

Summary/Conclusion: T2D was associated to low grade intravascular hemolysis, with more heme and EV in plasma. Heme-related absorbance could represent a novel circulating biomarker of peripheral nerve injury, whereas classical flow cytometry, sensitive to the largest EV, seems ill-adapted to demonstrate smaller EV in T2D, particularly those derived from glycated RBC. Therapeutic approaches limiting hemolysis of neutralizing RBC degradation products, like EV PS-neutalising molecules, could help protect blood vessels in T2D.

Funding: Fondation pour la Recherche Médicale

PF08.10 | Dynamic changes in Extracellular Vesicle-associated miRNAs and insulin sensitivity across gestation in normal and gestational diabetes mellitus pregnancies

Lilian Kessling[1]; Soumyalekshmi Nair[1]; Dominic Guanzon[1]; Andrew Lai[2]; Katherin Scholz-Romero[1]; David Simmons[3]; Gernot Desoye[4]; Mireille Van Poppel[5]; David McIntyre[1]; Carlos Salomon Gallo[1]

[1]The University of Queensland, Brisbane, Australia; [2]Exosome Biology Laboratory, University of Queensland Centre for Clinical Research, The University of Queensland, Brisbane, Australia; [3]University of Western Sydney, Sydney, Australia; [4]Medical University of Graz, Graz, Graz, Austria; [5]University of Graz, Graz, Austria

Introduction: Extracellular vesicles (EVs) are secreted from many cell types and contain bioactive molecules, including proteins, messenger (mRNA), and microRNA (miRNA). Notably, placental EVs from gestational diabetes mellitus (GDM) pregnancies are enriched in miRNAs that regulate glucose metabolism. GDM placental sEVs decrease insulin-stimulated glucose uptake in primary skeletal muscle cells, suggesting that placental EVs regulate insulin sensitivity during pregnancy. Here, we determine the association between EV-associated miRNAs and changes in insulin sensitivity across gestation.

Methods: Samples were obtained from a multicenter randomized controlled trial conducted from 2012 to 2014 [the DALI (vitamin D and lifestyle intervention for GDM prevention) lifestyle study]. EV-associated RNA were isolated from pregnant women (n=179, four times during pregnancy (716 total samples were analysed), longitudinal study design). Insulin sensitivity was determined using by Homeostatic model assessment (HOMA) index four times during gestation. Sequencing libraries were generated using the TruSeq® SmallRNA Library Prep Kit and processed for cluster generation and sequencing using NextSeq 500 High Output kit 75 cycles and Illumina NextSeq 500 sequencing platform.

Results: A total of 2822 miRNAs were analyzed using a small RNA library, and a total of 17 miRNAs that significantly correlates with changes in insulin sensitivity across gestation. Clustering analysis identified a total of 12 (hsa.let.7a.1.3p, hsa.let.7a.3.3p, hsa.mir.486.1.3p, hsa.mir.486.2.3p, hsa.mir.185.5p, hsa.mir.30c.1.5p, hsa.mir.30c.2.5p, hsa.mir.486.2.5p, hsa.mir.486.1.5p, hsa.mir.125b.1.5p, hsa.mir.125b.2.5p, and hsa.mir.183.5p), and 5 (hsa.mir.1260b, hsa.mir.101.1.3, hsa.mir.101.2.3p, hsa.mir.423.3p, hsa.mir.23b.3p) EV-associated miRNAs that positively and negatively correlates with HOMA across gestation, respectively.

Summary/Conclusion: The mechanisms associated with maternal insulin resistance are not fully elucidated. We suggest that miRNAs encapsulated in EV might be involved in this phenomenon. This new information will help us better understand the maternal metabolic adaptation to pregnancy, critical for normal fetal growth and development.

Funding: Lions Medical Research Foundation, Diabetes Australia, National Health and Medical Research Council (NHMRC; 1195451), and European Union EU FP7 (242187).

PF08.11 | Pig seminal extracellular vesicles (sEVs) load cytokines showing quantitative differences between sEV subsets

Lorena Padilla[1]; Isabel Barranco[2]; Ana Parra[1]; Xiomara Lucas[1]; Jordi Roca[1]
[1]*Department of Medicine and Animal Surgery, Faculty of Veterinary Science, University of Murcia, Murcia, Spain, Murcia, Spain;* [2]*Department of Veterinary Medical Sciences, University of Bologna, Ozzano dell'Emilia, Bologna, Italy, Bologna, Italy*

Introduction: Seminal plasma (SP) contains cytokines, which are involved in the immunoregulation of female genital tract environment, a key event for successful embryo development. This study aimed to (1) evaluate whether pig sEVs load cytokines and (2) assess putative quantitative differences between sEV subsets.
Methods: sEVs were isolated from five SP pools (three ejaculates per pool) from artificial insemination boars following the procedure described by Barranco et al. (2021), which includes SP centrifugation (20,000xg/30 min at 4°C), and SEC of resultant pellets (large sEVs) and supernatants after ultrafiltration (small sEVs). Fractions (7–10) were selected, mixed, concentrated and used for sEVs characterization (DLS, TEM and total protein concentration) and cytokine measurement (Luminex® Technology). Thirteen cytokines (GMCSF, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, TNF-α) were measured on intact and lysed sEVs (0.1% Triton and 0.1% Sodium Dodecyl Sulfate) and the difference between lysed and intact sEVs was considered the cytokine load in sEVs. Data are showed as mean±SD.
Results: Isolated structures showed the typical structure of EVs. The diameter and total protein concentration differed ($P^{<}0.001$) between large (237±12.01 nm and 83.96±53.92 μg/mL) and small sEVs (121.6±7.89 nm and 306.9±169 μg/mL). sEVs loaded all 13 cytokines, with amounts ranging from 2558±505.4 pg/mL for IFNγ to 0.25±0.08 pg/mL for IL-12. Small sEVs loaded higher ($P< 0.05$) than large sEVs for all cytokines except for IL-1ra.
Summary/Conclusion: Pig sEVs load cytokines, the amount being higher in small sEVs than in large ones. Then, pig sEVs would be involved in modulating the immune environment of the sow genital tract after mating or insemination, a role that would be played mainly by small sEVs, as they load higher amounts of most cytokines.
Funding: Spanish MCIN/AEI/10.13039/501100011033 (PID2020-113493RB-I00) and European Commission (H2020-MSCA-IF-2019-891382).

PF08.12 | Effects of extracellular vesicles derived from steroids-primed oviduct epithelial cells on porcine in vitro embryonic development

Seonggyu Bang; Xun Fang; Bereket Molla Tanga; Gyeonghwan Seong; Islam M. saadeldin; Sanghoon Lee; Jongki Cho
Chungnam national univ., Daejeon, Republic of Korea

Introduction: Extracellular vesicles (EVs) including exosomes and microvesicles provide a means of communication for delivering bioactive cargoes between cells. In the past decade, EVs have been shown to contribute to the transport of proteins, lipids, mRNA, and microRNA. In vivo, embryos receive nutrients and specific signals through the oviduct to enhance embryonic development.
Methods: Therefore, we aimed to isolate EVs from porcine oviduct epithelial cell (pOECs) that were primed with steroid hormones to mimic the in vivo conditions of reproductive cycle and studied their effects on the in vitro produced embryos development. pOECs were treated with formulated estardiol (E2) and progesterone (P4) combinations in two treatment groups: 50 pg/ml E2 + 0.5 ng/ml P4 (group H1), and 10 pg/ml E2 + 35 ng/ml P4 (group H2). The control group was not supplemented with hormones. Embryos were prepared after in vitro maturation and parthenogenetic activation. Embryos were randomly distributed into 4 groups: control, non-primed pOECs-derived EVs treated group (EV group), and two primed pOECs-derived EVs groups (H1 and H2 groups). EVs were isolated through targeted filtration commercial kits. Data were analyzed by ANOVA test and $P < 0.05$ was considered statistically different.
Results: pOECs-derived EVs were supplemented with embryo culture medium after measuring the protein concentration (260.6 μ/ml, 284.3 μ/ml, 283 μ/ml, 279.6 μ/ml, for EV, H1 and H2 groups, respectively). The number and concentration of the EVs was measured through nanoparticles tracking analysis. EVs uptake by the embryos was investigated after staining of the EVs with lipophilic fluorescent dye, PKH26. Results showed that EVs from hormone primed pOECs improved the blastocyst formation rate compared to the control group (20.3 ± 0.5 %, 22.8 ± 0.7, 25.0 ± 0.6, and 25.1 ± 1.1 %, for control, EV, H1 and H2 groups, respectively, $P < 0.05$). TUNEL assay showed that EVs-supplemented embryos contained less apoptotic cells when compared with the control group (11.8 ± 2.7 %, 6.9 ± 2.2 %, 6.09 ± 2.04 %, and 2.4 ± 0.7 %, for control EV, H1 and H2 groups, respectively, $P < 0.05$).
Summary/Conclusion: In conclusion, EVs derived from pOECs cultured in hormonal conditions that simulate the in vivo environment have a positive effect on porcine blastocysts formation and reduced the embryonic cell apoptosis, which would improve the porcine in vitro embryo production such as cloned animals.

PF08.13 | Extracellular vesicles as an efficient cargo delivery method to sperm cells

Mai Ahmed Mohamed Ibrahim; Alina Ivanova; Marcia Ferraz
Ludwig-Maximilians University, Munich, Germany

Introduction: A key challenge in editing the male germline is the delivery of gene editing tools into sperm's tightly packed DNA. To this end, biocompatibility and engineerability make extracellular vesicles (EVs) potential delivery vehicles. While EV-sperm interactions have been studied using dye-labeled EVs, results have been inconclusive. We thus investigated if a GFP-transgenic bovine fibroblast produced GFP-loaded EVs and whether the contents were delivered to sperm.
Methods: EVs were collected from conditioned media of bovine GFP-transgenic fibroblast or WT-fibroblast by ultrafiltration using an Amicon Ultra Filter (100kDa). EV isolation was validated by: (1) nanoparticle tracking analysis; (2) CD9 protein expression; (3) transmission electron microscopy; and (4) protein concentration. GFP cargo of EVs was evaluated by plate reader and ELISA. 10μg/mL of WT and GFP EVs were incubated with sperm and motility and viability were then measured over 90 min. At 90 min sperm were washed, fixed for confocal imaging, and lysed for ELISA. Data were analysed in R via linear or mixed-effects models.
Results: 6.5x109 EVs/mL enriched in CD9 were isolated from WT and GFP cells. We observed 37±19 and 42±33 ng of proteins in 1x106 EVs in GFP (n=11) and WT (n=6), respectively. GFP fluorescence in 1x106 EVs was higher in GFP (n=11) than WT (n=10) EVs (0.01±0.007 vs 0.006±0.001; p=0.006), which was confirmed by ELISA: 6±0.14 vs 2±0.07 pg/mL GFP in 1x106 EVs in GFP (n=9) and WT (n=6), respectively (p=0.005). Sperm viability and motility did not change with incubation nor time. GFP was detected in sperm (20±9.5 pg/mL of GFP in 0.5x106 sperm) incubated with GFP EVs, and not in WT (n=3, p=0.03). At 90 min all sperm incubated with GFP EVs showed GFP fluorescence by confocal analysis.
Summary/Conclusion: GFP-transgenic fibroblasts produced EVs with GFP cargo. These EVs delivered GFP to sperm with no measurable impact on viability and motility. This work demonstrates the potential of EVs as non-invasive delivery tools for sperm. We are now validating methods to load EVs with Cas9-RNP complexes for testing sperm gene editing.
Funding: This work was supported by the Alexander von Humboldt Foundation in the framework of the Sofja Kovalevskaja Award endowed by the German Federal Ministry of Education and Research

PF08.14 | Novel impacts of extracellular vesicles in the prepubertal testes in response to chemotherapy treatment – a previously unidentified impact of chemotherapy

Michael P. Rimmer[1]; Christopher D. Gregory[2]; Rod Mitchell[1]
[1]MRC Centre For Reproductive Health, University of Edinburgh, Edinburgh, United Kingdom; [2]University of Edinburgh, Edinburgh, United Kingdom

Introduction: Extracellular vesicles (EV) are released from the prepubertal testis but potential roles in the development of the spermatogonial stem cell (SSC) niche are poorly characterised. Damage to the SSCs from gonadotoxic compounds, eg the chemotherapeutic cisplatin, can lead to aberrant spermatogenesis and male infertility. This scenario would typically occur in a prepubertal boy with cancer, for whom there are no viable options for fertility preservation, as they do not produce sperm. We hypothesise cisplatin alters EVs in the testis and these EVs negatively impact the remaining SSCs and the supporting Sertoli cells.
Methods: EV characterisation from GC1-spg (mouse prepubertal SSC) and TM4 cells (mouse prepubertal Sertoli) was undertaken, growing cells in EV-depleted media, comparing cisplatin-treated cells to controls. Change in EV number was quantified using nanoparticle tracking analysis and impact on treatment-naïve recipient cells was assessed using in vivo cell imaging and detection of cleaved caspase-3. EV uptake and movement within target cells was examined using super-resolution microscopy.
Results: Mouse SSCs and Sertoli cells treated with cisplatin release twice as many EVs as control cells (p=0.004). EVs released by cisplatin-treated cells have greater uptake in treatment-naïve cells (p=0.002) and show peri-nuclear localisation. We identified EVs released by cisplatin-treated Sertoli cells induced higher rates of apoptosis in treatment-naïve Sertoli cells vs control EVs. However, when incubated with treatment-naïve SSCs, they resulted in lower rates of apoptosis vs control EVs (p= 0.02).
Summary/Conclusion: EVs released from prepubertal SSCs and Sertoli cells were significantly altered when incubated with cisplatin. Altered release, uptake and impact on treatment-naïve cells, are novel findings in the prepubertal testis. Damage to these cells in the testis may be a contributing factor to future male infertility and represent previously unidentified off-target effects of cisplatin.
Funding: MPR is funded by a UK Medical Research Council Centre for Reproductive Health Grant No: MR/N022556/1.

PF08.15 | Uptake of seminal extracellular vesicles (EVs) subsets by cumulus cells-oocyte complex in pigs

Yentel Mateo-Otero[1]; Marcella Spinaci[2]; Giovanna Galeati[2]; Marc Llavanera[1]; Jordi Roca[3]; Marc Yeste[1]; Diego Bucci[2]; Isabel Barranco[2]

[1]Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of Biology, Faculty of Sciences, Institute of Food and Agricultural Technology, University of Girona, Girona, Spain, Girona, Spain; [2]Department of Veterinary Medical Sciences, University of Bologna, Ozzano dell'Emilia, Bologna, Italy, Bologna, Italy; [3]Department of Medicine and Animal Surgery, Faculty of Veterinary Science, University of Murcia, Murcia, Spain, Murcia, Spain

Introduction: Seminal plasma (SP) is a fluid rich in EVs that act as key modulators of reproductive processes. While, in pigs, SP supplementation can improve in vitro fertility outcomes, there are no studies evaluating the effect of SP on in vitro oocyte maturation (IVM). This study is the first attempt to (1) examine the uptake of seminal EVs by cumulus-oocyte complexes (COCs) during IVM and (2) evaluate if they affect the viability of cumulus cells (CCs).
Methods: SP samples from five ejaculates (one per boar) were pooled and EVs were isolated by the SEC-based procedure (Barranco et al., 2021) that allows isolate small (S-) and large (L-) EVs. The EVs were characterized by protein concentration (MicroBCA), DLS and TEM. The conventional porcine two-day maturation protocol in the presence or absence of 0.2 mg/mL S-EVs and L-EVs was carried out (groups of 40 COCs). An aliquot of S-EVs and L-EVs was labeled (PKH67; uptake analysis), and another one remained unlabeled (CCs viability assessment). For uptake analysis of EVs, COCs were assessed by confocal microscopy and CCs were stained (0.4% Trypan Blue) for viability assessment.
Results: TEM showed that S-EVs were mostly spherical membranous vesicles (~30-130nm), whereas L-EVs were morphologically heterogeneous (~100-350nm). DLS revealed that S-EVs were smaller ($P < 0.001$) than L-EVs (124.1±7.7nm vs 303.9±9.6nm, respectively). No differences in protein concentration were found between EV subsets. S-EVs and L-EVs were able to bound COCs during IVM, as green fluorescent spots were observed in the membrane of CCs and not observed in control COCs (i.e., absence of EVs). No differences were found in CCs viability when incubated with S-EVs and L-EVs compared to control (93.78±5.17% and 94.91±6.55% vs. 100%, respectively).
Summary/Conclusion: Seminal EVs can be integrated by CCs, without interfering in their viability. Further studies are required to evaluate if seminal EVs may influence both oocyte nuclear and cytoplasmic maturation and further fertilization.
Funding: EC (H2020-MSCA-IF-2019-891382), MICINN/FEDER (AGL2017-88329-R) and MCIN/AEI/10.13039/501100011033 (PID2020-113493RB-I00), Spain and AGAUR, Catalonia (2020-FI-B-00412)

PF08.16 | MSC-derived secretome induced GLUT4 translocation in HepG2 insulin resistance model

Cynthia R. Sartika[1]; Tiana Milanda[2]; Ahmad Faried[3]

[1]PT. Prodia StemCell Indonesia, Kramat VII No. 11, 10430 Senen, Central Jakarta, Indonesia, Jakarta Pusat, Indonesia; [2]Universitas Padjajaran, Jatinangor, Indonesia; [3]Universitas Padjajaran, Bandung, Indonesia

Introduction: Diabetes mellitus (DM) is one of the most common metabolic disorders. By 2030, it is expected that more than 300 million individuals will be affected by diabetes. Glucose transporter type 4 (GLUT4) is a transmembrane protein that is involved in the elimination of glucose from the bloodstream. In addition to GLUT4, several investigations have shown that interleukin-6 (IL-6) plays an important role in DM. Recent evidence has indicated that IL-6 is a key regulator that affects glucose homeostasis. Interleukin-6 (IL-6) is a pleiotropic cytokine that can be discovered in the secretome of MSCs. The aim of this study is to examine whether IL-6 that was found in the Mesenchymal Stem Cells (MSC)-derived secretome could increase the GLUT4 translocation through AMPK activation in the HepG2-insulin resistance model.
Methods: MSC-derived secretome was produced by culturing the umbilical cord mesenchymal stem cells in a normoxic conditioned until it reach 80% confluency using DMEM High Glucose and supplemented with 5% Human Platelet Lysate. Upon it reached the confluency, the growth medium was discarded and changed with serum-free media, and incubated for 24 hours. The medium was collected and centrifuged for 500xg for 5 minutes and filtered using a 0.2μm filter membrane. The IL-6 concentration was confirmed using the MACSPlex Cytokine 12 kit. The insulin resistance model of HepG2 cells is established by adding 100nM of insulin for 24 hours. To detect the GLUT4 translocation. The insulin resistance model was fasted for 12 hours prior to the treatment using MSC-derived secretome for 24 hours. GLUT4 translocation was detected using immunofluorescence. HepG2 insulin-resistant model was fixed using methanol and cells were incubated with GLUT4 antibody-conjugated with Alexa488.
Results: The treatment of MSC-derived secretome increased the GLUT4 translocation in the HepG2 insulin model compared to the control ($p < 0.05$). This result corresponds with previous studies that show the expression of the GLUT4 gene is downregulated in T2DM. The expression of GLUT4 is lowered in diabetic patients due to blood glucose buildup. IL-6 plays an essential role in regulating glucose homeostasis by activating the GLUT4 translocation through AMPK activation. Given the critical roles of IL-6 that were found in the MSC-derived secretome, it could be a treatment strategy against diabetes.

Summary/Conclusion: MSC-derived secretome could upregulate the GLUT4 translocation.
Funding: The research is funded by Prodia Utama

PS01: Physiology and pathology: Immunity, autoimmunity, and inflammation

Chair: Susanne Gabrielsson – Division of Immunology and Allergy, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden

PS01.01 | Cancer cells release A to I edited RNA repeat elements into extracellular vesicles for reprogramming of the tumor microenvironment

Manideep Chowdary Pachva[1]; Peter Ruzanov[2]; Valentina Evdokimova[2]; Melanie Rouleau[1]; Hendrik Gassmann[3]; Uwe Thiel[4]; Stefan Burdach[4]; Lincoln D. Stein[5]; Laszlo Radvanyi[5]; Poul H. Sorensen[6]
[1]Department of Molecular Oncology, British Columbia Cancer Research Centre, Vancouver, Canada, Vancouver, Canada; [2]Ontario Institute for Cancer Research, Toronto, ON, Canada, Toronto, Canada; [3]Translational Pediatric Cancer Research Action - Institute of Pathology, TUM School of Medicine, Munich, Germany, Munich, Germany; [4]3Translational Pediatric Cancer Research Action - Institute of Pathology, TUM School of Medicine, Munich, Germany, Munich, Germany; [5]Ontario Institute for Cancer Research, Toronto, ON, Toronto, Canada; [6]Department of Molecular Oncology, British Columbia Cancer Research Centre, Vancouver, Vancouver, Canada

Introduction: Ewing sarcoma (EwS) is a highly aggressive cancer and the second most common malignant bone tumor in children and young adults with high propensity for metastasis. Patients with metastasis have a poor long-term outcome. Novel targeted therapeutic strategies that are more efficacious and less toxic are therefore desperately needed. Intercellular communication within the tumor microenvironment (TME) is emerging as a crucial mechanism for cancer cells to establish immunosuppressive and cancer-permissive environment. Extracellular vesicles (EVs) offer a candidate mechanism as they are actively released by tumor cells and enriched with proteins and RNAs to communicate with other cells in the TME.
Methods: For EV purification, Conditioned medium (CM) was subjected to sequential centrifugation at 2000 g for 10 min and 10,000 g for 20 min. CM was then concentrated using the Tangential Flow Filtration Easy columns, passed through 0.22 μm filter, diluted with equal volumes of PBS and subjected to ultracentrifugation (UC) at 100,000 g for 4hrs. EV pellets were then re-suspended in 3 ml of PBS and pelleted again by UC. Purified EVs were dissolved in 500 μl PBS. EVs were quantified using Nanoparticle Tracking Analysis. Purified EVs were tested for presence of exosome markers (CD63, CD9, and CD81) using western blot and using R-PLEX Human CD63/CD81/CD9 (EV) Antibody Set. Cytokine response from EwS ADAR1+/- cells and their EV treated fibroblasts was analyzed using the customized U-plex inflammatory panel (MSD). droplet-based digital PCR (ddPCR) and RNA-seq were used to analyze the expression of repeat elements in cells and their EVs.
Results: In our recent study involving whole transcriptome RNA sequencing, it was found that EVs secreted by EwS cell lines as well as those detected in the plasma of EwS patients are selectively enriched with Adenosine to Inosine (A to I) edited RNAs. A high proportion of these A-to-I edited transcripts are derived from diverse long and short interspersed retrotransposon elements (LINEs and SINEs), human endogenous retroviral elements (HERVs) and pericentromeric genomic regions, where their abundance in plasma was associated with metastatic progression. A to I conversion is catalyzed by the ADAR1 enzyme. We therefore performed ADAR1 knock-down (KD) in EwS cells, which accumulated cellular levels of these repeat RNAs in donor cells and limited their packaging into EVs. Moreover, we observed secretion of cytokines characteristic of a type 1 interferon response in ADAR1 KD cells compared to wild-type cells. Notably, treatment of fibroblasts with EwS-derived EVs resulted in intracellular accumulation of HSAT2 RNAs, but accumulation was reduced when EVs were derived from ADAR1 KD compared to control EwS cells.
Summary/Conclusion: These results suggest that tumor cells secrete EVs enriched in A to I edited RNAs derived from various repeat elements that can target non-tumor host cells, with functional consequences remaining to be elucidated.

PS01.02 | Characterization of neutrophil-derived ectosomes (NDEs) produced by nitrogen cavitation and their effects on neutrophil survival and IL-8 release

Maria J. Hurtado-Gutierrez[1]; Frédérick Allard[1]; Patrick P. McDonald[2]
[1]FMSS. Dept of Immunology and Cell Biology. Université de Sherbrooke, Sherbrooke, Canada; [2]FMSS. Université de Sherbrooke, Sherbrooke, Canada

Introduction: Neutrophils, the most abundant circulating leukocytes, influence both innate and adaptive immunity by modulating their survival and by releasing cytokines and ectosomes. Neutrophil-derived ectosomes (NDEs) carry components of the membrane and cytoplasm of the parent cell that could affect the functional response of the receiving cell.
Methods: Neutrophils were isolated from healthy donors by a dextran-Ficoll procedure under endotoxin-free conditions. NDEs were produced by nitrogen cavitation (350 psi, 10 min, on ice). Cavitates were submitted to serial centrifugations (1000g and 4000g, both 15 min, 4°C). The final pellet was resuspended in cold PBS and NDEs were analyzed by FACS for vesicular integrity (calcein blue AM fluorescence), phosphatidylserine externalization (Annexin-V), as well as parent cell identity and plasma membrane origin (CD66b). Different amounts of NDEs were then incubated with stimulated neutrophils to investigate their effects on cell survival (by FACS) and release of IL-8 (ELISA).
Results: Neutrophil exposure to high amounts of NDEs significantly decreased their survival at 20 h, independently of the presence of GM-CSF, a pro-survival stimulus. The lower dose of NDEs (1 per 5 cells) increased survival as much as the cytokine alone (positive control).
Co-incubation of TNFα-stimulated neutrophils with NDEs for 6h dose-dependently inhibited the release of IL-8.
Summary/Conclusion: We have established reliable methods for NDE production and characterization. Treatment of neutrophils with NDEs had a mostly anti-inflammatory effect which might serve to limit tissue damage.
Funding: MEDTEQ and CIHR.

PS01.03 | Differential Secretagogue Function of Extracellular Vesicles Released by Ocular Epithelial Cells in Health Compared to Inflammation

Changrim Lee; Darlene Dartt
Department of Ophthalmology, Harvard Medical School, Boston, USA

Introduction: Goblet cell functions especially secretion of mucins are relatively well studied owing to their protective role on the ocular surface; however, little attention is given to the other secretory products, such as extracellular vesicles (EVs). The purpose of this study is to examine the function of goblet cell secreted EVs, especially their secretagogue activity, and test whether EVs produced by vehicle (health) or inflammatory stimuli (disease) will cause distinct actions on recipient goblet cells.
Methods: Nanoparticle tracking analyzer and western blotting were used to analyze the size and the identity of the EVs isolated from conjunctival goblet cells. Serum starved primary human conjunctival goblet cells (HCGCs) grown from conjunctiva explants were incubated for 4 h either with the allergic mediator histamine (His, 10-5 M) to induce inflammation or HBSS (non-treated control) to indicate normal cells. EVs isolated from collected media using Total Exosome Isolation Reagent (Invitrogen) were denoted as EVs-His and EVs-NT, respectively. To examine secretagogue activity, first passage cells (trypsinized and passaged primary cells) were treated with EVs diluted to 1, 10, 100, 1000 ng/mL) for 4 h in HBSS. The amount of secreted high molecular weight proteins (HMWP) in the cell culture supernatants was measured using an enzyme-linked lectin assay (ELLA).
Results: The median and the mean diameter of isolated EVs was 138.3 and 163.6 nm, respectively. EVs were positive for CD9, ALIX and CD81 but negative for GM130 (a golgi marker) and calnexin (an ER marker). HCGCs stimulated with His secreted 4-fold higher amount of total EV proteins into the media compared to that of the untreated cells, which confirmed that the primary cells are effectively stimulated with His. For secretagogue activity of EVs, addition of 10 ng/mL EVs-His to HCGCs induced secretion of HMWP by 4.8-fold. The same concentration of EVs-NT caused a 1.6-fold increase. For both EV-His and EV-NT, an increase of 1.0∼1.9-fold was observed at the other four concentrations; however, all lower than the activity seen at 10 ng/mL. Fold-change of secretion in His treated (positive cntl) and untreated cells (negative cntl) was 2.2 and 1.0, respectively.
Summary/Conclusion: We conclude that HCGCs EVs have differential secretagogue activity when stimulated with inflammatory mediator compared to under control (healthy). EVs have an indispensable role in regulating goblet cell secretion at the extracellular level, which warrants in-depth investigation into their molecular properties and mechanism of action.
Funding: NIH R01EY029789, NIH R01EY019470

PS01.06 | Heparin-functionalized adsorbents eliminate platelets and platelet-derived extracellular vesicles that carry platelet factor 4 and are mediators of immunothrombosis

Marie Ebeyer-Masotta[1]; Tanja Eichhorn[1]; René Weiss[1]; Vladislav Semak[1]; Lucia Lauková[1]; Michael B. Fischer[2]; Viktoria Weber[1]
[1]Department for Biomedical Research, Center for Biomedical Technology, Danube University Krems, Krems, Austria, Krems, Austria; [2]Department for Biomedical Research, Center for Biomedical Technology, Danube University Krems, Krems, Austria; Clinic for Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Vienna, Austria, Krems, Austria

Introduction: Inflammation and thrombosis are closely linked in numerous disorders, including sepsis and coronavirus disease 2019 (COVID-19), where deregulated immunothrombosis can lead to microthrombus formation and multiple organ failure.

Platelets are effectors of thrombosis and mediators of the innate immune response. Upon activation, they release extracellular vesicles (pEVs), which support coagulation, in particular in the presence of tissue factor expressed on activated endothelial cells, monocytes, and monocyte derived EVs. Next to EVs, activated platelets release PF4, a strongly cationic chemokine displayed on the surface of activated platelets and pEVs, which has been shown to trigger immunothrombosis. Due to its positive charge, PF4 exerts high affinity for heparin. We assessed the ability of heparin-functionalized adsorbents to bind PF4+ platelets, PF4+ EVs, as well as soluble PF4.

Methods: Medical grade platelet concentrate was activated with thrombin receptor activated peptide-6 and recirculated over cartridges containing Heparin Sepharose for 2 h. Flow cytometry was used to characterize platelets and pEVs at baseline and after 30, 60 and 120 min (Cytoflex LX; CD41, platelet marker; CD62P and PF4, markers of platelet activation; Anx5 to detect phosphatidylserine). Calibration was performed with fluorescent silica beads, the triggering signal for EVs was set to violet side scatter, and the EV gate was set below the 1μm bead cloud. Platelets were identified as CD41+ cells and pEVs were identified as CD41+Anx5+ events. Platelets bound to the adsorbent were visualized using confocal microscopy.

Results: Activated platelets and pEVs were efficiently adsorbed, with preferential binding of PF4+ platelets and pEVs (66% vs. 10% PF4+ pEVs at 0 vs.120 min). Soluble PF4 was significantly depleted, as well.

Summary/Conclusion: Extracorporeal therapies using heparinized adsorbents can be a beneficial intervention to alleviate immunothrombosis in sepsis or COVID-19.

PS01.07 | Increased levels of small extracellular vesicles released upon stimulation of endothelial cells with antiphospholipid antibodies

Ula Štok[1]; Marija Holcar[2]; Metka Lenassi[3]; Snezna Sodin-Semrl[1]; Saša Čučnik[1]; Polona Žigon[1]
[1]University Medical Centre Ljubljana, Department of Rheumatology, Ljubljana, Slovenia, Ljubljana, Slovenia; [2]Institute of Biochemistry and Molecular Genetics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, Ljubljana, Slovenia; [3]Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, Ljubljana, Slovenia

Introduction: Antiphospholipid syndrome (APS) is a systemic autoimmune disorder, characterized by thrombosis and/or obstetric complications in the presence of antiphospholipid antibodies (aPL). Extracellular vesicles (EVs) are associated with several thrombotic disorders, including APS. Very little information on small EVs released upon in vitro stimulation of endothelial cells with aPL is available. Therefore, we sought to determine levels of small EVs (sEVs) after stimulation of endothelial cells with aPL, mimicking pathological conditions of an acute phase of APS.

Methods: Human umbilical vein endothelial cells (HUVEC) were stimulated with lipopolysaccharide (LPS) in the presence or absence of the IgG fraction from pooled sera of 12 APS patients or 12 HBDs for 24 hours. HUVEC activation was determined by measuring ICAM-1 surface expression by flow cytometry. Cell number and viability was determined using an automated cell counter and staining with Trypan blue. Small EVs were isolated from cell culture supernatants by serial centrifugation at 3,000 × g 10 min and 10,000 × g 20 min followed by size exclusion chromatography. The concentration and size of sEVs were analyzed by nanoparticle tracking analysis.

Results: HUVEC showed significant activation when treated with pooled IgG from APS patients (mean MFI +/- SD 18.27 +/- 3.80) compared to control IgG (mean MFI +/- SD 3.45 +/- 0.19, p=0.0025). Cell number and viability was not significantly different between stimulations with apoptosis rates not exceeding 5%. A significantly higher number of sEVs (mean 1.11 × 10^8 sEVs/mL) was detected in cell supernatant of cells stimulated with IgG isolated from APS patients than in IgG isolated from HBDs-stimulated cells (mean 3.80 × 10^7 sEVs/mL). In comparison, untreated cells and cells treated with LPS only secreted even lower levels of sEVs in cell surroundings (mean < 1.25 × 10^7 sEVs/mL). The isolated sEVs had similar sizes between stimulations.

Summary/Conclusion: Our study shows that activation of HUVEC with aPL isolated from APS patients with a history of thrombosis leads not only to an altered adhesion profile but also to increased cell membrane vesiculation.

PS01.08 | Induction of erythroid progenitor cells by melanoma derived extracellular vesicles

Doste Mamand[1]; Safa Bazaz[1]; Oscar Wicklander[1]; Samir El Andaloussi[2]; Manuchehr Abedi-Valugerdi[3]
[1]Department of laboratory medicine, Karolinska Institutet, Stockholm, Sweden; [2]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden, Huddinge, Sweden; [3]Laboratory of medicine, Karolinska Institutet, Stockholm, Sweden

Introduction: Extracellular vesicles (EVs) are lipid enclosed particles containing various biomolecules and are dynamically released by different cell types including tumor cells. Tumor cell EVs are shown to exert activities that lead to tumor progression and metastasis. It has also been suggested that EVs play a pivotal role in the development of tumor immune escape. Based on this suggestion, the current study was designed to explore the immunosuppressive potential of EVs derived from melanoma cells.
Methods: EVs from B16F10 melanoma cells were isolated and characterised employing size exclusion chromatography (SEC), nanoparticle tracking analysis (NTA) and flow cytometry, respectively. Biomolecular components of the isolated EVs were characterized using commercially available angiogenesis and cytokine array kits. C57BL/6 mice were injected (i.p.) with 1011 melanoma cell EVs in PBS every third days for 3 weeks. Control mice received only PBS. At the end of the experiment, the spleen, bone marrow and liver were analysed for different immune cell populations and histological alterations employing flow cytometry H&E staining, respectively.
Results: Our SEC, NTA and flow cytometry analysis on isolated EVs revealed that about 95% EVs were of exosome size (125 nm) and expressed CD63. Moreover, protein array profiling demonstrated that the isolated EVs contained high levels of angiogenic factors including ADAMTS1, Endoglin, Fractalkine, Osteopontin, Tissue Factor, TIMP-1 and VEGF. Mice receiving melanoma cell derived EVs displayed extramedullary erythropoiesis characterised by increased number of erythropoietic foci in the spleen and elevated numbers of splenic CD71+ and CD71+TER119+ cells which are known as potent immunosuppressive cells.
Summary/Conclusion: Our findings clearly show that melanoma cell derived EVs can contribute to melanoma-induced immunosuppression by promoting the generation of immunosuppressive erythroid progenitors.

PS01.09 | Study of extracellular vesicles in idiopathic inflammatory myopathies to evaluate their role as potential disease and treatment response biomarkers

Chiara Franco[1]; Anna Ghirardello[1]; Loris Bertazza[2]; Luca Iaccarino[1]; Mariele Gatto[1]; Andrea Doria[1]
[1]Department of Medicine (DIMED), Rheumatology Unit, University of Padova, Padova, Italy; [2]Department of Medicine (DIMED), Endocrinology Unit, University of Padova, Padova, Italy

Introduction: Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of autoimmune disorders with distinct phenotypes. Extracellular vesicles (EVs) act in autoimmune diseases pathogenesis and have been proposed as disease biomarkers. This study aims at characterizing and investigating EVs as potential biomarkers of IIM.
Methods: EVs were isolated from platelet-free plasma of 45 IIM patients and 45 age/sex-matched healthy donors (HDs) through size exclusion chromatography and ultrafiltration steps. EVs were observed by transmission electron microscopy (TEM), quantified by nanoparticles tracking analysis (NanoSight NS300), and immuno-characterized by imaging flow cytometry (Amnis ImageStream MkII) for the tetraspanins CD63, CD81, CD9, and integrin CD11c. Informed consent and local ethics committee approval were obtained.
Results: TEM images showed intact small roundish particles (Ø range: 30-150 nm). Mean EV concentration was slightly higher in IIM than in HDs (1.95x10^10 ± 1.47x10^10 SD [EVs/mL] vs. 1.45x10^10 ± 7.82x10^9, p=0.0478) with mode size of 150.9 ± 19.9 nm and 154.5 ± 14.8 nm, respectively. EV levels were increased in patients with interstitial lung disease (n=24) (2.20x10^10 ± 1.64x10^10, p=0.0416) and significantly higher in patients with cancer-associated myositis (CAM) (n=8) (3.28x10^10 ± 2.84x10^10, p< 0.0001) compared to HDs. Moreover, EVs concentration was higher in IIM patients with CAM than in those without (n=35) (1.59x10^10 ± 6.12x10^9, p=0.0004). EVs concentration in pharmacologically untreated patients (n=13) (2.77x10^10 ± 2.36x10^10) was increased compared to treated ones (n=31) (1.63x10^10 ± 7.12x10^9, p=0.0092) and HDs (p=0.0011). CD63+ EVs were those most frequently detected both in IIM (n=16) (3.04x10^8 ± 9.67x10^7) and HDs (n=16) (2.70x10^8 ± 1.19x10^8).
Summary/Conclusion: EVs seem to be increased in IIM and may be associated with some disease phenotypes. Moreover, IIM therapy seems to normalize their concentration. These results suggest EVs as useful potential biomarkers of IIM.

PS01.10 | Unveiling novel mechanisms of immune tolerance loss through small RNA-seq of CD4+ T cell-derived extracellular vesicles in multiple sclerosis

Alessandra Colamatteo[1]; Silvia Garavelli[2]; Anna Lise Ferrara[3]; Antonio Pezone[4]; Dario Di Silvestre[5]; Ilaria Giusti[6]; Vincenza Dolo[6]; Fabio Buttari[7]; Giorgia Maniscalco[8]; Giuseppe Matarese[9]; Diego Centonze[10]; Veronica De Rosa[2]; Paola de Candia[1]
[1]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università Degli Studi di Napoli "Federico II", Naples, Italy, Naples, Italy; [2]Laboratorio di Immunologia, Istituto di Endocrinologia e Oncologia Sperimentale, Consiglio Nazionale delle Ricerche (IEOS-CNR), Naples, Italy, Naples, Italy; [3]Dipartimento di Scienze Mediche Traslazionali, Università degli Studi di Napoli "Federico II", Naples, Italy, Naples, Italy; [4]Dipartimento di Biologia, Università Degli Studi di Napoli "Federico II", Naples, Italy, Naples, Italy;

[5]*Istituto di Tecnologie Biomediche, Consiglio Nazionale delle Ricerche (ITB-CNR), Milan, Italy, Milano, Italy;* [6]*Department of Life, Health and Environmental Sciences- University of L'Aquila, L'Aquila- Italy, L'Aquila, Italy;* [7]*Unit of Neurology and Unit of Neurorehabilitation, IRCCS Istituto Neurologico Mediterraneo (INM) Neuromed, Pozzilli, Isernia, Italy;* [8]*Dipartimento di Neurologia, Centro Regionale Sclerosi Multipla, Azienda Ospedaliera "A. Cardarelli", Naples, Italy, Naples, Italy;* [9]*Treg Cell Lab, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università Degli Studi di Napoli "Federico II", Naples, Italy, Naples, Italy;* [10]*Unit of Neurology and Unit of Neurorehabilitation, IRCCS Istituto Neurologico Mediterraneo (INM) Neuromed, Pozzilli, Isernia, Italy, Rome, Italy*

Introduction: An impairment of immune tolerance is a determining factor in relapsing-remitting multiple sclerosis (RR-MS) and defects in CD4+ regulatory T (Treg) cell function are believed to be a major pathogenic factor. While CD4+ conventional T (Tconv) cells are known to substantially contribute to the autoimmune and inflammatory processes occurring in MS, the biological effect of extracellular vesicles released by Tconv cells (Tconv-EVs) on the differentiation and function of Treg cells remains uncharacterized. This study aimed at filling this gap in knowledge.
Methods: Human Tconv cells isolated from blood of naïve to treatment RR-MS patients and healthy controls were in vitro stimulated and Tconv-EVs isolated by size exclusion chromatography and characterized by electron microscopy, flow cytometry and nanoview's technology. Small RNA-seq was used to profile Tconv-EV RNA cargo. Then, the transcriptome of human Treg cells in vitro treated with Tconv-EVs was quantified by RNA-seq. Relevant experimental data have been submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV210300).
Results: Tconv-EVs from RR-MS patients demonstrated a significantly different molecular content in terms of small RNA moieties, compared with healthy controls. Through a bioinformatic approach, some of these molecules, in particular belonging to the microRNA family, could be linked to the differential effect of these two types of Tconv-EVs on Treg cell transcriptome, in particular on the transcripts involved in Treg cell growth and regulatory function.
Summary/Conclusion: Our results have unveiled novel molecular mechanisms which may causally link the Tconv cell dysregulation in MS with a potential suppressive effect of their released EVs on Treg cell-mediated maintenance of self-tolerance in these patients.
Funding: This work is supported by Fondazione Italiana Sclerosi Multipla (FISM, grant 2018/R/4) to P.d.C. and V.D.R.

PS01.11 | Extracellular vesicles as potential clinical markers of mortality

Nicole Noren Hooten; Stephanie Lazo; Nicolle Mode; Alan Zonderman; Ngozi Ezike; Michele K. Evans
NIA, NIH, Baltimore, USA

Introduction: Despite medical advances and high per capita health care spending in the United States, life expectancy is lower compared to other high-income countries. Disparate health outcomes and premature mortality persist among minority groups and individuals with low socioeconomic status. Thus, there is substantial clinical interest in non-invasive biomarkers that can potentially identify individuals at risk for early mortality. Extracellular vesicles play key roles as intercellular communicators and contain bioactive cargo that may provide blood-based clues to tissue and organ pathophysiology. Here we examined whether EVs and their associated cargo were associated with mortality.
Methods: We examined the association of EVs and mortality in a prospective, racially, and socioeconomically diverse middle-aged cohort. EVs were separated from plasma among 76 African American and White individuals who died within five years and 76 matching alive individuals. The cohort also was matched on sex and poverty status. We measured EV characteristics and cargo including EV associated mitochondrial DNA and inflammatory protein levels.
Results: EV concentration, size, or EV associated mtDNA levels were not significantly different between individuals that died or their alive counterparts. However, significant differences were observed in EV inflammatory proteins. We report that mortality was associated with several of these EV inflammatory proteins including: CCL23, CSF-1, CXCL9, GDNF, MCP-1, STAMBP, and 4E-BP1. There was a significant association between mortality status and the presence of IL-10RB and CDCP1 in EVs. Furthermore, we observed that EV inflammatory proteins varied with poverty status, race, and sex.
Summary/Conclusion: These data provide evidence that plasma EV inflammatory proteins may provide information that can differentiate future disparate health outcomes and mortality.
Funding: Intramural Research Program of the National Institute on Aging, National Institutes of Health

PS02: Physiology and pathology: Cancer metastasis and tumor angiogenesis

Chair: Hidetoshi Tahara – Department of Cellular and Molecular Biology, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan

PS02.04 | Cancer cell invasion changes the protein content of extracellular vesicles

Jens C. Luoto; Leila S. Coelho Rato; Sara H. Bengs; Jannica Roininen; John E. Eriksson; Lea Sistonen; Eva Henriksson
Åbo Akademi University, Turku, Finland

Introduction: Extracellular vesicles (EVs) are important in intercellular communication and mediate local and long-range signals in cancer metastasis. The onset of invasion is the key step of the metastatic cascade, but the secretion of EVs at that phase has remained unexplored due to technical challenges. In this study, we present a platform to track and characterize EVs over the course of invasive development of tumor spheroids by utilizing in vivo-mimicking 3D cultures.
Methods: Human prostate cancer PC3 cells were grown in extracellular matrix based 3D cultures or in standard 2D culture conditions. EVs were isolated with differential centrifugation or high-resolution iodixanol gradient centrifugation. The isolated EVs were characterized with nanoparticle tracking analyses, electron microscopy, immunoblotting and mass spectrometry (MS). EV-TRACK ID: EV200156.
Results: Using this ECM-based cell culture method combined with proteomic profiling, we show that PC3 human prostate cancer spheroids secrete EVs with previously undefined protein cargo. Upon invasive transition of the spheroids, an increase in EV amounts and extensive changes in EV protein composition was detected. Importantly, chemical inhibition of invasion suppressed these changes.
Summary/Conclusion: Our results demonstrate that novel EV proteins can be identified with in vivo-mimicking 3D culture platforms. The use of cancer invasion simulating platform showed that EV cargo proteins change according to the invasive status of the prostate cancer organoids. These results highlight the necessity of using in vivo-mimicking conditions for the discovery of novel cancer-derived EV components linked to tumor invasion and metastasis.
Funding: Åbo Akademi University, Academy of Finland, the Medical Research Foundation Liv och Hälsa, Sigrid Jusélius Foundation, Cancer Society of Finland, Otto A. Malm Foundation, Magnus Ehrnrooth Foundation, Swedish Cultural Foundation, K. Albin Johansson Foundation, and the Victoria Foundation.

PS02.05 | Defining the role of prostate cancer-secreted small EVs in lymph node pre-metastatic niche formation

Elena Castellano Sanz[1]; Vanesa Santos[1]; Pedro Matos Filipe[2]; Pilar Ximénez Embún[1]; Javier Muñoz[1]; Lola Martinez[1]; Lucia R. Languino[3]; David Olmos[1]; Arkaitz Carracedo[4]; Veronica Torrano[4]; Judith Farrés[2]; Héctor Peinado Selgas[1]
[1]Spanish National Cancer Research Center (CNIO), Madrid, Spain; [2]Anaxomics Biotech, Barcelona, Spain; [3]Thomas Jefferson University, Philadelphia, USA; [4]CIC bioGUNE, Vizcaya, Spain

Introduction: Prostate cancer (PCa) represents a major global health problem. PCa cells present metastatic organotropism primarily to lymph nodes (LN) and bones. Metastatic spread accounts for the deadliest phase of cancer progression. Previous studies from our laboratory have demonstrated a crucial role for melanoma-derived sEVs in LN and lung premetastatic niche (PMN) formation. In PCa, the mechanisms underlying early metastatic dissemination are still poorly understood. We hypothesize that PCa-derived sEVs distribute through the lymphatic vasculature promoting the re-education and reprogramming of the microenvironment in the LNs and bones, thus favoring early metastatic dissemination.
Methods: To identify protein signatures associated with metastatic progression, mass spectrometry analysis was performed on tumor-derived sEVs from a panel of PCa models. We performed a bioinformatic study to select those candidates that were significantly enriched and were related to the PMN formation process.
We have analyzed the biodistribution of PCa-derived sEVs in mice after footpad and orthotopic injection. SEVs were purified, fluorescently labeled, and then injected in mice. Biodistribution of sEVs was tracked and measured by multispectral imaging. We studied the main cell types taking up PCa-derived sEVs in the LNs by flow cytometry and immunofluorescence.
In order to define the mechanisms involved in PCa early metastatic dissemination, we are analyzing the extracellular matrix and immune cell composition of LNs derived from patients and mouse PCa models by immunohistochemistry and flow cytometry.
Results: We found that PCa-derived sEVs can reach the sentinel LN and the pelvic bone. Interestingly, sEVs derived from the LN metastatic model (C4-2B) reached more efficiently LNs, while those derived from bone metastasis (PC3) were significantly increased in the bone. We found that lymphatic endothelial cells (LECs), fibroblastic reticular cells (FRCs), and macrophages were the main cell types taking up sEVs in the sentinel LNs.

Proteomic analysis showed around 600 proteins differentially expressed when comparing sEVs derived from LNCaP and C4-2B lymph node metastatic models. We are currently analyzing the signatures associated to the highly metastatic model C4-2B.
Summary/Conclusion: Our data show that sEVs derived from LN and bone metastatic models home more efficiently to lymph nodes and bone respectively, suggesting their involvement in PMN formation in these organs.
Detailed analysis showed that LECs, FRCs, and macrophages are the main cell types in the sentinel LNs taking up sEVs.
Our results support that PCa-derived sEVs play a role in PCa lymph node PMN formation. We are currently analyzing the main signatures associated with LN metastasis and the main changes in metastatic sentinel LNs.
Funding: ProEVLifeCycle, EU Horizon 2020, Marie Skłodowska-Curie grant No 860303.

PS02.06 | Developing liver spheroids for elucidating the role of colorectal cancer-derived small extracellular vesicles in the pre-metastatic niche formation

Ornella Urzì[1]; Marzia Pucci[1]; Marta Moschetti[2]; Marco Loria[1]; Alice Conigliaro[1]; Maria Cristina Guerrera[3]; Riccardo Alessandro[1]; Simona Fontana[1]
[1]University of Palermo, Dep. of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), Palermo, Italy; [2]University of Palermo, Dep. of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), palermo, Italy; [3]University of Messina, Dep. of Veterinary Sciences, Messina, Italy

Introduction: Colorectal cancer (CRC) is the third most common tumor in the world frequently associated with liver metastasis causing unfavorable prognosis. A recent study performed in our laboratory has demonstrated that CRC small extracellular vesicles (SEVs) induce an epithelial to mesenchymal transition (EMT) of hepatocytes (heps) driving them to actively participate in the pre-metastatic niche formation, probably contributing to form a liver fibrotic microenvironment. Since 2D cell cultures partially reflect the structural complexity of the in vivo microenvironment to give more power to our functional model, we switched to use hepatocyte spheroids (HeSPHs), which can give us more proper information on the consequences of CRC_SEV-induced hepatocytes EMT on cell-cell interactions and extracellular matrix remodelling which can drive and support the invasion of tumor cells.
Methods: We isolated SEVs from SW480 CRC cells, through differential centrifugation followed by ultracentrifugation. HeSPHs were obtained by seeding normal human liver cells (THEL-2) in ultra-low attachment 96 well plates. After treating HeSPHs with CRC_SEVs, we analyzed the modulation of expression of structural and functional hepatocyte markers. By co-culturing HeSPHs with SW620-GFP cells, we evaluated the ability of CRC_SEV to increase the invasion potential of tumor cells.
Results: Our data on HeSPHs confirmed the ability of CRC_SEVs to alter the expression of hepatocyte structural and functional markers (ApoE, albumin and cytokeratins 8/18) observed in the 2D model. Moreover, we found that in CRC-SEVs-treated HeSPHs the invasive capability of tumor cells increased, indicating that injured-SEV heps can have driving and supporting tumor liver colonization.
Summary/Conclusion: Overall, the HeSPHs represent a promising model to study the role that tumor-derived SEVs can have in rendering heps able to actively drive the formation of an environment conducive to metastasis.

PS02.07 | Electrical Monitoring of Cell Epithelial-to-Mesenchymal Transition Induced by Tumor-Derived Extracellular Vesicles using Bioelectronic Technologies

Walther Traberg-Christensen; Roisin Owens
University of Cambridge, Cambridge, United Kingdom

Introduction: Tumor cell-derived EVs (TDEs) have recently been reported to play an active role in tumorigenesis and metastasis owing to their ability to transmit oncogenes. One hypothesis is that TDEs contribute to metastasis by inducing the epithelial-to-mesenchymal transition (EMT), characterized by the loss of barrier function in barrier tissue-forming cells.
Methods: This study aims to elucidate the role and mechanisms of TDE uptake by investigating the process by which TDEs "infect" normal cells inducing EMT prior to metastasis. Current strategies for investigating the spatial and temporal aspects of EV uptake, often optical, lack the ability to obtain quantitative data in real time. We have shown that organic electronic devices based on the conducting polymer poly(3,4-ethylenedioxythiophene): poly(styrenesulfonate) (PEDOT:PSS) can be interfaced with biological systems of varying complexity allowing for quantitative real-time monitoring of biological interactions. The optical transparency of our PEDOT:PSS-based devices provides the unique advantage of dual transduction increasing the credibility of our platform and enriching the biological information obtained.
Results: We have shown that CD63-positive TDEs derived from the triple-negative breast cancer cell line MDA-MB-231 can induce an EMT-like process in MCF-10A non-tumorogenic breast epithelial cells as demonstrated by changes to cell morphology,

loss of apico-basal polarity, reorganization of filamentus actin, and gain of mesenchymal proteins, such as vimentin and N-cadherin. An integral feature of EMT is the dissolution of tight junctions and the consequential loss of lateral cell-cell adhesion leading to a phenotype with increased motility and invasiveness. This decrease in barrier integrity of the cell monolayer was monitored electrically using organic electrochemical transistors, gaining of truly quantitative insight into TDE-induced EMT with higher temporal resolution than conventional, orthogonal methods.

Summary/Conclusion: These combinatorial optical and electrical measurements of TDE interactions with cell monolayers provide invaluable information for developing strategies that may inhibit TDE interactions by e.g. blocking specific surface markers, thereby preventing TDE-induced EMT with implications for preventing cancer metastasis. Moreover, the compatibility of electronic devices with microfabrication methods allows for high throughput studies.

Funding: This work is funded by a research grant from King Abdullah University of Science and Technology and The Cambridge Trust.

PS02.08 | Mesothelial cells- derived extracellular vesicles promote angiogenesis via angiopoietin-2

Shelly Loewenstein[1]; Fabian Gerstenhaber[2]; Talya Kalfon[2]; Stav Leibou[2]; Osnat Sher[2]; Guy Lahat[2]
[1]Tel Aviv Sourasky Medical Center, Tel-Aviv, Israel; [2]Tel Aviv Sourasky Medical Center, Tel Aviv, Israel

Introduction: Peritoneal carcinomatosis is orchestrated by a reciprocal interplay between invading cancer cells and resident normal peritoneal cells. Within that process, angiogenesis constitutes an important control point of cancer progression. We investigated the role of mesothelial cells derived extracellular vesicles (EVs) in angiogenesis.

Methods: Endothelial, mesothelial, and gastric cancer cell lines were used for in vitro experiments. Gastric cancer and mesothelial extracellular vesicles (EVs) were produced from cell cultures. Proliferation, migration, and invasion assays were used to evaluate the phenotypic behavior of treated endothelial cells. Matrigel tube- and plug- formation assays were used to demonstrate angiogenesis in vitro and in vivo. Angiogenesis protein array was used to identify proangiogenic proteins in mesothelial EVs. Finally, Lenti viral shRNA were used for gain and loss of function experiments to investigate specific signaling pathways.

Results: Initially, we demonstrate a robust uptake of mesothelial and gastric cancer EVs by endothelial cells. We show that these vesicles enhance endothelial cell proliferation, migration and invasion and that mesothelial- derived EVs are more effective when compared with gastric cancer- derived EVs. We also demonstrate that mesothelial EVs significantly augment in vitro and in vivo tube formation. Angiogenesis array identified 43 differently expressed regulators of angiogenesis within mesothelial EVs. Based on the results of the array, we specifically investigated Angiopoietin-2 (Ang2) -Tie2 pathways. We show that the above noted effects of the mesothelial EVs were mediated by Ang2 through Tie2, PI3K, and Akt activation, whereas inhibition of Ang2 significantly decreased the pro-angiogenic effect of mesothelial EVs.

Summary/Conclusion: We demonstrate for the first time uptake of mesothelial EVs by endothelial cells and the induction of angiogenesis by these EVs. We also demonstrate that these effects are mediated by Ang2 trough the PI3K/AKT pathway. Taken together, our data imply that mesothelial EVs are important regulators of angiogenesis, thus playing an active role in the progression of peritoneal metastasis.

Funding: Cancer Biology Research Center

PS02.09 | NFkB activation by hypoxic sEV drives oncogenic reprogramming in a breast cancer microenvironment

Irene Bertolini; Michela Perego; Jagadish Ghosh; Dario Altieri
The Wistar Institute, Philadelphia, USA

Introduction: Small extracellular vesicles (sEV) are produced by all types of cells and have emerging roles in cell-to-cell communication. Cancer cells produce higher levels of EV compared to normal cells, and this response is further augmented by stress conditions, such as hypoxia. Intra-tumoral hypoxia is a common occurrence in advanced breast cancer, correlating with increased risk of metastasis and poor prognosis. We have recently demonstrated that sEV produced by cancer cells in hypoxic, but not normoxic conditions, induce oncogenic changes in normal epithelial cells in vitro. Mechanistically, hypoxic sEV deliver ILK to recipient normal mammary epithelial cells resulting in heightened mitochondrial dynamics and increased cell motility and invasion. In this study, we sought to elucidate the ILK signaling axis activated by hypoxic sEV.

Methods: We isolated sEV from two different human breast cancer cells (MCF7 and MDA-231) and one normal mammary epithelial cell (MCF10A) maintained in hypoxic (sEVHYP) or normoxic (sEVNORM) conditions (1% or 5% O2 respectively for 24 h). sEV were isolated using differential centrifugation followed by size exclusion chromatography (SEC, Izon). sEV

characterization was carried out according to MISEV guidelines. sEV were quantified after each isolation using the ZetaView system and the same number of sEV was applied to recipient MCF10A cells in every co-culture experiment.
Results: We found that breast cancer-derived sEVHYP, but not sEVNORM, potently activate NFκB gene expression in recipient MCF10A cells. In turn, this increases the production and release of inflammatory cytokines, heightens mitochondrial dynamics and stimulates cell motility, disrupting epithelial acini morphogenesis in 3D cultures. Mechanistically, pharmacologic or genetic targeting of ILK or p65 NFkB abolishes the effect of sEVHYP on recipient mammary cells.
Summary/Conclusion: sEV released by breast cancer cells under hypoxic conditions activate NFκB in normal mammary recipient cells. This pathway may drive multiple pro-tumorigenic steps of heightened inflammation, deregulated mitochondrial dynamics and aberrant 3D epithelial morphogenesis in a breast cancer microenvironment.

PS02.10 | Role of CD133-containing EVs in aggressive cancers

Mireia Gomez Duro[1]; Graça Raposo[2]; Sarah Watson[3]; Gisela D´angelo[2]
[1]Institut Curie, PARIS 16, France; [2]Institut Curie CNRS, PARIS 05, France; [3]Institut Curie INSERM, PARIS 05, France

Introduction: The spread of cancer cells from the primary tumor into surrounding tissues and metastasis to distant organs is the primary cause of cancer morbidity and mortality. Recent data suggest that EVs released by cancer cells may stimulate tissue invasion and dissemination of tumor cells to target tissues. The invasive and metastatic character of several cancer types is associated with the presence of CD133 and CD44, which predict poor outcomes of cancer patients. CD133 is among one of the molecular signatures of EVs. CD44 has been reported to be associated with EVs, but its role in signal-mediated EVs is not known. The major goal is to correlate the expression of CD133 and CD44, to EV secretion, their EV-mediated invasive and metastatic properties with a long-term goal for the development of innovative EV-based therapeutic approaches.
Methods: EVs were isolated by size exclusion chromatography (SEC) and by ultracentrifugation from breast cancer (MDA-MB468, MCF7) and osteosarcoma (U20S, SAOS) cell lines and characterized by WB, NTA, and EM to evaluate particle size, EV morphology, and composition.
Results: We found that all EV samples isolated by SEC represent a heterogeneous population categorized into three prominent sub-populations non-vesicular particles (< 50 nm), and two subpopulations consisting of small-EVs (50-100 nm) and large-EVs (150-250 nm). Interestingly, in osteosarcoma cell lines, the range and the abundance of EVs were lower as compared to breast cancer cells lines. EVs specific markers (CD9, CD81, CD63, Alix, Tsg101) were detected in the four cell lines. Although breast cancer and osteosarcoma cell lines produce EVs loaded with CD133 and CD44, their expression levels are higher in the triple-negative breast cancer cell line (MDA-MB468).
Summary/Conclusion: Our results thus reveal that the expression of biomarkers of interest does not occur equally in all cell types, which may influence their metastatic capacity and the development of the disease.

PS02.11 | Role of plasma extracellular vesicles-miRNAs in lung cancer pre-metastatic niche formation

Francesca Pontis[1]; Ilaria Petraroia[1]; Paola Suatoni[2]; Ugo Pastorino[2]; Mattia Boeri[1]; Fabio Maiullari[3]; Roberto Rizzi[3]; Claudia Bearzi[3]; Gabriella Sozzi[1]; Orazio Fortunato[1]
[1]Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy, Milan, Italy; [2]Fondazione IRCCS Istituto Nazionale dei Tumori , Milan, Italy, Milan, Italy; [3]Istituto Nazionale Genetica Molecolare INGM "Romeo Ed Enrica Invernizzi", Milan, Italy, Milan, Italy

Introduction: Lung cancer is the deadliest cancer worldwide, mostly due to the metastatic spread. Extracellular vesicles (EVs) are key players in cancer progression thanks to their ability to deliver biofunctional molecules as miRNAs and proteins to recipient cells.
We aim to elucidate the role of patient's plasma-EVs in lung cancer progression and identify new prognostic biomarkers.
Methods: Plasma-EVs were obtained by ultracentrifugation from 20 early stages patients survived at 5 years (ESA-EVs) and 20 died within two years (ESD-EVs). EV's characterization was performed by Nanoparticles tracking analysis, Flow Cytometry and TEM. For uptake experiments PKH26-labeled-EVs were used. Functional experiments were carried out in vitro in 2D and 3D-bioprinting co-cultures models. Nanostring was used to investigate the EV's miRNA cargo.
Results: Patient's plasma-EVs were sized between 100 and 150 nM and possess the EVs markers (CD9, CD81, CD63). Notably, FC analysis revealed that ESD-EVs were enriched in CD31 compared to ESA-EVs. Moreover, In vitro experiments showed that plasma-EVs were mostly incorporated by stromal cells; mainly by endothelial cells followed by macrophages and fibroblasts rather than epithelial cells. Strikingly, ESD-EVs treatment increased endothelial VCAM1, CXCR4 and CXCL1 levels compared to ESA-EVs in 2D and in 3D models. Interestingly, in 3D-bioprinted co-cultures, ESD-EVs treatment induced endothelial activation and increased fibroblast (α-SMA and CXCL12) and epithelial (E-cadherin and EpCAM) gene expression. Furthermore, ESD-EVs

also stimulated the macrophages phenotypic shift towards M2 polarization in vitro as noted by the increment of IL10 and CD206 levels. Importantly, 90 EV-miRNAs were detected in plasma-EVs, 5 of them differentially expressed between ESA- and ESD-EVs.
Summary/Conclusion: Our findings suggested that ESD-EVs generate a pre-metastatic milieu by endothelial activation and the modulation of stromal cells phenotype. Moreover, EV-miRNAs could be used as prognostic biomarkers in early stages lung cancer patients.
Funding: The study was supported by grants from Italian Ministry of Health RF-2018-12367824 to G.S.

PS02.12 | Transglutaminase-2, RNA-binding proteins andmitochondrial proteins selectively traffic toMDCK cell-derived microvesicles following H-Ras-induced epithelial–mesenchymal transition

Adnan Shafiq
La Trobe University, PRESTON, Australia

Introduction: pithelial–mesenchymal transition (EMT) describes an evolutionary conserved morphogenic process defined by loss of epithelial characteristics and acquisition of mesenchymal phenotype, and altered patterns of intercellular communication, leading to functional changes in cell migration and invasion. In this regard, we have previously reported that oncogenic H-Ras induced EMT in Madin-Darby Canine Kidney (MDCK) cells (21D1 cells) trigger changes in the protein distribution pattern in cells, exosomes, and soluble protein factors (secretome) which modulate the tumor microenvironment.
Methods: EVs were isolated using differential centrifugation including OptiPrep density gradient and characterised by NTA and western blot analysis.
Results: Proteomic analysis revealed that the protein spectra of RNA-binding proteins and mitochondrial proteins in 21D1-sMVs differ profoundly compared to those of exosomes, likewise proteins associated with suppression of anoikis. We show that 21D1-sMVs promote cell migration, confer anchorage independent growth, and induce EMT in parental MDCK cells. An unexpected and novel finding was the selective sorting of tissue transglutaminase-2 (TGM2) into 21D1sMVs; there was no evidence of TGM2 in MDCK-sMVs. Prior treatment of 21D1-sMVs with neutralizing anti-TGM2 or anti-FN1 antibodies attenuates the invasive capability of fibroblasts. These finding suggest that microvesicle-associated TGM2 may play an important contributory role in the EMT process and warrants further investigation
Summary/Conclusion: Collectively, our findings suggest that MDCK-sMV cargo changes that occur following oncogenic H-Ras transformation are sufficient to confer cellular migration, anchorage-independent growth capabilities and induce EMT in recipient MDCK cells. Among the important question for the future will be to better understand how the cargo of different EV classes (microvesicles and exosomes) along with the secretome act ina coordinated fashion to mediateEMT. It's also important to explore further whatthe impacts the sMVmakeonthe cancermicroenvironments as anenablerof cancerprogression. Harnessing this knowledge will open avenues for future EV studies aimed at furthering our understanding of the role of sMVs in the EMT program and the targeting of EVs as potential druggable molecules for therapeutic application.

PS02.13 | Tumor-derived microvesicles are a critical determinant for successful metastatic colonization in colorectal cancer

Suganja Sivaloganathan[1]; Raquel Blazquez[2]; Tobias Pukrop[2]; Darius Wlochowitz[3]; Tim Beißbarth[3]; Simone König[4]; Matthias Schulz[3]; Claudia Binder[5]; Kerstin Menck[6]; Annalen Bleckmann[6]
[1]University Hospital Münster, Münster, Germany; [2]University Hospital Regensburg, Regensburg, Germany; [3]University Medical Center Göttingen, Göttingen, Germany; [4]Core Unit Proteomics, University of Münster, Münster, Germany; [5]University Medical Center Göttingen, Göttingen, Göttingen, Germany; [6]Dept. of Medicine A, Haematology, Oncology, and Pneumology, University Hospital Münster, Münster, Germany

Introduction: The formation of distant metastases is the major obstacle in treating cancer. Metastatic spread requires a cancer-induced modulation of the surrounding environment, which can be mediated via the release of tumor-derived extracellular vesicles (T-EV). Using a syngeneic mouse model for colorectal cancer (CRC), we identified two variants of the murine CRC cell line CMT93, which differed significantly in their metastatic colonization in vivo. The aim of this study was to identify the molecular mechanisms responsible for the effect.
Methods: The two CMT93 variants were characterized regarding their basic characteristics using proliferation, invasion, migration and adhesion assays. T-EV released by the cells were collected via differential ultracentrifugation at 17,000 g for larger microvesicles (MV) and 143,000 g for small EV. The obtained EV were characterized by immunoblotting, nanoparticle tracking analysis, transmission electron microscopy and proteomics.

Results: Analysing the cell-intrinsic basic characteristics of the cells revealed no major difference between the two CMT93 variants. Thus, we extended our analysis to the released secretome, which revealed a major difference in the amount and composition of released T-EV. While T-EV released by the malignant CMT93 variant induced tumor invasion, this effect was not observed with T-EV from the less aggressive CMT93 variant. Intriguingly, this functional difference was more prominent with MV than with small EV. To identify the molecular characteristics responsible for this difference, the MV released by both CMT93 variants were compared by proteomic analysis. The results revealed that the MV released by the malignant CMT93 variant were enriched in proteins associated with adhesion. Using immunoblotting, we were able to validate an upregulation of the proteins Itga3 and Fascin1 on MV released by the malignant variant. Analysing microarray data, we identified that these proteins were upregulated in human primary and metastatic CRC in contrast to normal colon tissue, highlighting the translational relevance of our findings.
Summary/Conclusion: Conclusively, we identified MV as the critical determinants for successful tumor invasion in CRC and attributed this effect to the enrichment of tumor-supporting adhesion proteins on the vesicles. These results shed further light on the molecular mechanisms underlying EV-mediated metastatic colonization and open new options for targeted therapy.

PS03: Physiology and pathology: Cancer pathogenesis

Chair: Elena S. Martens-Uzunova – Department of Urology, Erasmus MC, University Medical Center Rotterdam

Chair: Marco Falasca – Curtin University

PS03.01 | Cancer cells shuttle extracellular vesicles containing oncogenic mutant p53 proteins to the tumor microenvironment

Bibek Bhatta[1]; Ishai Luz[2]; Christian Krueger[3]; Fanny Xueting Teo[3]; David P. Lane[4]; Kanaga Sabapathy[3]; Tomer Cooks[2]
*[1]Ben Gurion University, Beersheva, Israel; [2]The Shraga Segal Department of Microbiology and Immunology, Ben Gurion University of the Negev, beer sheva, Israel; [3]Division of Cellular & Molecular Research, Humphrey Oei Institute of Cancer Research, National Cancer Centre Singapore, Singapore 169610, Singapore, Singapore, Singapore; [4]p53 Laboratory (p53Lab), Agency for Science, Technology, and Research (A*STAR), Singapore 138648, Singapore, Singapore, Singapore*

Introduction: Most cancers harbor mutations in the TP53 gene (encoding for the p53 tumor suppressor protein). Furthermore, gain-of-function (GOF) mutation in p53 imparts an aggressive traits to the cells when compared to the cancer cells harboring inactivating mutations or wild-type (WT)-p53. Notably, multiple studies have delineated the presence of GOF-mutant p53 protein in untransformed cells or in stromal compartments of tumor microenvironment (TME). In recent years, the involvement of extracellular vesicles (EVs) in cell-to-cell communication has emerged as a major route by which cancer cells can interact and educate immune, and non-immune cells in TME to become tumor supportive. To this end, we hypothesize that mutant p53 protein can be shuttled via EVs to TME cells thus shedding light on a novel non-cell autonomous role of mutant p53 cancers
Methods: EVs were isolated from various cancer cell lines (pancreas, lung, colon) differing by their p53 status and the effect on neighboring cancer cells and TME cells was studied in vitro and in-vivo. We also utilized the human colorectal Colo-320DM cancer cell xenograft model, which expresses the R248W p53 mutant. FFPE sections of subcutaneous tumors derived from the Colo-320DM xenografts were stained for p53 using the DO-1 antibody that specifically recognizes human p53.
Results: Our data demonstrated that mutant p53 protein can be selectively sorted into EVs; that mutant p53 in EVs can be taken up by neighboring cancer cells and macrophages that do not harbor mutant p53 protein. Evident of macrophage education was seen with the increased expression and secretion of pro-inflammatory cytokines. Notably, mutant p53 expression was also found in non-tumor cells in both human cancers, and in non-human tissues in human xenografts.
Summary/Conclusion: Cancer cells harboring GOF p53 mutants, can package mutant p53 proteins in EVs, and deliver them to neighboring cancer cells and to the TME.

PS03.02 | Differential profile analysis of microRNAs in exosomes from prostate cancer cells. Target prediction

Antonio Altuna Coy[1]; Silvia Sanchez Martin[1]; Marina Mendieta Homs[1]; Xavier Ruiz-Plazas[2]; José Segarra Tomás[2]; Helena Ascaso-Til[3]; Marta Alves Santiago[2]; Manuel Prados-Saavedra[3]; Matilde R. Chacón[1]
[1]Pere Virgili Health Research Institute, Tarragona, Spain; [2]Joan XXIII University Hospital/Pere Virgili Health Research Institute, Tarragona, Spain; [3]Joan XXIII University Hospital, Tarragona, Spain

Introduction: Prostate cancer (PCa) is a commonly diagnosed pathology. A critical role of cell-to-cell communication during tumorigenesis has been proven, allowing to re-program the tumor microenvironment to facilitate its progression. In this sense, exosomes, small extracellular vesicles released to the extracellular and their microRNAs content (exomiRNAs) has been shown regulate gene expression and PCa development.
Thus, our aim was to investigate a differential exomiRNA cargo in two PCa cell lines PC-3 (representing the most aggressive stage) and LNCaP (less aggressive stage) versus non-cancerous prostate cell line RWPE-1, and to identify their putative targets and their possible role on the PCa development.
Methods: PC-3, LNCaP and RWPE-1 cell lines were cultured during 24h in their respective culture medium. Exosomes were isolated from 8 ml culture medium by exoEasy Maxi Kit (Qiagen) and characterized by western blot and transmission electron microscopy. ExomiRNAs were isolated by ExoRNeasy Maxi Kit (Qiagen) and qRT-PCR was performed by using miRCURY LNA miRNA miRNome PCR Panel I+II V5 (Qiagen), that included 752 mature human cancer-related miRNAs. Pathway and functional enrichment analysis on putative targets of the selected exomiRNAs was performed by using miRNet, Reactome and STRING softwares.
Results: Microarray analysis revealed a total of 38 exomiRNAs differentially express in PC-3 and LNCaP versus RWPE-1 cell line. MiRNet analysis showed 41 putative genes regulated, at least, by 20 of the 38 differentially expressed exomiRNAs. Reactome and STRING analysis demonstrated that MDM2, TNRC6A, TNRC6B and AGO1 were common targets of the putative miRNA-regulated pathways with an important role on miRNA expression regulation.
Summary/Conclusion: PCa secreted exomiRNAs can modulate tumor microenvironment gene expression and may be crucial to the progression of cancer.
Funding: This study is funded by PI20/00418 Instituto de Salud Carlos III and Co-founded by European Union (ERDF/ESF "A way to make Europe/Investing in your future")

PS03.03 | Effects of extracellular vesicles on vemurafenib sensitivity in syngeneic melanoma cell lines

Afrodité Németh[1]; Tamás Kós[1]; Gréta Lilla Bányai[1]; Zoltán Varga[2]; Anikó Gaál[2]; Magdolna Dank[3]; István Takács[3]; Attila Marcell Szász[3]; Tamás Garay[1]
[1]Pázmány Péter Catholic University Faculty of Information Technology and Bionics, Budapest, Hungary; [2]Biological Nanochemistry Research Group, Institute of Materials and Environmental Chemistry, Research Centre for Natural Sciences, Budapest, Hungary; [3]Division of Oncology, Department of Internal Medicine and Oncology, Semmelweis University, Budapest, Hungary

Introduction: Recently, more and more evidence is found that extracellular vesicles (EVs) are linked to therapy resistance. The majority of malignant melanoma cases harbor oncogenic BRAF mutations. BRAF inhibitors, like vemurafenib, show remarkable initial responses, however, resistance emerges in almost all patients. Our aim was to investigate the role of EVs in the evolvement and spread of therapy resistance in an in vitro setting using sensitive-resistant syngeneic melanoma cell pairs, which were isolated before and after the vemurafenib treatment from each patient.
Methods: Melanoma cells were kept in DMEM supplemented with 1% EV-depleted FBS for at least three days before collecting media. ExoQuick exosome precipitation reagent was used to isolate EVs. Total protein content was analyzed using the Qubit Protein Assay Kit. EVs' size distribution and concentration were determined using Microfluidic Resistive Pulse Sensing (MRPS). Baseline vemurafenib sensitivity and EVs capacity to transfer therapy-resistance were evaluated by SRB viability assay and videomicroscopy, cell proliferation and migration were quantified.
Results: Treatment with the EVs produced by the resistant syngeneic cell line diminished the effect of vemurafenib treatment. Similarly, treatment with self-produced EVs showed a tumor-promoting effect. However, the observed effects were cell line dependent.
Summary/Conclusion: Our result indicates that the effects of extracellular vesicles on vemurafenib sensitivity, proliferation and migration are cell line dependent. EVs potentially contribute to resistance against the BRAF inhibitor vemurafenib.
Funding: Supported by the ÚNKP-21-3 New National Excellence Program of the Ministry for Innovation and Technology from the source of the National Research, Development and Innovation Fund.

PS03.04 | Effects of small extracellular vesicles derived from prostate cancer cells on a salivary gland epithelium barrier model

Ana Špilak; Andreas Brachner; Ulrike Kegler; Christa Nöhammer; Winfried Neuhaus
AIT Austrian Institute of Technology, Vienna, Austria

Introduction: Besides potentially having a major role in cancer metastasis, small extracellular vesicles (sEVs) released by cancer cells can serve as liquid biopsy biomarkers for detection in bodily fluids such as saliva (PMID: 34087328). However, the

mechanisms of how sEVs interact with biological barriers are not clarified. We investigated the effects of prostate cancer (PCa) sEVs on a human submandibular salivary gland barrier (SSGB) model in vitro.

Methods: sEVs were isolated from conditioned serum-free medium after 48h collection with size exclusion chromatography from PCa DU145 cell line in normoxic and hypoxic conditions and characterized with NTA for particle count, size distribution and zeta potential, microBCA and western blots for CD81, CD9, ALIX and GM130. sEVs were applied to the basolateral (blood) side of a SSGB Transwell model based on HTB-41 B2 cells (PMID: 32842479) and barrier integrity was assessed with the transepithelial electrical resistance (TEER) measuring CellZscope device in one-hour intervals. After 40 hours apical and basolateral media as well as HTB-41 B2 lysates were collected. sEVs in media were measured with NTA, mRNA of 96 barrier relevant targets were quantified by high-throughput qPCR (Fluidigm).

Results: Experiments with PCa sEV in serum-containing or serum-free SSGB medium revealed serum dependent effects on TEER over time, highlighting the importance of optimized experimental settings for sEV/barrier interaction studies. sEVs showed minor effects on TEER compared to controls, but addition of hypoxia PCa sEVs resulted in an increase of apically found sEV particles in comparison to sEVs from normoxic conditions. The found effects correlated to mRNA regulation of selected barrier targets in HTB-41 B2 cells.

Summary/Conclusion: Our results indicated that the different PCa sEV fractions influenced either the sEV secretion of SSGB cells or possessed different permeation properties. Current studies aim to elucidate the origin of the apically found sEVs and the underlying mechanisms for these differences.

Funding: The project is a part of ProEVLifeCycle network, funded by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 860303.

PS03.05 | EVs secreted by AML cells mediate suppression of immune effector cell function

Nicole Maeding[1]; Heide-Marie Binder[1]; Balazs Vari[2]; Anna Raninger[3]; Martin Wolf[1]; André Cronemberger Andrade[1]; Thomas Heuser[4]; Astrid Obermayer[5]; Gabriele Brachtl[1]; Lisa Pleyer[6]; Richard Greil[6]; Katharina Schllmoser[7]; Dirk Strunk[1]

[1]Cell Therapy Institute, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI - TReCS), Paracelsus Medical University (PMU), Salzburg, Austria, Salzburg, Austria; [2]Experimental and Clinical Cell Therapy, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCITReCS), Paracelsus Medical University, Salzburg, Austria, Salzburg, Austria; [3]Cell Therapy Institute, Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI-TReCS), Paracelsus Medical University, Salzburg, Austria, Salzburg, Austria; [4]Vienna Biocenter Core Facilities, Medical University, Vienna, Vienna, Austria; [5]Dept. of Biosciences, Paris Lodron University Salzburg, Salzburg, Austria; [6]3rd Medical Dept. with Hematology, Medical Oncology, Rheumatology and Infectiology, Paracelsus Medical University, Salzburg, Salzburg, Austria; [7]Department of Transfusion Medicine and SCI-TReCS, PMU, Salzburg, Austria, Salzburg, Austria

Introduction: Extracellular vesicles (EV) are enriched in leukemia patients and were found to promote migration, invasion and chemoresistance of acute myeloid leukemia (AML) cells, and quiescence of hematopoietic stem cells. We asked if AML-EVs can also target immune cell functions to facilitate immune escape and/or disease progression.

Methods: AML cell lines HL-60, KG-1, OCI-AML3 and MOLM-14 were cultured in particle-free medium under large-scale culture conditions to obtain EV-conditioned medium. AML-EVs were purified and enriched using two sequential rounds of tangential flow filtration (TFF) and characterized by electron microscopy, western blotting and bead-based flow cytometry, confirming double-membrane morphology, purity and identity. AML patient plasma-derived EVs were isolated by size exclusion chromatography. Purified AML-EVs were used to modulate T cell proliferation and NK cell-dependent cytotoxicity.

Results: Purified AML-EVs showed a significant dose-dependent inhibition of PHA-stimulated T cell proliferation. Furthermore, primary AML patient and cell line-derived EVs reduced NK cell-mediated lysis of K-562 target cells in a dose-dependent manner. Also, osteogenic differentiation capacity of bone marrow stromal cells was increased by AML-EVs. Analysis of EV surface marker expression by MACSPlex compared to flow cytometry for AML cells showed a comparable pattern reflecting the AML cell origin. RNA cargo analysis of AML-EVs revealed small RNA species (e.g. miRNAs) as well as longer RNA molecules, albeit to a lesser extent.

Summary/Conclusion: We show that AML-EVs inhibit T cell proliferation and NK cell functionality while enhancing osteogenic differentiation capacity of bone marrow stroma. These effects could contribute to the creation of a leukemia-permissive niche and immune escape of AML mediated by their EVs. The underlying molecular mechanisms need further investigation.

Funding: The presented work received funding from the European Union's Horizon 2020 research and innovation program and by Land Salzburg in the framework of EV-TT, EV-TT – Bpro, and Cancer Cluster Salzburg.

PS03.07 | Extracellular vesicles orchestrate prostate cancer progression by modulating a complex interaction network with adipocytes

Fabrizio Fontana[1]; Monica Marzagalli[1]; Martina Anselmi[1]; Emanuela Carollo[2]; Patrizia Sartori[1]; Patrizia Procacci[1]; David R F. Carter[2]; Patrizia Limonta[1]
[1]University of Milan, Milan, Italy; [2]Oxford Brookes University, Oxford, United Kingdom

Introduction: It is known that an association exists between obesity and risk of prostate cancer (PCa). A crosstalk between adipocytes and PCa has been demonstrated; however, the study of this dialog has been limited to soluble factors, although emerging evidence points to a key role of extracellular vesicles (EVs) in the control of tumor progression.
Methods: After isolation by SEC, EVs were characterized by NTA, TEM and Western blot analysis (TSG101, Hsc70, Alix, calnexin and cytochrome c). In 3T3-L1 adipocytes, the effects of PCa EVs on lipolysis were assessed by flowcytometry and colorimetric assay, while changes in adipokine production were determined by ELISA assay; Akt and MAPK phosphorylation was analyzed by Western Blot. In PC3 and DU145 PCa cells, the effects of EV-treated adipocyte conditioned media on tumor proliferation and survival were evaluated by Trypan blue exclusion assay, while cell metastatic potential was investigated by scratch test and Boyden chamber assay.
Results: We demonstrated that PCa EVs can promote both lipolysis and adipokine (interleukin 6, MCP-1 and TNFα) production in adipocytes, accompanied by Akt, ERK1/2 and p38 activation. Interestingly, conditioned media from EV-treated adipocytes stimulated PC3 and DU145 cell growth, migration/invasion and docetaxel resistance.
Summary/Conclusion: Overall, these data indicate that an EV-mediated crosstalk exists between PCa and adipocytes, endowing the latter with pro-tumor properties. Further studies will be performed to confirm this evidence in vivo and to identify the EV molecular cargo responsible for the modulation of the interactions between adipose tissue and PCa.
Funding: Fabrizio Fontana was awarded with an AIRC Fellowship for italy.

PS03.08 | Extracellular vesicles released by differentiating colorectal cancer stem cells induce a switch from oxidative to glycolytic metabolism in cancer-associated fibroblasts

Giulia Artemi[1]; Donatella Lucchetti[2]; Filomena Colella[2]; Claudio Ricciardi-Tenore[2]; Federica Calapà[3]; Giacomo Lazzarino[4]; Micol Eleonora Fiori[3]; Federica Vincenzoni[5]; Andrea Urbani[6]; Barbara Tavazzi[6]; Ruggero De-Maria[7]; Alessandro Sgambato[8]
[1]Department of Translational Medicine and Surgery, Faculty of Medicine, Catholic University of the Sacred Heart, Rome, Italy, Celleno, Italy; [2]Department of Translational Medicine and Surgery, Faculty of Medicine, Catholic University of the Sacred Heart, Rome, Italy, Roma, Italy; [3]Department of Oncology and Molecular Medicine, IstitutoSuperiore di Sanità, Rome, Italy, Roma, Italy; [4]UniCamillus-Saint Camillus International University of Health Sciences, 00131 Rome, Italy, Roma, Italy; [5]Department of Basic Biotechnological Sciences, Intensive and Perioperative Clinics, Catholic, Roma, Italy; [6]Department of Basic Biotechnological Sciences, Intensive and Perioperative Clinics, Catholic University of the Sacred Heart of Rome, 00168 Rome, Italy;Fondazione Policlinico Universitario "A. Gemelli"-IRCCS, 00168 Rome, Italy, Roma, Italy; [7]Department of Translational Medicine and Surgery, Faculty of Medicine, Catholic University of the Sacred Heart, Rome, Italy; Fondazione Policlinico Universitario "A. Gemelli"-IRCCS, 00168 Rome, Italy, Roma, Italy; [8]Department of Translational Medicine and Surgery, Faculty of Medicine, Catholic University of the Sacred Heart, Rome, Italy;IRCCS-Centro di Riferimento Oncologico della Basilicata (CROB), Rionero in Vulture (PZ), Italy, Roma, Italy

Introduction: Reciprocal relationships between cancer cells and the surrounding non-neoplastic cells are critical important in several aspects of tumor development. It has been known,for a while, that cancer cells are metabolically distinct from other non-transformed cells. This metabolic phenotype is not peculiar to cancer cells but reflects tumor microenvironment characteristics. Recently, it has been shown that Extracellular Vesicles (EVs) are involved in the metabolic switch occurring in cancer and tumor-stroma cells and sustain the growth of tumor cells. Our previous studies showed that differentiation process affects the content of EVs released by Colorectal Cancer Stem Cells (CR-CSCs). In this regard, this study aims to analyze the effects of EVs on Cancer Associated Fibroblast (CAFs) metabolism reprogramming.
Methods: Proteomic profile of EVs content was performed by a classical bottom-up platform followed by high-resolution nano-HPLC-ESI-MS/MS (VelosOrbitrap-Elite). Metabolomic content was analyzed by the ion-pairing HPLC method in recipient cells (CAFs). Evaluation of glycolytic-related genes and proteins, lactate production and glucose uptake was investigated by RT-PCR, western blot (WB), lactate and glucose assays respectively, to confirm metabolomic data.
Results: Proteomic analyses demonstrate that EVs released by CRC cells (HT29 cell line) treated with sodium butyrate differentiating agent are enriched in glycolytic enzymes related to cancer metabolism compared to undifferentiated counterpart. Moreover, we showed by metabolomic analysis that CAFs treated with EVs released by differentiated CRC-CSCs switch from oxidative to glycolytic metabolism. RT-qPCR and WB analyses confirmed the increase of significant genes and proteins involved in glycolytic metabolism.

Summary/Conclusion: This study revealed that EVs released by CR-CSCs, subjected to differentiation, induce a metabolic reprogramming of CAFs. This process triggers a metabolic change of stromal cells in support of CR-CSCs and tumor progression that could explain the failure of differentiated cancer therapies.

PS03.09 | Melanoma-derived extracellular vesicles mediate chemokine production in the tumour microenvironment

Stephanie Bollard[1]; Cristina Casalou[2]; Jane Howard[3]; Desmond J. Tobin[2]; Pamela Kelly[1]; Amanda McCann[1]; Shirley M. Potter[4]
[1]University College Dublin, Dublin, Ireland; [2]Charles Institute of Dermatology, University College Dublin, Belfield, Dublin 4, Ireland, Dublin, Ireland; [3]UCD School of Medicine, University College Dublin., Dublin 4, Ireland; [4]School of Medicine & UCD Conway Institute of Biomolecular & Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland, Dublin, Ireland

Introduction: Extracellular Vesicles (EVs) are tiny lipid bilayer bound nanoparticles, known to play a role in melanoma progression. Their cargo can drive several specialised functions in cancer, including the control of tumour proliferation and pre-metastatic niche formation. In other malignancies, EVs have been shown to alter chemokine secretion in the tumour microenvironment (TME) favouring tumour progression. This study aims to assess the EV-mediated interaction between melanoma cells and fibroblasts within the TME.
Methods: Metastatic melanoma (A375, RPMI-7951) and normal dermal fibroblast (Malme-3) cell lines were maintained in culture. EVs were harvested from melanoma cell lines using differential ultracentrifugation and characterised according to MISEV2018 guidelines. A co-culture experiment in which EVs derived from melanoma cell lines were applied to Malme-3 was performed, and the resulting secretome was analysed after 48 hours using a chemokine array (Abcam, ab169812). Data were analysed using GraphPad Prism v.9 for macOS. Welch t-test was used for comparison between secretion profiles, $p< 0.05$.
Results: The secretion of several chemokines was increased significantly with the addition of EVs in comparison to Malme-3 cells alone. When Malme-3 fibroblasts were co-cultured with EV's from A375 melanoma cells there was a significant increase in the secretion of CCL5 (36.20-fold, p=0.001), CXCL1 (6.41-fold, $p< 0.001$), CXCL10 (3.03-fold, p=0.007), CXCL11 (1.96-fold, p=0.001), CXCL8 (13.25-fold, 0.004) and CXCL9 (1.05-fold, p=0.002). Similarly, when co-cultured with EV's from RPMI-7951 melanoma cells, there was an increased secretion of CCL2 (1.42-fold, p=0.018), CXCL1+2+3 (0.25-fold, p=0.029), CXCL10 (0.80-fold) and CXCL8 (1.71-fold) by Malme-3 dermal fibroblasts.
Summary/Conclusion: The EVs derived from metastatic melanoma cell lines significantly altered the secretome of normal dermal fibroblasts when co-cultured. These results give insights into the mechanisms of paracrine signalling between cell populations in the melanoma TME and highlight potential targets for further study to investigate the mechanisms of melanoma progression within the TME.
Funding: This work was performed within the Irish Clinical Academic Training (ICAT) Programme, supported by the Wellcome Trust and the Health Research Board (Grant Number 203930/B/16/Z), the Health Service Executive National Doctors Training and Planning and the Health and Social Care, Research and Development Division, Northern Ireland, and was also supported by Science Foundation Ireland (19/FFP/6752).

PS03.11 | Periprostatic adipose tissue exomicroRNA profile in prostate cancer patients with different degree of aggressiveness

Silvia Sanchez Martin[1]; Antonio Altuna Coy[1]; Xavier Ruiz-Plazas[2]; José Segarra Tomás[2]; Helena Ascaso-Til[3]; Marta Alves Santiago[2]; Manuel Prados-Saavedra[3]; Joan Francesc Garcia Fontgivell[3]; Xana Bernal Escoté[3]; Matilde R. Chacón[1]
[1]Pere Virgili Health Research Institute, Tarragona, Spain; [2]Joan XXIII University Hospital/Pere Virgili Health Research Institute, Tarragona, Spain; [3]Joan XXIII University Hospital, Tarragona, Spain

Introduction: From the first steps of prostate cancer (PCa) initiation, tumours are in contact with the most-proximal adipose tissue called periprostatic adipose tissue (PPAT). The secretion of extracellular vesicles by PPAT may provide new evidence for the understanding of the interactions between adipocytes and tumours. Extracellular vesicles, in particular exosomes, are important carriers of non-coding RNA such miRNAS (exomiRNAS) that are crucial for cellular communication. Analyzing the exomiRNA-PPAT content can be of importance for the understanding of its progression and aggressiveness.
Methods: A total of 24 samples of human PPAT and abdominal adipose tissue (ABAT) were used. Explants (2.5 g) were cultured 24 h on M199 media and exosomes were isolated by using the exoEasy Kit (Qiagen). Exosomes were characterized by western blot and transmission electron microscopy (TEM). ExomiRNAs were extracted, and expression profiling of n=4 samples of PPAT and 4 samples of ABAT tissue was performed by using miRCURY LNA miRNA miRNome PCR Panel, that included 752 mature

human cancer-related miRNAs. Deregulated exomiRNAS were validated the 24 AT samples. Pathway and functional enrichment analysis on putative targets of the selected exomiRNAs was performed.
Results: Western blot and TEM revealed characteristics of exosomes in AT samples. Nine miRNAs were found in the pilot study to be differentially express between PPAT and ABAT samples. Validation analysis showed that hsa-miR-18a-5p, -20a-5p, -18b-5p, -93-5p, -17-5p,160b-5p, -126-3p were increased in low-risk PCa PPAT when compared with ABAT, while when comparing PPAT regarding aggressiveness, only miR-18a-5p was significantly altered. The RORA gene (a RAR Related Orphan Receptor A) was identified as a common target of the putative miRNA-regulated pathways.
Summary/Conclusion: PPAT microRNA content is linked with PCa and could be envisage as new possibility to be addressed for therapeutic strategies.
Funding: This study is funded by PI20/00418 Instituto de Salud Carlos III and Co-founded by European Union (ERDF/ESF "A way to make Europe/Investing in your future")

PS04: Biomarkers: EV nucliec acids II (other diseases)

PS04.01 | Alpha-1 acid glycoprotein and microRNA miR-21-5p in urinary extracellular vesicles as potentials biomarkers of primary aldosteronism

Cristian A. Carvajal; Jorge A. Pérez; Alejandra Tapia-Castillo; Carlos E. Fardella
Pontificia Universidad Catolica de Chile, Santiago, Chile

Introduction: Primary aldosteronism (PA) is the most common cause of secondary arterial hypertension (HTN), reaching a prevalence of 6-10%. Several efforts have been done in order to identify novel PA biomarkers to support its early detection and other reported effects (inflammation, endothelial dysfunction, kidney damage and oxidative stress) by the aldosterone excess, which can modify the levels and expression of specific proteins (eg lipocalins NGAL and Alpha-1 acid glycoprotein (AGP1)), miRNAs (eg miRNA-21, Let-7i), and the concentration and content of extracellular vesicles (EVs). The current aim is to evaluate the lipocalins AGP1 and NGAL, as well the miRNAs miR-21-5p and Let-7i-5p associated to urinary EVs as potential biomarkers of PA.
Methods: A cross-sectional cohort study in 41 adult subjects with similar age, gender, and BMI, classified as normotensive controls (CTL), essential hypertensives (EH) and subjects with positive screening of PA. Plasma aldosterone levels, plasma renin activity (PRA) and the aldosterone to renin ratio (ARR). Inflammatory parameters such as C-reactive protein were evaluated ultrasensitive PCR (usPCR), PAI-1, MMP9, IL-6, and also lipocalins AGP1 and NGAL by immunoassay. Urinary EVs were isolated of all subjects by ultracentrifugation and characterized by nanotracking analysis (NTA), transmission electron microscopy (TEM) and western blot (CD63). The expression of miR-21 and Let-7i in uEVs by qPCR-Taqman. Statistical analyzes and ROC curves were performed using SPSS v15 and Graphpad Prism v9.
Results: We observed an increase in AGP1 levels in PA subjects with respect to the EH and CTL group ($p < 0.05$). We did not observe significant differences in usPCR, PAI-1, MMP9, IL-6 and NGAL. We detected significant associations of AGP1 with aldosterone (rho = 0.34 $p < 0.05$), PRA (rho = -0.44 $p < 0.01$) and with ARR (rho = 0.38 $p < 0.05$). We did not observe differences in both the concentration of uEVs and the expression of Let-7i-5p between groups. We detected lower levels of miR-21-5p in the uEVs of PA subjects ($p < 0.05$). The ROC curve analysis showed that AGP1 (AUC 0.90; CI 95 [0.79-1.00]; $p < 0.0001$) and AGP1 + miR-21-5p (AUC 0.94; CI 95 [0.85 - 1.00]; $p < 0.001$) can discriminate PA.
Summary/Conclusion: We observed a higher concentration of circulating AGP1 and lower levels of miR-21-5p in uEVs in PA subjects. The associations of AGP1 with aldosterone, PRA and ARR together with the discriminatory ability of AGP1 and miR-21-5p to identify the PA, suggesting a potential role as biomarkers of PA.
Funding: Partially supported by grants ANID-FONDECYT 1212006 and 3200646; CONICYT-FONDEQUIP EQM150023; ANID–Millennium Science Initiative Program-IMII P09/016-F, ICN09_016; SOCHED 2019-09.

PS04.02 | APOE genotype differentially effects the microRNA cargo of cerebrospinal fluid extracellular vesicles in females with Alzheimer's disease compared to males

Ursula S. Sandau[1]; Trevor J. McFarland[2]; Sierra J. Smith[2]; Douglas R. Galasko[3]; Joseph F. Quinn[2]; Julie A. Saugstad[1]
[1]Oregon Health & Science University, PORTLAND, USA; [2]Oregon Health & Science University, Portland, USA; [3]University of California, San Diego, San Diego, USA

Introduction: Multiple biological factors including age, sex, genetics, and family history influence Alzheimer's disease (AD) risk. Of the 6.2 million Americans living with Alzheimer's dementia, 3.8 million are women and 2.4 million are men. The strongest

genetic risk factor for sporadic AD is apolipoprotein E-e4 (APOE-e4). Female APOE-e4 carriers develop AD more frequently than age matched males, and have more brain atrophy and memory loss. Consequently, biomarkers that are sensitive to biological risk factors may improve AD diagnostics and provide insight to underlying mechanistic changes that could drive disease progression. Here we assessed the effects of sex- and APOE-e4-dependent on the miRNA cargo of cerebrospinal fluid (CSF) extracellular vesicles (EVs) in AD.

Methods: Ultrafiltration combined with size exclusion chromatography was used to enrich for CSF EVs (e.g., Flotillin+, but depleted for proteins and lipoproteins not associated with EVs). CSF EVs were isolated from females and males AD or controls (CTLs) that were either APOE-e3,4 or -e3,3 positive (n=7/group, 56 total). MiRNA expression levels were quantified using a custom TaqMan array that assayed 190 miRNAs previously found in CSF, including 25 miRNAs that we previously validated as candidate AD biomarkers.

Results: We identified changes in the EV miRNA cargo that were affected by both AD and sex. Four miRNAs (miR-454-3p, -409-3p, -331-3p, -16-5p) were significantly increased in AD vs. CTL and three miRNAs (miR-150-5p, -342-3p, -146b-5p) were significantly increased in females vs. males. Furthermore, we found that APOE-e4 status affects different subsets of CSF EV miRNAs in females vs. males. The predicted gene targets of the four miRNAs increased in AD identified highly relevant pathways (e.g., senescence and autophagy).

Summary/Conclusion: These studies demonstrate the complexity of the biological factors associated with AD risk, and their impact on EV cargo, which may play a role in AD pathophysiology.

Funding: Oregon Health & Science University Center for Women's Health Circle of Giving

PS04.03 | Association of decreased microRNA-93 expression from neuronal-enriched extracellular vesicles and dysregulated brain interoceptive processing in major depressive disorder

Kaiping Burrows[1]; Jennifer L. Stewart[1]; Leandra K. Figueroa-Hall[1]; Rayus Kuplicki[1]; Ahlam M. Alarbi[2]; Chibing Tan[2]; Victoria B. Risbrough[3]; Brett A. McKinney[4]; Rajagopal Ramesh[5]; Jonathan B. Savitz[1]; T. Kent Teague[2]; Sahib S. Khalsa[1]; Martin P. Paulus[1]
[1]Laureate Institute for Brain Research, TULSA, USA; [2]University of Oklahoma, TULSA, USA; [3]University of California, San Diego, La Jolla, USA; [4]University of Tulsa, TULSA, USA; [5]University of Oklahoma, Oklahoma City, USA

Introduction: This investigation combined functional Magnetic Resonance Imaging (fMRI) and Neuronally Enriched (NE) Extracellular Vesicle (EV) technology to identify cellular processes underlying insula dysfunction during interoception in major depressive disorder (MDD). MicroRNA-93-5p (miR-93) is a putative depression biomarker targeting genes highly expressed in the brain that regulate neuronal axogenesis. We investigated whether: 1) MDD patients differed from healthy comparisons (HC) in NEEV miR-93 expression and 2) groups differed in associations between brain activation and NEEV miR-93 expression.

Methods: The study was approved by the Western Institutional Review Board, performed in accordance with the Declaration of Helsinki, and participants provided informed consent. 41 MDD and 35 HC completed an interoceptive attention task during fMRI and provided blood. EV were separated from plasma using a polymer-based kit. NEEV were enriched by a magnetic streptavidin bead immunocapture kit against the neural adhesion marker biotinylated antibody. NEEV specificities were confirmed by flow cytometry and western blot; size and concentration were determined by nanoparticle tracking analysis. NEEV small RNAs were purified and sequenced. EV-TRACK ID EV210507.

Results: MDD exhibited lower NEEV miR-93 expression than HC ($p=.037$, $d=.48$). Groups differed in bilateral insula-NEEV correlations (left: $F1,71 = 6.34$, $p=.014$; $z=2.57$, $p=.010$; right: $F1,71=9.75$, $p=.003$; $z=3.49$, $p<.001$), such that within HC but not MDD, miR-93 expression was positively correlated with left ($r=.34$, $p=.047$) and right ($r=.54$, $p=.001$) insula signal.

Summary/Conclusion: MDD is associated with decreased miR-93 expression but this expression is unrelated to insula activation, raising the possibility of abnormal NEEV regulation in depression. Neuroimaging-EV integration provides an exciting opportunity to discover novel cellular disease targets for depression.

Funding: This work was supported by The William K. Warren Foundation, and the National Institute of General Medical Sciences Center Grant Award (P20GM121312).

PS04.04 | Combined exosomal and plasma non-coding RNA signature associated with urinary albumin excretion in hypertension

Olga Martinez-Arroyo[1]; Ana Ortega[1]; Angela Riffo-Campos[2]; Javier Perez-Hernandez[3]; Ana Flores-Chova[1]; Josep Redon[1]; Raquel Cortes[4]
[1]Cardiometabolic and Renal Risk Research Group, Biomedical Research Group Clinic Hospital - INCLIVA, Valencia, Spain; [2]Millennium Nucleus on Sociomedicine (SocioMed); and Vicerrectoría Académica, Universidad de La Frontera,, Temuco, Chile;

[3]T-cell tolerance, Biomarkers and Therapies in Type 1 Diabetes Team, Université de Paris, Institut Co-chin, CNRS, INSERM,, Paris, France; [4]Cardiometabolic and Renal Risk Research Group, Biomedical Research Institute Clinic Hospital - INCLIVA, Valencia, Spain

Introduction: Non-coding RNA (ncRNA), released into circulation or packaged into exosomes, play important roles in many biological processes in the kidney. The purpose of the present study is to identify a common ncRNA signature from exosomes, urine and plasma associated with early renal damage and its related molecular pathways by constructing a RNA-based transcriptional network.
Methods: This is an observational case-control study which included twenty-one patients with essential hypertension (n=21) and twenty-two without persistent elevated urinary albuminuria (UAE) (≥30 mg/g urinary creatinine). Three individual libraries (plasma and urinary exosomes and total plasma) were prepared from each hypertensive patient for ncRNA sequencing analysis. Next, a RNA-based transcriptional regulatory network was constructed.
Results: The three RNA biotypes with the greatest number of differentially expressed transcripts were long-ncRNA (lncRNA), microRNA (miRNA) and piwi-interacting RNA (piRNA). We identified a common 24 ncRNA molecular signature related to hypertension-associated albuminuria, of which lncRNA was the most representative. In addition, the transcriptional regulatory network analysis showed five lncRNA (LINC02614, BAALC-AS1, FAM230B, LOC100505824 and LINC01484), and the miR-301a-3p to play a significant role in network organization and to target critical pathways regulating filtration barrier integrity, tubule reabsorption and systemic endothelial dysfunction.
Summary/Conclusion: Our study found a combined ncRNA signature associated with albuminuria, independently of biofluid origin (urine or plasma, circulating or in exosomes) that identifies a handful of potential targets involved in filtration barrier, tubule reabsorption and endothelial function that may be utilized to treating hypertension-associated albuminuria and cardiovascular damage progression
Funding: "Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III" [PI12/02615; PI18/01405; PI19/01796 (FIS Projects for J.R. and R.C.), CD18/00166 (Sara Borrell A.O.), "Ministerio de Ciencia e Innovación" (PID2020-117114GB-I00 for A.L.R.-C.), Maria Zam-brano grant for the requalification of the Spanish University system - NextGeneration EU for A.L.R.-C., ANID - Millennium Science Initiative Program (NCS2021_013 – SocioMed for A.L.R.-C.) and the European Regional Development Fund (ERDF).

PS04.05 | EVs DNA from different biological fluids

Alisa Petkevich[1]; Aleksandr Abramov[1]; Vadim Pospelov[2]
[1]Peoples' Friendship University of Russia (RUDN), Moscow, Russia; [2]RUDN University, Moscow, Russia

Introduction: Extracellular vesicles (EVs) and its molecular cargo may serve as a valuable predictive and prognostic biomarker for cancer. Apparently, there are four main informative molecules containing in EVs – proteins, microRNA, mRNA and DNA. In some researches, it is assumed, that EV DNA may be a more sensitive and informative option in terms of cancer prognosis and prediction compare to cell free circulating DNA despite isolation of the latter may be more yielded than of EV DNA. The aim of this study was to compare the sequencing data and quantity of double-stranded DNA isolated from EVs of different human biological fluids – blood serum, plasma and urine.
Methods: Biological fluids were obtained from healthy volunteers (n=17, male=9, female=8, mean age=45 (30 - 61)). Blood was taken to EDTA K2 tubes (4 ml) and centrifugated immediately after collection (15 minutes, 2000 g, 4°C) to get plasma, blood was taken to SiO2 tubes (4 ml) and centrifugated immediately after collection (15 minutes, 2000 g, 4°C) to get serum, 20 ml of "mid-stream" urine was centrifugated immediately after collection (30 minutes, 2000 g, 4°C); all samples were stored at +4°C for around 24 hours before EVs isolations. Samples were filtered 0,45 μm and then EVs were isolated with differential ultracentrifugation protocol. Size of isolated EVs was measured with NTA, samples were treated with DNAse before DNA isolation, DNA was isolated with DNeasy (Qiagen, Germany), DNA was eluted in 50 mcl of AE buffer and stored at -20°C. DNA quantity was determined with Nanodrop® 1000 (Thermo Fisher Scientific). Double-stranded DNA (ds DNA) was determined with Agilent 7500 DNA Kit (Agilent Technologies). Library preparation was performed with TruSight Tumor 170, sequencing was performed with HiSeq™ 4000, data processing was performed with Illumina software.
Results: Size of isolated EVs ranged from 150 nm to 350 nm, DNA quantity after DNAse treatment was more than 10 ng in every sample. The least quantity of ds DNA was 8 ng and was identified in EVs from urine. Sequencing error rate varied from 0,2x10-5 to 6 x10-5, median coverage rate for allele variant of 0,6% frequency was at least 1200x in all samples types.
Summary/Conclusion: There is a slight difference in ds DNA concentration and length from EVs of different biological fluids. Working with DNA from EVs of cancer patients it is may be reasonable to perform normalization depending on the source of EVs.

PS04.06 | Exhaled small-EVs provide a unique and valuable resource to assess microRNAs representative of deep lung pathologies non-invasively

Megan I. Mitchell[1]; Miao Shi[2]; Ali Sadoughi[2]; Chirag Shah[2]; Taha Siddiqui[2]; Ahamefule Okorozo[2]; Kenny Ye[2]; Simon Spivack[2]; Olivier Loudig[1]
[1]*Center for Discovery and Innovation, Hackensack Meridian Health, Nutley, USA;* [2]*Albert Einstein College of Medicine, Bronx, USA*

Introduction: The development and validation of surrogate tissue assays for non-invasive evaluation of the lung could provide a practical means for routine assessment of lung pathologies, as well as for early detection of disease pathogenesis/risk. We have developed a method for precisely evaluating and enriching deep lung tissue microRNA signals from exhaled breath condensate (EBC), which we compared to miRNA signatures from four different upper and lower airway levels, collected simultaneously during routine care, or clinically indicated (bronchoscopy) from the same patients.
Methods: Eighteen individuals undergoing routine bronchoscopy for various clinical reasons had non-invasive upper airway specimens collected; namely, mouth rinse (MR), buccal brushings (BuBr), and EBC, followed by the collection of lower airway bronchial brushings (BrBr) and bronchioalveolar lavage (BAL). Bronchial brushings and lavages were accomplished at mainstem and lobar standard locations, remote from any focal pathology, such as a nodule, or an infiltrate. Small-EV isolations from both BAL and EBC samples were performed using our ultra-sensitive anti-CD63-based small-EV purification assay (EV-CATCHER). Small-RNA next-generation sequencing (NGS) was then performed on MR, BuBr, whole EBC, EBC-small-EVs, BrBr, whole BAL and BAL-small-EVs.
Results: Small EV isolations from whole EBC samples increased the EBC microRNA read count depth by 10-fold ($p < 0.01$) when compared to whole EBC read counts. We observed the strongest correlation in miRNA signatures between EBC and deep lung specimens (BAL and BrBr) when compared with those of upper airway (MR, BuBr).
Summary/Conclusion: Exhaled microRNAs encapsulated within small-EVs provide a robust representation of deep lung tissue processes. Targeted isolation of exhaled lung tissue small-EVs enhances microRNA NGS quantitation and provides a novel avenue for non-invasive biomarker identification of deep lung pathologies.
Funding: NIH-R33HL156279

PS04.07 | Identifying Biomarkers for Inflammatory Bowel Disease using Extracellular Vesicles from Intestinal Fluid

Kanchana K. Ayyar[1]; Ria Shah[2]; Jean Mendez[3]; Sharmeel K. Wasan[2]; Alan C. Moss[4]
[1]*Boston Medical Center, East Boston, USA;* [2]*Boston Medical Center, Boston, USA;* [3]*Massachusetts General Hospital, Boston, USA;* [4]*Boston Medical Center, boston, USA*

Introduction: The incidence of Inflammatory Bowel Disease (IBD) is increasing since 2000, 1 in 200 individual suffers from IBD. Diagnosis of IBD involves endoscopy, colonoscopy, CT, MRI. Early detection and treatment can help patients as IBD is a progressive and destructive disease which strongly affects the quality of life. There is a need for novel measures that can assess disease severity with minimal risk and inconvenience to patients. Extracellular vesicles (EV) are tiny packets of protein and nucleic acids that are released by most cell types, including inflamed intestinal epithelial cells. EVs can be found in most biological fluids including intestinal fluid. We sought to characterize these signals in patients with IBD.
Methods: Intestinal fluid was collected from healthy individuals and IBD patients during colonoscopy. EVs were isolated by differential ultracentrifugation. Isolated EVs were characterized using nanoparticle tracking analysis on the Nanosight NS300 instrument. Tetraspanin markers were identified on isolated EVs using Exoview platform. RNA was extracted from 1011 EVs and converted to cDNA. A Taqman array plate containing 96 different genes was used to quantify gene expression using qRT-PCR.
Results: The mean and mode size of particles isolated from intestinal fluid was 171.9nm and 111.7nm (n=111). The average concentration of EVs isolated from intestinal fluid was 2.52x1012 particles/ml (n=111). EVs isolated from intestinal fluid express CD63 and Syntenin. CD81 and CD9 expression is moderate to low. qPCR identified 14 genes that were differentially expressed between healthy and IBD group, including CCL2, IFN-γ and TNF. Most of the differentially expressed genes are involved in the Th17 pathway and play a role in inflammation.
Summary/Conclusion: Intestinal fluid contains abundant EVs, with RNA cargo. Gene expression studies illustrate differences in RNA profile between controls and patients with IBD. Further studies to identify IBD biomarkers are warranted.
Funding: This study is funded by the Helmsley Charitable Trust.

PS04.08 | In-depth characterization of human brain pericyte-derived small extracellular vesicles and their modified composition after exposure to pro-inflammatory cytokines

Camille Menaceur[1]; Paulo Marcelo[2]; Andreas Karner[3]; Ricardo Figueiredo[4]; Giulio G. Muccioli[5]; Rémi Chassagnon[6]; Ioannis Karamanos[7]; Yasuteru Sano[8]; Fumitaka Shimizu[8]; Takashi Kanda[8]; Nadine Millot[6]; Björn Rotter[4]; Jaroslaw Jacak[3]; Fabien Gosselet[7]; Laurence Fenart[7]; Julien Saint-Pol[7]

[1]Laboratoire de la Barrière Hémato-Encéphalique (LBHE), Univ. Artois, UR2465, LENS, France; [2]ICAP Platform, Centre Universitaire de Recherche en Santé (CURS), Amiens, France; [3]Institute of Applied Physics, Upper Austrian University of Applied Sciences, Linz, Austria; [4]GenXPro GmbH, Frankfurt am Main, Germany; [5]UCLouvain, Bruxelles, Belgium; [6]Laboratoire Interdisciplinaire Carnot de Bourgogne, UMR 6303 CNRS, site uB, Université Bourgogne Franche-Comté, Dijon, France; [7]Laboratoire de la Barrière Hémato-Encéphalique (LBHE), Univ. Artois, UR2465, Lens, France; [8]Department of Neurology and Clinical Neuroscience, Yamaguchi University Graduate School of Medicine, Ube, Japan

Introduction: The brain homeostasis is maintained by the blood-brain barrier (BBB) located in brain microvessel endothelial cells (ECs). This barrier is formed during brain development and matures after birth thanks to cell-cell communications between ECs and neighbor cells particularly brain pericytes (BPs). Indeed, they (i) induce the pro-angiogenic mechanisms in ECs and promote barriergenesis, (ii) structure the junctional complexes between the ECs, (iii) restrict aspecific vesicular transport, and (iv) are essential for the maintenance of the BBB main features after birth and throughout life. An extracellular vesicle (EV)-mediated cell-cell communication between ECs and BPs is hypothesized as observed in bone marrow where BP-derived EVs promote the ECs differentiation. The objectives of this study are to characterize in depth the composition of BPs-derived EVs and particularly the small EVs (sEV) composition, and to study their modifications once exposed to pro-inflammatory cytokines such as TNF-α, IL-1ß and IFN-γ, mimicking a neurodegenerative context.

Methods: EVs features are evaluated by microscopy approaches (transmission electronic microscopy (TEM), atomic force microscope (AFM)), dynamic light scattering (DLS) and ζ potential measurements once isolated by differential ultracentrifugation (2K, 10K and 100K fractions). Protein and lipid compositions are checked by mass spectrometry, completed by a large nucleic acids sequencing (miRNAs, long non-coding RNAs, …). Impact of cytokines (10 ng/mL each) on BP-derived small EVs is evaluated in terms of (i) concentration of EVs/mL and (ii) protein composition by mass spectrometry.

Results: Expected size and shape of BP-derived sEV (diameter of 100nm, and ζ potential around -20mV) have been validated by AFM and TEM. BP-derived sEV lipid composition is close to the multivesicular body endomembrane and is enriched in ceramides. Their protein composition (i) presents "exosome-related markers" such as CD9, CD81, CD63, syntenin-1 and the absence of calnexin and (ii) is modified in pro-inflammatory conditions and (iii) seems to be cytokine-dependent.

Summary/Conclusion: This study opens discussions about the role of these sEV in (i) establishing and maintaining the BBB main features and (ii) their pathological impact on ECs in pro-inflammatory conditions.

Funding: Camille Menaceur is a recipient of a doctoral grant from the Hauts-de-France Region and the University of Artois. This work is financed by the Agence Nationale de la Recherche (ANR-21-CE14-0002) and by the Hauts-de-France Region (CPER, MOSOPS).

PS04.10 | Role of miR-21 on MSC-derived EV and their paracrine effect

Miriam Morente-López[1]; Rocío Mato-Basalo[1]; María Del Pilar Miranda-Quintana[1]; Sergio Lucio-Gallego[1]; Juan Antonio Fafián Labora[1]; María C. Arufe[2]

[1]Grupo de investigación en Terapia Celular y Medicina Regenerativa, Departamento de Fisioterapia, Medicina y Ciencias Biomédicas, Facultad de Ciencias de la Salud, Universidade da Coruña, INIBIC- Complejo Hospitalario Universitario A Coruña (CHUAC), CICA, A Coruña, Spain, A Coruña, Spain; [2]University of La Coruña, A Coruña, Spain

Introduction: Mesenchymal stem cell-derived extracellular vesicles have significant age-dependent differences in their immune profiles and the miR-21-5p has been shown to play a key role in the regulation of immune and inflammatory responses. The senescence-associated secretory phenotype (SASP) is being seen to offer a new perspective as a marker for the progression of many age-related diseases. Being able to give us an idea of the state of inflammation and complexity of multiple diseases associated with age from multiple pathologies.

For this reason, in this work we studied the paracrine function of miR-21-5p on extracellular vesicles behaviour and SASP. Understanding all these properties of extracellular vesicles makes them especially attractive for the development of new therapeutic approaches based on gene therapy

Methods: Stable and transient transfections of mesenchymal stem cells were performed to inhibit or overexpress miR-21-5p respectively. Their extracellular vesicles were then extracted using ultracentrifugation. The concentration and density of EV in the transfected mesenchymal stem cells as well as the expression of different SASP markers using NTA and RT-PCR respectively.

Finally, these extracellular vesicles from the transfected cells were used to treat mesenchymal stem cells and to verify the paracrine effect of miR-21-5p through evaluation of SASP components using RT-PCR in the the recipient mesenchymal stem cells.
Results: The inhibition of miR-21-5p produced a significant decrease in the amount of extracellular vesicles produced and its overexpression produced the opposite effect in cells. Inhibition of miR-21-5p significantly decreased the expression of SASP (IL-6; HMGB1; S100A4; S100A6; IL-1B). Furthermore, this effect on SASP transmission was significative increased in the mesenchymal stem cells treated with extracellular vesicles from overexpression miR-21-5p mesenchymal stem cells and the opposite effect in the extracellular vesicles from MSCs with the inhibition of miR-21-5p.
Summary/Conclusion: The immunomodulatory properties of mesenchymal stem cells and their ability to release extracellular vesicles has been modified by miR-21-5p. Extracellular vesicles would be acting as a vehicle for cell-cell communication to influence cellular activities in receptor cells, regulating the expression of your target genes related with SASP.
Funding: JFL was funded Xunta de Galicia, grant number ED481D-2021-020. MCA was granted by the Spanish National Health InstituteCarlos III (PI20/00497)

PS05: Biomarkers EV proteins

Chair: Alicia Llorente – Department of Molecular Cell Biology, Institute of Cancer Research, Oslo University Hospital, The Norwegian Radium Hospital

PS05.01 | "Characterization of podocyte-derived microparticles in IgA nephropathy"

Negin Farzamikia
Kidney Research Center, Tabriz University of Medical Sciences, Tabriz, Iran;, Tabriz, Iran

Introduction: Microparticles(MP) are plasma membrane-originated vesicles formed in conditions like cell activation, stress, and apoptosis and released into the extracellular environment. The size of MPs is 100–1000 nm globular-like structures, contains different bioactive molecules such as proteins, lipids, signaling molecules, mRNA, microRNA, long non-coding RNAs, and even DNA.MPs are released by most cell types and they can be found in all biological fluids. Urine is the source of podocyte-derived MPs, the contents and the amount of them changed in renal injury conditions. So they have been considered as biomarkers in many Kidney diseases with podocyte injury like IgA nephropathy. In this study, we have isolated and characterized the podocyte-derived MPs in the urine of patients with IgAN to use them as diagnostic biomarkers as a noninvasive method versus invasive renal biopsy
Methods: In this study, 15 biopsy-proven IgAN patients and 15 healthy people participated. Early morning urine was collected, The samples were centrifuged by differential centrifugation. First, centrifuge at 300g for 10 minutes to remove all live cells and second the collected supernatant of first step centrifuge at 25000g for 20 minutes at 4°C, the MP-containing pellet was resuspended in 4X PBS before characterization. To size the isolated MPs, they are visualized by scanning electron microscope (SEM). Then we use the western blot method to quantify the 6 podocyte-specific protein markers Nephrin, Podocalyxin, flotillin, CD-9, TSG101, ALiX, and HIST1H as a negative marker in both IgAN patients and healthy group.
Results: the Images of SEM indicate that the mean size of MPs isolated from urine sample of IgAN patients is 125.8 nm and by western-blot, we had observed that the 5 podocyte-specific proteins Nephrin, Podocalyxin, flotillin, TSG101, and ALiX present in two groups but the density of them are higher in IgAN patients versus healthy group but the density of CD19 was almost the same in two IgAN positive IgAN negative samples. The absence of protein HIST1H as a negative marker confirms the presence of podocyte-derived MPs in samples.
Summary/Conclusion: Characterization of MPs indicates that podocyte-derived MPs exist in the urine of IgAN patients. This investigation provides the study of early IgAN diagnosis with specific biomarkers as a noninvasive method in the future.

PS05.02 | Circulating GLAST+ Extracellular Vesicles (EVs) are new biomarker for Amyotrophic Lateral Sclerosis (ALS)

Beatrice Vilardo[1]; Davide Raineri[2]; Fabiola De Marchi[3]; Letizia Mazzini[4]; Giuseppe Cappellano[5]; Annalisa Chiocchetti[6]
[1]Università del Piemonte Orientale - Immunomics Laboratory, Novara, Italy; [2]Università del Piemonte Orientale, Novara, Italy; [3]Ospedale Maggiore della Carità Novara, novara, Italy; [4]Ospedale Maggiore della Carità - Novara, Novara, Italy; [5]Università del Piemonte Orientale, novara, Italy; [6]Università del Piemone Orientale, Novara, Italy

Introduction: Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease whose triggering factors are still poorly understood. ALS is a "silent" disease compared to other neurodegenerative disorders, manifesting earlier cognitive defects

or postural instability. No reliable biomarker is available either for ALS diagnosis, for the monitoring of disease progression or assessment of response to therapy. Literature showed that astrocytes derived-extracellular vesicles (EVs) carrying on the surface GLAST (GLAST+ EVs) were increased in ALS preclinical model but not data were available in the humans. Nowadays, EVs are recognized as biomarker of several human diseases, and they transfer their cargo to the recipient cells by affecting their functions. GLAST is the transporter of glutamate, the main neurotransmitter in the central nervous system. An excessive accumulation of glutamate is responsible for neuronal death. The aim of this pilot project was to search for GLAST+ EVs in ALS patients.
Methods: We enrolled 25 ALS patients at the Hospital Maggiore della Carità and 17 healthy age-matched controls. GLAST-EVs were quantified in plasma by flow cytometry using a fast (1hr) method, recently validated by our laboratory.
Results: We found that GLAST+ EV counts were significantly higher in ALS patients compared to HC. Importantly, by stratifying ALS patients in fast and slow progressors, we found that GLAST-EVs counts were even higher in fast patients compared with slow ones.
Summary/Conclusion: We identified for the first time a neuronal tissue specific EV in ALS patients which allows, from the clinical point of view to discriminate fast and slow progressor patients. We applied a method which can be easily transferred to the hospitals, already equipped with a flow cytometer, thus entering the diagnostic process. Our results are preliminary and will be validated in a larger cohort.
Funding: This project is funded by Italian Ministry of Education, University and Research (MIUR) program "Departments of Excellence 2018–2022" (MINDFUL and AGING Project).

PS05.03 | Investigation of metabolic changes by EVs derived from endothelial and beta-cells under hyperglycemic conditions

Ewa Ł Stępień[1]; Magdalena E. Marzec[2]; Carina Rząca[1]; Andrzej Wróbel[1]; Julia Nizioł[3]; Mariusz Kępczyński[4]; Agata Żak[4]; Paweł Moskal[5]
[1]Department of Medical Physics, M. Smoluchowski Institute of Physics, Faculty of Physics, Astronomy and Applied Computer Science, Jagiellonian University, Kraków, Poland, Kraków, Poland; [2]Department of Medical Physics, M. Smoluchowski Institute of Physics, Faculty of Physics, Astronomy and Applied Computer Science, Jagiellonian University in Krakow, Poland, Krakow, Poland; [3]Department of Experimental Particle Physics and Applications, Marian Smoluchowski Institute of Physics, Faculty of Physics, Astronomy and Applied Computer Science, Jagiellonian University, Krakow, Poland; [4]Faculty of Chemistry, Jagiellonian University, Krakow Poland, Kraków, Poland; [5]Department of Experimental Particle Physics and Applications, Marian Smoluchowski Institute of Physics, Faculty of Physics, Astronomy and Applied Computer Science, Kraków, Poland

Introduction: Hyperglycemia causes a deleterious effect on vascular cells and stimulates pancreatic beta-cells to insulin secretion. Dyslipidemia is a significant manifestation of a persistently elevated glucose concentration in diabetic patients. Extracellular vesicles are lipid and protein rich entities and their molecular content reflect cell metabolism, structure and membrane composition. We aimed to collect and in-depth analyze data on the quantitative and qualitative composition of proteins and lipids transported by EVs.
Methods: We applied novel techniques, developed in material science and nanotechnology, to characterize EVs content: SIMS – Secondary Ion Mass Spectrometry, PALS - Positronium Annihilation Lifetime Spectroscopy with the 22Na isotope as a positron emitter, and TRAMS - time-resolved fluorescence anisotropy measurements with DPH probes. Exosomes and larger subpopulations of EVs were isolated from preconditioned media of human microvascular endothelial and beta-cells for our investigation.
Results: SIMS allowed us to define lipids including sterols, prenols, and sphingomyelins, and characterize the abundance of amino acids in different subpopulations of EVs. We observed altered lipid composition of EVs under hyperglycemic conditions, in both cell lines and different EV subpopulations. Such changes were associated with lower lipid membrane fluidity in EVs obtained from hyperglycemic cells, which was investigated by PALS and TRAMs.
Summary/Conclusion: We conclude that hyperglycemic conditions have an impact on EV formation and their molecular content. Such metabolic alterations can be characterized on the molecular level to define molecular content and structure combining complementary information obtainable by SIMS, PALS, and TRAMs methods.
Funding: Study supported by the National Science Centre grant OPUS 17 to E. Stępień No. 2019/33/B/NZ3/01004 and the Jagiellonian University via project CRP/0641.221.2020.

PS05.04 | L1CAM is associated with neuron derived extracellular vesicles

Dimitrios Kapogiannis[1]; Erden Eren[2]; Carlos J. Nogueras-Ortiz[2]; Maja Mustapic[2]
[1]National Institutes of Health, Baltimore, USA; [2]Laboratory of Clinical Investigation, National Institute on Aging, Baltimore, USA

Introduction: Isolating neuronal-derived extracellular vesicles (NDEV) in blood and studying their cargo may help us understand cellular processes and follow their evolution during neurological disorders. Recent publications have challenged the claims that neuronal cell adhesion molecule L1CAM is EV-associated and that targeting it with immunocapture effectively isolates NDEVs from human plasma. Here we provide evidence that some plasma EVs carry L1CAM on their surface and, therefore, L1CAM may be utilized for isolation of NDEVs from plasma.
Methods: We isolated EVs from 10 ml of plasma of three healthy individuals using qEV10 70 nm size-exclusion chromatography columns and collected 13 fractions. We replicated the published SIMOA assay for L1CAM developed by Norman et. al. and measured L1CAM levels in each fraction. This assay uses L1CAM (5G3) as capture antibody and L1CAM (UJ127) as detector. We also developed a novel homebrew SIMOA assay to detect L1CAM on intact EVs (NDEV assay) in each fraction. We used L1CAM (5G3) as capture antibody, mixed biotinylated CD9, CD81 and CD63 antibodies as detectors and human iPSC-derived neuronal EVs as calibrator during assay development.
Results: In agreement with findings of Norman et. al., using the L1CAM SIMOA assay, we detected L1CAM levels close to LLOQ in fractions 1-4 but detected increasing levels of L1CAM from fraction 5 to 10; L1CAM levels declined gradually between fractions 11 and 13. However, using our novel homebrew NDEV SIMOA assay in the same fractions, we found that L1CAM+ EVs were present in highest levels in fractions 1 to 5 and in declining levels in fractions 6 to 13
Summary/Conclusion: Our study provides evidence that L1CAM is a surface marker of EVs of variable size, making it plausible that L1CAM may be used for immunocapture of an EV sub-population of neuronal origin.
Funding: This research was supported by the Intramural Research Program of the National Institute on Aging, NIH.

PS05.05 | Matrix metalloproteinase-7 in urinary extracellular vesicles predicts disease progression in polycystic kidney disease

Martijn H. van Heugten; Charles J. Blijdorp; Hester van Willigenburg; Mahdi Salih; Ewout J. Hoorn
Erasmus Medical Center, Rotterdam, Netherlands

Introduction: Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited form of kidney disease and often leads to kidney failure. However, disease progression is highly variable and unpredictable. Here, our aim was to identify a protein biomarker in urinary extracellular vesicles (uEVs) to differentiate rapid from slow disease progression.
Methods: Patients were selected from the clinical DIPAK trial (informed consent, medical ethics approval). We created a discovery (n=10) and confirmation cohort (n=10) including patients with rapid and slow disease progression (eGFR decline $\geq$4 or $<$ 2 mL/min/1.73 m2/year). uEVs were isolated from 50 mL spot urines by a three-step differential ultracentrifuge protocol, and uEV-proteins were mass tag labeled before tandem mass spectrometry. A third validation cohort (n=24) was created to validate the mass spectrometry findings with immunoblotting. All patients were matched for established risk factors for disease progression, including age, sex, baseline eGFR, and genetic mutation, and had equal total kidney volume.
Results: We identified 2,727 and 1,115 unique uEV-proteins with over 60% annotated for the extracellular exosome (Benjamini $<$ 0.01). In the discovery and confirmation cohort, a significantly different uEV-protein abundance was found for 65 and 36 proteins, respectively. Matrix metalloproteinase 7 (MMP-7), a protein previously implicated in kidney disease progression, was consistently higher by 47% and 64% in patients with rapid disease progression in both cohorts ($p < 0.05$). Pathway analysis showed enrichment of Wnt-signaling (q-value $<$ 0.05) of which MMP-7 is a downstream mediator. In the validation cohort, MMP-7 was also higher by 120% in uEVs of patients with rapid disease progression ($p < 0.05$).
Summary/Conclusion: uEV-associated MMP-7 distinguishes patients with slow or rapid ADPKD progression independently of established disease progression markers. MMP-7 is a novel and biologically plausible urinary biomarker for ADPKD which we are currently validating in a larger cohort of patients using a high-throughput assay.
Funding: Dutch Kidney Foundation.

PS05.06 | Proteomic analysis of EVs derived from 3D trophoblast cells spheroids improves understanding of EVs mediated trophoblast endometrial communication

Norhayati Liaqat Ali Khan[1]; Keerthie Dissanayake[2]; Kasun Godakumara[2]; Getnet B. Midekessa[2]; Subhashini Muhandiram[3]; Aneta Andronowska[4]; Paul R. Heath[5]; Alireza Fazeli[6]
[1]Academic Unit of Reproductive and Developmental Medicine, Department of Oncology and Metabolism, The Medical School, University of Sheffield, Sheffield, UK, Sheffield, United Kingdom; [2]Department of Pathophysiology, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu, 14B Ravila, 50411 Tartu, Estonia, Tartu, Estonia; [3]Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia, Tartu, Estonia; [4]Department of Hormonal Action Mechanisms, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland, Olsztyn, Poland;

[5]*Sheffield Institute for Translational Neuroscience (SITRAN), University of Sheffield, Sheffield, UK, Sheffield, United Kingdom;* [6]*Unit of Reproductive and Developmental Medicine, Department of Oncology and Metabolism, The Medical School, University of Sheffield, Sheffield, UK, Sheffield, United Kingdom, United Kingdom*

Introduction: Extracellular vesicles (EVs) are involved in cell to cell communication. In vitro, JAr (human choriocarcinoma cell line) cell spheroids (3D) are used as mimics of embryo trophoblasts when studying EV mediated trophoblast-endometrial communications. However, the differences of the proteomic profile EVs from 3D JAr spheroids compared to 2D JAr monolayer cultures are unknown. Therefore, we investigated the proteomic profiles of EVs derived from 3D compared to 2D in vitro trophoblast cell culture systems.
Methods: JAr cells were cultured and trophoblast spheroids (3D) were formed. JAr monolayer cultures were used as 2D cell culture system. FBS-free culture media conditioned by these JAr 2D cultures or 3D spheroids for 6 hours were collected. Following the sequential centrifugation to remove cells and cell debris, EVs were isolated by size exclusion chromatography and subsequently characterized by nanoparticle tracking analysis (NTA), electron microscopy and mass spectrometry shot-gun based quantitative proteomics. Differential enrichment of proteins in EVs from 3D spheroids compared to 2D JAr cultures and pathway enrichment analysis were performed.
Results: EVs enrichment in our purified samples were verified with all the methods, including enrichment of EV markers such as CD9, CD63 and CD81 in the samples. 1509 proteins were identified. 65 of proteins, including LAMB1, LAMC1, COL4A2, LAMA1, HSPG2, LAMA5 and VWF were differentially abundant in 3D culture samples. KEGG pathway analysis showed the enrichment of Extracellular matrix receptor interaction and focal adhesion pathways in 3D JAr cell culture system.
Summary/Conclusion: EVs derived from 3D JAr cell culture system provide a different proteomic profile compared to the 2D cultures. Hence, the culture system affects the biological function of these cells in vitro.
Funding: This work was supported by the Ministry of Education (Higher education Malaysia) and Universiti Teknologi MARA (UiTM), Malaysia and the European Union's Horizon 2020 research and innovation program under grant agreement No 857418 (COMBIVET)

PS05.07 | Proteomic profiling of microglial derived exosomes in Alzheimer's disease

Charisse N. Winston[1]; Sonia Podvin[2]; Vivian Hook[2]; Robert Rissman[2]
[1]*UC San Diego, Lemon Grove, USA;* [2]*UC San Diego, La Jolla, USA*

Introduction: Introduction: Recent studies suggest neuronal and astrocyte-derived exosomes may serve as the source of biomarkers for many neurodegenerative diseases, include AD. However very few studies have characterized the biomarker potential of microglial derived exosomes (MDEs). Here, we employed the use of unbiased mass spectrometry (MS) to characterize the molecular composition of MDEs derived from healthy controls and pathologically confirmed Alzheimer's disease patients.
Methods: Methods: Plasma exosomes were extracted, precipitated, and enriched against a microglial source (TMEM119) using magnetic immunocapture and fluorescence-activated cell sorting (FACS) sorting. MDEs were characterized by size (Nanosight) and shape (TEM). Exosome marker profiling was done by western blot. Proteomic profiling was conducted for novel biomarker identification.
Results: Result: Blood-based MDEs demonstrate similar size distributions and shape to previously reported exosome preparations. Western Blot demonstrated that MDEs were positive for exosome marker Flotilin-1; microglial markers CD68 and IBA1 and negative for astrocyte marker, GLAST. Out of 143 total proteins identified by MS, only 1 protein, thrombospondin, was unique to the control group as compared to the AD group.
Summary/Conclusion: Conclusion: MDEs can be successfully isolated from human blood. MDE cargo are associated with lipid metabolism (APOE), the complement cascade (Clusterin, C3, C5), and DNA translation and repair (CDK12). Further characterization is required to determine functional protein network analyses of MDE cargo and to determine the biomarker potential of MDE cargo in AD.
Funding: MOSAIC K99AG070390-02

PS05.08 | Raman spectral signatures of plasma-derived extracellular vesicle-enriched isolates may support the diagnosis of different cancerous diseases

Matyas Bukva[1]; Edina Gyukity-Sebestyen[2]; Timea Boroczky[1]; Yasmin Ranjous[3]; Maria Harmati[2]; Gabriella Dobra[4]; Adrienn Jenei[5]; Laszlo Szivos[6]; Katalin Hideghety[7]; Krisztina Budai[8]; Judit Olah[9]; Peter Horvath[2]; Gyorgy Lazar[8]; Tamas Biro[10]; Zoltan Konya[3]; Pal Barzo[6]; Almos Klekner[5]; Krisztina Buzas[11]

[1]Laboratory of Microscopic Image Analysis and Machine Learning, Institute of Biochemistry, Biological Research Centre, Szeged, Hungary; Doctoral School of Interdisciplinary Medicine, University of Szeged, Szeged, Hungary; Department of Immunology, Albert Szent-Györgyi Medical School, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary, Szeged, Hungary; [2]Laboratory of Microscopic Image Analysis and Machine Learning, Institute of Biochemistry, Biological Research Centre, Szeged, Hungary, Szeged, Hungary; [3]Department of Applied and Environmental Chemistry, University of Szeged, Szeged, Hungary, Szeged, Hungary; [4]Laboratory of Microscopic Image Analysis and Machine Learning, Institute of Biochemistry, Biological Research Centre, Szeged, Hungary; Doctoral School of Interdisciplinary Medicine, University of Szeged, Szeged, Hungary, Szeged, Hungary; [5]Department of Neurosurgery, Clinical Centre, University of Debrecen, Debrecen, Hungary, Debrecen, Hungary; [6]Department of Neurosurgery, Albert Szent-Györgyi Medical School, University of Szeged, Szeged, Hungary, Szeged, Hungary; [7]ELI-ALPS, ELI-HU Non-Profit Ltd., Szeged, Hungary; University of Szeged, Albert Szent-Györgyi Medical School, Department of Oncotherapy, Szeged, Hungary, Szeged, Hungary; [8]Department of Surgery, Albert Szent-Györgyi Medical School, University of Szeged, Szeged, Hungary, Szeged, Hungary; [9]University of Szeged, Albert Szent-Györgyi Medical School, Department of Oncotherapy, Szeged, Hungary, Szeged, Hungary; [10]Department of Immunology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary; Monasterium Laboratory, Münster, Germany, Debrecen, Hungary; [11]Laboratory of Microscopic Image Analysis and Machine Learning, Institute of Biochemistry, Biological Research Centre, Szeged, Hungary; Department of Immunology, Albert Szent-Györgyi Medical School, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary, Szeged, Hungary

Introduction: Thorough examination of the molecular composition of small extracellular vesicles (sEVs) by spectroscopic methods is a promising but hitherto barely explored approach for diagnosing cancerous diseases, especially central nervous system tumors. We attempt to reveal the potential role of plasma-derived sEVs in diagnosing seven different patient groups through Raman spectroscopic analyses using a relevant number of clinical samples.
Methods: The study is conducted in accordance with the Declaration of Helsinki, informed consent forms are collected and the study was approved by a national ethics committee. Up to 490 plasma samples will be obtained from seven patient groups (glioblastoma multiforme, meningioma, melanoma and non-melanoma brain metastasis, colorectal tumors, melanoma and a control group). SEV isolation is performed through differential centrifugation. The isolates are characterized by Western Blot, transmission electron microscopy and nanoparticle tracking analysis. Principal Component Analysis–Support Vector Machine algorithm is performed on the Raman spectra for classifications. Classification accuracy, sensitivity, specificity and the Area Under the Curve (AUC) value are used to evaluate the classification performance.
Results: According to preliminary results, the patient groups are distinguishable with 80–95% sensitivity and 80–90% specificity. AUC scores of 0.82–0.9 suggest excellent classification performance.
Summary/Conclusion: Our results support that Raman spectroscopic analysis of sEV-enriched isolates from plasma is a promising approach for developing non-invasive, cost-effective methods for the clinical diagnosis of different cancerous diseases.
Funding: Supported by GINOP-2.2.1–15-2017–00052, ÚNKP-20–3-New National Excellence Program of the Ministry for Innovation and Technology from the National Research, Development and Innovation Fund; University of Szeged, Faculty of Medicine, Szent-Györgyi Albert Research Fund (2021); MEC-R 141410, MEC-R 141412.

PS05.09 | Urinary extracellular vesicles as early predictive biomarkers in urinary bladder cancer

Loïc Steiner[1]; Rosanne E. Veerman[1]; Maria Eldh[1]; Amir Sherif[2]; Susanne Gabrielsson[1]
[1]Division of Immunology and Allergy, Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden, Stockholm, Sweden; [2]Umeå University, Umea, Sweden

Introduction: Urinary Bladder Cancer (UBC) is the 4th most common cancer type in men and associated with a poor survival rate and a bad prognostic. At the time of diagnosis, one third of detected cancers are already muscle-invasive and start invading other organs. Due to their abundance in urine, Extracellular Vesicles (EVs) make ideal biomarker candidates for early diagnosis of UBC. Thus, we characterized the protein content of urinary EVs to determine an EV protein signature of UBC and link it to some clinical features.
Methods: Urine was collected from 40 bladder cancer patient at different stages and 5 age-matched healthy controls. EVs were enriched from urine by centrifugation (300g, 3000g and 10 000g), followed by tangential flow filtration (100kDa column) and a final ultracentrifugation step at 120 000g. After characterization of EVs by nanoparticle tracking analysis, bead-based flow cytometry and western blot, the vesicles were lysed and their protein content was analyzed by proximity elongation assay (Olink, immuno-oncology panel).
Results: The proteomic analysis highlighted important changes in the protein composition of EVs. Firstly, urinary EVs from UBC patients greatly differed from healthy controls establishing an EV protein signature of urinary bladder cancer. We also compared matched samples of urinary EVs and bulk urine (not EV-enriched) from UBC patient and discovered a set of proteins exclusively found on purified EVs, strengthening our rationale for focusing on isolated EVs for biomarker discovery. Additionally,

the protein profile was correlated with clinical data such as invasiveness or response to chemotherapy and sets of proteins were identified as predictors of such features.

Summary/Conclusion: Taken together, these results suggest the great potential of using isolated urinary EVs as a biomarker for UBC that can then be employed to predict clinical markers and better orient the care of the patients at an early stage of the disease.

PS05.10 | Highly sensitive system for FACS profiling of histological markers on EVs

Victoria O. Shender[1]; Polina V. V. Shnaider[1]; Vassili N. Lazarev[1]; Irina Yu. Petrushanko[2]; Elena Khomyakova[3]
[1]*Federal Research and Clinical Center of Physical-Chemical Medicine of Federal Medical Biological Agency, Moscow, Russia;* [2]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia;* [3]*Exosome Analytics, Evry-Courcouronnes Cedex, France*

Introduction: Cancer takes the lives of hundreds of thousands of people every year. Most cancer deaths result from the metastatic propagation of drug- resistant cells, thus the biomarkers capable of predicting tumor progression and therapeutic response are required. A major cause of chemotherapy failure is attributed to subpopulation of self-renewing, drug-resistant cells suspected to originate from the stem cells.

Liquid biopsy is a promising approach to characterize tumor phenotype. EVs are potential sources of tumor biomarkers since they carry the same membrane histological markers as a tumor. However, a major challenge faced while analyzing histological and particularly stem cell markers on EVs is their low quantity compared to CD81/CD9/CD63 antigens that make them difficultly detectable since the signal is often under the limit of detection of commonly used techniques.

We develop a highly sensitive system for FACS profiling of low-represented EVs membrane markers. The system was validated by analysis of EpCAM, CD166 and CD117 expression on EVs isolated from cell culture supernatants and biological fluids of cancer patients.

Methods: EVs were isolated from HT29 and SK-OV-3 cell cultures by ultrafiltration. EVs from clinical samples of ovarian cancer patients were isolated using the adapted procedure of ultrafiltration. Pre-purified EVs were incubated with CD9 beads followed by CD81, EpCam, CD166 and CD117 staining and flow analysis.

Results: Limit of detection (LOD) of the system was determined on HT29 and SK-OV-3 derived EVs and estimated to be 1e+5 for CD81, 5e+5 for EpCam (HT29), 2e+8 for CD166 (HT29), and 1e+8 for CD117 (SK-OV-3). Signal calibration method was proposed for evaluation of tumor differentiation degree and validated using forced HT29 cells differentiation and clinical samples of cancer patients.

Summary/Conclusion: A highly sensitive system for profiling of low-represented small EVs isolated from cell cultures and physiological fluids has been developed.

PS05.11 | Multi-omic analysis of extracellular vesicles in plasma following an acute bout of exercise

Lone Peijs[1]; Naja Z Jespersen[2]; Sarah Heywood[2]; Diana Samodova[1]; Bente K Pedersen[2]; Atul S Deshmukh[1]
[1]*Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Denmark, Copenhagen, Denmark;* [2]*Trygfondens Centre for Physical Activity Research, Rigshospitalet, Denmark, Copenhagen, Denmark*

Introduction: Exercise facilitates beneficial effects on peripheral tissues, and extracellular vesicles (EVs) may play an important role in mediating these effects.

In this study, we aim to map the circulating cargo of EVs in plasma following an acute bout of exercise using a multi-omic approach. We will use liquid chromatography mass spectrometry (LC-MS/MS) for characterization of EV-associated proteins and small-RNA sequencing for analysis of miRNAs.

Methods: Twenty-five healthy males undertook a 45 min acute exercise bike intervention. Blood plasma samples were collected at just prior to, and immediately after cessation of the exercise intervention. Moreover, blood samples were collected 1 hrs and 3 hrs into recovery as well as 24 hrs after the exercise intervention. As an initial test, plasma samples were pooled from all individuals at each timepoint and EVs were isolated in technical triplicates using size-exclusion chromatography (SEC) with a 70 nm pore size. The EV isolation protocol was qualitatively evaluated using transmission electron microscopy (TEM) and quantitatively evaluated using nanoparticle tracking analysis (NTA). Protein cargo analysis was performed using LC-MS/MS

Results: TEM images showed successful isolation of intact rounds vesicles in size ranges ~30-200 nm. Preliminary NTA analysis showed increased concentration of EVs in the recovery period. Mass spectrometry-based EV preliminary proteome analysis led to quantification of 834 protein, among these were known protein EV-markers such as CD9, CD63 and CD81.

Summary/Conclusion: The preliminary NTA and MS-based analysis emphasizes that acute exercise triggers release of EVs that might convey important signals to multiple organs. The upcoming experiments directed at using integrative approach of multi-omics data may enhance the understanding of the molecular dynamics of systemic beneficial effects mediated by exercise.
Funding: This project was supported by EFSD/Novo Nordisk Foundation Future Leaders Awards Programme to A.S.D. (NNF19SA058976)

PS06: Biomarkers: EV nucleic acids I (Cancer)

Chair: Charlotte Proudhon, Institut Curie / INSERM U934

PS06.01 | Age-related vesicular miRNA comparison proposes potential biomarkers for triple-negative young breast cancer in a Brazilian cohort

Alexis Germán Murillo Carrasco[1]; Silvina Odete Bustos[2]; Tainara Francini Felix[3]; Patricia Pintor Dos Reis[3]; Luciana Nogueira de Sousa Andrade[1]; Roger Chammas[1]
[1]Center for Translational Research in Oncology (LIM24), Instituto do Cancer do Estado de Sao Paulo (ICESP), Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo (HCFMUSP), Sao Paulo CEP 01246-000, SP, Brazil, Sao Paulo, Brazil; [2]Center for Translational Research in Oncology (LIM24), Instituto do Cancer do Estado de Sao Paulo (ICESP), Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo (HCFMUSP), Sao Paulo CEP 01246-000, SP, Brazil, São Paulo, Brazil; [3]Department of Surgery and Orthopedics, Faculty of Medicine, São Paulo State University (UNESP), Botucatu, SP, Brazil, São Paulo, Brazil

Introduction: Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer. Since young TBNC patients (< 40 years) show a shorter survival time, this age-related group could have different molecular behavior promoted by microRNAs (miRNAs). Then extracellular vesicles (EVs) arise as a minimally invasive option for searching disease biomarkers. Herein, we compared vesicular miRNA levels from plasma of young (n=5) and elderly (n=6) Brazilian TNBC patients.
Methods: All samples were retrieved from the Academic Biobank for Cancer Research at the University of São Paulo. The informed consent and an epidemiological questionnaire (Ethics Committee Approval N° 3.007.737/18) were obtained from all patients enrolled in the study. EVs were collected from plasma samples (1mL) using size-exclusion chromatography, quantified by nanoparticle tracking analysis, and characterized by transmission electron microscopy as well as the expression of CD63, CD9 and/or flotillin by western blot. EVs were treated with proteinase K and RNase A before RNA extraction using a silica column method. Finally, vesicular miRNA content was detected and quantified through a digital barcode technology.
Results: We did not find statistical differences in EVs plasma concentration between young and elderly patients with TNBC ($p=0.792$). However, we found 37 differentially expressed miRNAs (DEmiRs) between these groups ($p< 0.05$ and $|\text{fold change (FC)}| > 1.2$). Among these DEmiRs, hsa-miR-409-3p (FC=2.2, p=0.004), hsa-miR-3180-5p (FC=1.9, p=0.002), hsa-miR-10a-5p (FC=1.9, p=0.008), hsa-miR-933 (FC=1.9, p=0.008), hsa-miR-367-3p (FC=1.7, p=0.009), and hsa-miR-133b (FC=-2.7, p=0.001) showed remarkable differences.
Summary/Conclusion: Our preliminary study indicates that vesicular miRNAs show the potential to identify new biomarkers and therapeutic targets in an age-related manner in TNBC patients.
Funding: Grant #2019/05583-0, São Paulo Research Foundation (FAPESP); Grant # 25000.069252/2015-79, Programa Nacional de Apoio à Atenção Oncológica (PRONON).

PS06.03 | Combination of tissue biopsy and EV-based liquid biopsy achieves improved prognosis in melanoma

Susana Garcia-Silva[1]; Cristina Vico-Alonso[2]; Lisa Meyer[3]; Mikkel Noerholm[3]; Johan Skog[4]; José-Luis Rodríguez-Peralto[5]; Pablo L. Ortiz-Romero[2]; Héctor Peinado Selgas[6]
[1]Spanish National Cancer Research Centre (CNIO), Madrid, Spain; [2]Department of Dermatology, Medical school, Universidad Complutense de Madrid, Instituto i+12, Hospital Universitario 12 de Octubre, Madrid, Spain, Madrid, Spain; [3]Exosome Diagnostics, a Bio-techne brand, Martinsried, Germany., Martinsried, Germany; [4]Exosome Diagnostics, a Bio-techne brand, Waltham, MA, Waltham, USA; [5]Department of Pathology, Medical school, Universidad Complutense de Madrid, Instituto i+12, Hospital Universitario 12 de Octubre, Madrid, Spain, Madrid, Spain; [6]Spanish National Cancer Research Center (CNIO), Madrid, Spain

Introduction: Liquid biopsy provides a suitable option with progressively improved sensitivity and specificity to complement or to substitute the information obtained by regular biopsy. Extracellular vesicles (EVs) are emerging as powerful tools for providing

information about the tumor and its microenvironment. The detection of specific mutations in EV-loaded nucleic acids has shown similar or improved sensitivity and specificity compared to cell free DNA analysis. Our group has shown that detection of BRAFV600E mutation in EV-associated nucleic acids (EV-NAs) isolated from lymphatic drainage from melanoma patients has prognostic significance. In this work, we have determined BRAF status (BRAFV600E mutation or BRAF wild type) in melanoma patients using an EV-NA-based liquid biopsy test in plasma and compared it to tissue biopsy information.
Methods: Plasma was collected from a cohort of 18 melanoma patients (stages II-IV). Samples were collected in EDTA tubes, inverted several times, spun at 500 xg and frozen until subsequent analysis. BRAF status in tissue samples was determined using Cobas assay (Roche). Plasma samples were analyzed using the ExoDx BRAF V600E/K test by Exosome Diagnostics. Co-isolation of EV RNA and DNA along with any cfDNA present (EV-NA) was purified using the ExoLution Plus extraction technology (Exosome Diagnostics). Samples were subjected to reverse transcription and a highly sensitive quantitative PCR for determination of BRAF wt and BRAFV600E alleles.
Results: From 18 patients, 5 were detected as BRAF mutant carriers by tissue biopsy. In two of those, BRAF mutation was detected also in plasma. Additionally, in 2 more patients, BRAF mutation was present in the plasma but not detected in the original tissue. The mutant allele frequency spanned between 0.01-3.43%. Twelve patients developed distant metastasis and presence of BRAFV600E mutation in either tissue or liquid biopsy predicted a shorter overall survival (log rank test, p= 0.0411). BRAF mutation analysis only in tissue biopsy or in liquid biopsy tests did not stratified patients adequately (p=0.5786 and p=0.3373).
Summary/Conclusion: Combination of information from tissue and EV-NA-based liquid biopsy analyzing BRAF mutational status achieves prognostic significance whereas only tissue biopsy is not informative enough. This study also adds evidence about the relevance of the analysis of BRAF in melanoma patients in their visit to the dermatologist to improve their prognosis.
Funding: LABAE19027PEIN Fundación AECC, Beca Leonardo 2021 Fundación BBVA, Severo Ochoa Excellence Center grant.

PS06.04 | Detection of circulating extracellular vesicle-associated mutant DNA in the plasma of metastatic colorectal cancer patients

Thupten Tsering[1]; Alexander Laskaris[1]; Amélie Nadeau[1]; Stephanie Petrillo[1]; Anthoula Lazaris[1]; Peter Metrakos[1]; Julia Burnier[2]
[1]RIMUHC, Montreal, Canada; [2]RI-MUHC/McGill University, Montreal, Canada

Introduction: Liquid biopsy is a non-invasive approach for early detection of cancer and to monitor real-time tumor evolution and treatment response. Circulating tumor (ct)DNA Recently, extracellular vesicles (EVs) have attracted tremendous interest as biomarkers in many pathological diseases including cancer. EVs are a highly heterogenous population of naturally occurring vesicles released by all cell types. EVs act as a messenger between cells by carrying biomolecules (DNA, RNA, lipids and proteins). We embarked on this study to explore EVs as a liquid biopsy approach to detect tumor-derived DNA in the blood of metastatic colorectal cancer patients.
Methods: Informed consent was obtained from all the participants and the study was approved by a ethics committee.
EVs were isolated from plasma of metastatic colorectal cancer (mCRC) patients (n=5) and patients with no disease (n=10) using ultracentrifugation and size exclusion chromatography. EVs were further characterized using nanoparticle tracking analysis, transmission electron microscopy and western blots. EV-associated DNA (EV-DNA) was isolated using ethanol precipitation methods. We employed droplet digital PCR (ddPCR) to detect single nucleotide polymorphism in the KRAS genes. Data was processed using QuantaSoft v.1.6 (Bio-Rad). The tumour mutations were verified using next generation sequencing.
Results: The total EV-DNA of mCRC patients was significantly higher compared to healthy controls ($P < 0.005$). In addition, we successfully detected KRAS G12D mutant copies in the plasma of one of the five mCRC patients. Interestingly, the patient who was positive for mutant KRAS EV-DNA showed tumour allele frequencies (AF) of 60%, whereas the remaining four patient had AF below 20%. We further demonstrated that the majority of double stranded (ds) DNA was present on the surface of EVs by using DNAse I treatment and confocal microscopy.
Summary/Conclusion: We showed higher levels of total EV-DNA in all cancer patients compared to the control cohort. The data supports the use of EV-DNA measured by ddPCR to detect tumour-specific DNA in clinical samples.

PS06.05 | Exosomal miR-1246 promotes rhabdomyosarcoma tumor progression through paracrine signaling

Farah Ramadan[1]; Nader Hussein[2]; Farah Ghamloush[3]; Pascale Cohen[4]; Philippe Clezardin[4]; Bassam Badran[2]; Rita Khoueiry[5]; Zdenko Herceg[5]; Raya saab[3]; Sandra Ghayad[6]
[1]University of Lyon Claude Bernard, INSERM, UMR1033 LYOS, Lyon, France., Saida, Lebanon; [2]Department of Cancer Biology Stem Cells and Molecular Immunology, Faculty of Science I, Lebanese University, Hadath, Lebanon., Beirut, Lebanon; [3]Department of Pediatric and Adolescent Medicine, American University of Beirut, Beirut, Lebanon., Beirut, Lebanon; [4]University of Lyon Claude

Bernard, INSERM, UMR1033 LYOS, Lyon, France., Lyon, France; [5]Epigenetics Group, International Agency for Research on Cancer (IARC), 69372 Lyon Cedex 08, France., Lyon, France; [6]Aix-Marseille Université, C2VN (Centre de recherche en CardioVasculaire et Nutrition), Inserm 1263, INRAe 1260, Marseille, France., Marseille, France

Introduction: Rhabdomyosarcoma (RMS) is a pediatric soft-tissue sarcoma classified into two major histologic subtypes: alveolar (ARMS) and embryonal (ERMS). Exosomes are extracellular vesicles that carry and deliver protein and nucleic acid cargo capable of modulating recipient cell function and influencing cancer progression. Previously, we identified miR-1246 as a commonly enriched miRNAs within all RMS-derived exosomes based on microarray results. Cellular and exosomal miR-1246 have been implicated in promoting cancer metastasis and can therefore contribute to RMS progression.
Methods: We downregulated miR-1246 in RMS cells and subsequently isolated their corresponding exosomes. To explore the paracrine signaling induced after cellular miR-1246 knockdown we treated normal fibroblasts with these exosomes and investigated their proliferation, invasive and metastatic ability and by Hoechst staining and transwell migration and invasion assays.
Results: Consistent with our previous results, treatment with RMS-derived exosomes resulted in a significant increase in recipient fibroblasts proliferation, migration, and invasion. Knockdown of cellular miR-1246 in RMS cells and subsequent treatment of fibroblasts with the exosomes reversed these effects indicating a potentiating role of exosomal miR-1246 in promoting progression of RMS through modulating recipient cell functional behavior. Analysis of miR-1246 targets expression level revealed an upregulation of GSK3b and JARID2 following miR-1246 inhibition, indicating possible roles in promoting Wnt signaling pathway and epigenetic regulation of recipient cells.
Summary/Conclusion: Our results demonstrate pro-tumorigenic effects of miR-1246 in RMS cells that can be mediated through paracrine signaling via exosomes. miR-1246 is therefore a promising biomarker and possible therapeutic target in RMS that should be further studied.
Funding: This work was funded by a research grant from the Lebanese University, a research grant from PHC-Cedre and by a Ligue Contre le Cancer grant.

PS06.06 | Exosomal miRNA and mRNA signatures as biomarker for head and neck cancer (HNSCC)

Linda Hofmann[1]; Jasmin Ezic[1]; Simon Laban[1]; Patrick J. Schuler[1]; Beate Niesler[2]; Cornelia Brunner[1]; Thomas K. Hoffmann[1]; Marie-Nicole Theodoraki[1]
[1]Department of Otorhinolaryngology, Head and Neck Surgery, Ulm University Medical Center, Ulm, Germany, Ulm, Germany; [2]nCounter Core Facility, Institute of Human Genetics, University of Heidelberg, Heidelberg, Germany, Heidelberg, Germany

Introduction: Head and neck squamous cell carcinomas (HNSCC) are highly immunosuppressive. Exosomes are small extracellular vesicles that mediate intercellular communication. HNSCC patient's plasma is enriched in immunomodulatory exosomes which correlate with clinical parameters. We investigate the miRNA and mRNA signatures of plasma-derived exosomes as potential biomarkers for HNSCC.
Methods: Exosomes were isolated from plasma of 16 HNSCC patients and 12 healthy donors (HD) by ultrafiltration and differential (ultra)centrifugation (EV-Track ID 210344). Primary tumor cells were obtained from the same HNSCC patients. Total exosomal and tumor RNA was used for targeted profiling of 798 miRNAs and 730 mRNAs. Differential presence and discriminatory potential of exosomal RNAs between HNSCC and HD were analyzed by multiple Mann-Whitney test and unsupervised hierarchical clustering. Ingenuity pathway analysis was applied to predict downstream effects of miRNAs and identify related pathways.
Results: Of all detected exosomal RNAs, 55 % of miRNAs and 31 % of mRNAs were HNSCC-exclusive, while 8 % of miRNAs and 48 % of mRNAs were HD-exclusive. 91 miRNAs and 347 mRNAs were significantly differentially present between HNSCC and HD exosomes. Both exosomal RNA signatures could successfully assign samples to "Tumor" or "Healthy". 165 miRNAs and 146 mRNAs overlapped between corresponding tumor and exosomes and were considered as tumor-originating RNAs. These were filtered to 23 miRNAs inversely targeting 17 mRNAs, e.g. upregulated miR-421-3p targeting downregulated anti-tumor IL15. Identified core hubs of tumor-originating RNAs were related to TLR, NF-κB and IFN signaling.
Summary/Conclusion: Exosomal miRNA and mRNA signatures have high discriminatory potential between HNSCC patients and HD. Final RNA candidates are currently validated in an independent cohort. Identified RNAs were related to pathways of immune regulation, inflammatory response and cellular development which highlights their relevance for disease pathogenesis.
Funding: Deutsche Forschungsgemeinschaft (DFG) grant TH 2172/2–1

PS06.07 | Extracellular vesicles profiling for the identification of circulating prostate cancer biomarkers

Vera Mugoni[1]; Yari Ciani[2]; Federico Vannuccini[2]; Orsetta Quaini[2]; Simone Tomasini[2]; Angela Martinelli[2]; Antonello Veccia[3]; Vito Giuseppe D'Agostino[4]; Orazio Caffo[3]; Gerhardt Attard[5]; Dolores Di Vizio[6]; Caterina Nardella[2]; Francesca Demichelis[2]
[1]Department of Cellular, Computational and Integrative Biology - CIBIO; UNIVERSITY OF TRENTO, Trento (Italy), Italy; [2]Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, 38123 Trento, Italy, Trento, Italy; [3]Department of Medical Oncology, Santa Chiara Hospital, Trento, Italy, Trento, Italy; [4]Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy; [5]University College London Cancer Institute, London, United Kingdom, London, United Kingdom; [6]Department of Surgery, Division of Cancer Biology and Therapeutics, Cedars-Sinai Medical Center, Los Angeles, CA, USA, Los Angeles, USA

Introduction: A growing body of evidence supports the hypothesis that extracellular vesicles (EVs) and their associated RNAs (EV-RNAs) represent informative circulating material for liquid biopsies in prostate cancer (PCa) patients. However, details on the EVs populations specifically released by PCa into the blood, as well as on the PCa-related EVs-associated transcripts have not been fully elucidated yet.
Methods: We evaluated the detection of PCa biomarkers in EVs samples isolated through three methodological approaches. Specifically, EVs were isolated from cancer cell lines as well as from the plasma of healthy donors and PCa patients (from the PRIME consortium study) by using ultracentrifugation, size exclusion chromatography, and a charge–based isolation method. We profiled the EVs by nanoparticle tracking analysis technology, imaging flow cytometry, and western blotting analysis to characterize the heterogeneity of their size distribution and the expression of protein markers. Finally, we performed RNA-Seq to investigate whether the detection of PCa-specific EV-RNAs was dependent on the EV isolation method.
Results: By imaging flow cytometry and western blotting analysis, we observed similar EV subpopulations and PCa biomarkers (i.e. AR and PSMA) on EVs isolated from both cell lines and plasma. Furthermore, for all sequenced EV-RNAs we identified fragments of coding transcripts (i.e. mRNA) as well as of non-coding transcripts (including miRNAs, lncRNA and a variety of miscellaneous small RNA) with comparable composition of RNA species. Furthermore, by ddPCR we were able to detect PCa-associated transcripts (such as MYC) on EV-RNAs derived from PRIME PCa patients.
Summary/Conclusion: The evaluated EVs isolation methods represent valid approaches to investigate PCa biomarkers towards the exploitation of EV-RNA in liquid biopsies.
Funding: Accelerator Award 2018 (AIRC; ID 22792, CRUK; ID A26822); Fondazione Caritro; Prostate Cancer SPORE grant P50 CA211024-01A1.

PS06.08 | Investigation of cancer-specific mutations in DNA isolated from human melanoma tissue-derived extracellular vesicles

Rossella Crescitelli[1]; Stefan Filges[2]; Nasibeh Karimi[3]; Tamara Alonso Agudo[1]; Anders Ståhlberg[2]; Jan Lötvall[3]; Cecilia Lässer[3]; Roger Olofsson Bagge[1]
[1]Sahlgrenska Center for Cancer Research and Wallenberg Centre for Molecular and Translational Medicine, Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, Gothenburg, Sweden; [2]Sahlgrenska Center for Cancer Research and Wallenberg Centre for Molecular and Translational Medicine, Department of Laboratory Medicine, Institute of Biomedicine, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden, Gothenburg, Sweden; [3]Krefting Research Centre, Institute of Medicine at Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, Gothenburg, Sweden

Introduction: Liquid biopsies in patients with cancer have recently emerged as a tool for early detection and residual disease monitoring. The most studied source has been total circulating DNA where cancer-specific mutations or profiles have been analyzed. However, extracellular vesicles (EVs) containing tumor-derived materials could potentially be used as source of cancer-specific DNA. The aim of this study was to investigate and compare DNA from human melanoma-derived EVs and total circulating plasma DNA.
Methods: EVs were isolated from human melanoma metastatic tissues (n=6) using enzymatic treatment followed by ultracentrifugation. Both large and small EVs were pooled and bottom loaded on a density cushion. Tissue-derived EVs were characterized by transmission electron microscopy (TEM) and EV markers analysis using ExoView™ and Western blot. Using the ultra-sensitive SiMSen-seq technique and a panel of 34 melanoma-related genes, DNA from the patients' tumors as well as DNA from corresponding tumor-derived EVs and patient plasma was isolated and investigated.

Results: TEM pictures showed presence of round elements with typical EV size (80-200 nm). Western blot and ExoView™ analysis confirmed the presence of CD63, CD9, CD81, Flotillin-1 as well as ADAM10 and Mitofilin. Moreover, a total of six mutations were identified (in BRAF, NRAS, CDKN2A, STK19, PPP6C and RAC genes) and one or more were detectable in all tumors, tumor-derived EVs and patient plasma. Interestingly, the allele mutation frequency was higher in the tumor-derived EVs compared to both tumors and plasma (tumor mean: 34.9%, tumour-derived EVs mean: 39.9% and plasma mean: 6.7%).
Summary/Conclusion: The allele mutation frequency was higher when analyzing tissue-derived EVs compared to total circulating plasma DNA, suggesting a potential role of EVs as future biomarkers in melanoma.

PS06.09 | Multi-omic approach for studying molecular components of serum-derived sEV and tumor tissue of rectal cancer patients with different response to neoadjuvant radiotherapy

Urszula Strybel[1]; Lukasz Marczak[1]; Marcin Zeman[2]; Mykola Chekan[3]; Ewa Zembala-Nozynska[4]; Łukasz Mielańczyk[5]; Monika Pietrowska[2]; Anna Wojakowska[1]
[1]Institute of Bioorganic Chemistry Polish Academy of Sciences, Poznan, Poland; [2]Maria Sklodowska-Curie National Research Institute of Oncology, Gliwice Branch, Gliwice, Poland; [3]Maria Sklodowska-Curie National Research Institute of Oncology, Gliwice Branch, Gliwice, Poland;, Gliwice, Poland; [4]Maria Sklodowska-Curie National Research Institute of Oncology, Gliwice Branch, Gliwice, Poland, Gliwice, Poland; [5]3 Department of Histology and Cell Pathology, Faculty of Medical Sciences in Zabrze, Medical University of Silesia, Katowice, Poland, Katowice, Poland

Introduction: Molecular composition of small extracellular vesicles (sEV) could be used as biomarkers for the prediction and monitoring of response to radiotherapy, yet knowledge about proteomic and metabolomic components of such vesicles is very limited in locally advanced rectal cancer. Here we applied a combined proteomic and metabolomic MS-based approach for studding molecular components of serum-derived sEV and tumor tissue to reveal pathways connected with different response to neoadjuvant radiotherapy (neo-RT) in rectal cancer patients.
Methods: Tissue and serum samples were collected from 20 rectal cancer patients treated with neo-RT (study approved by the local Ethics Committee; approval no. KB/430-50/12). Samples were divided into two groups, depending on their TRG scale and according to the percentage of cancer cells in resected tumor specimens. sEV were isolated from serum using SEC and characterized according MISEV2018 criteria. LC-MS/MS-based approach was used for proteomic profiling, while metabolites were analyzed by the GC-MS.
Results: Proteomic approach allowed identification of nearly 4,000 proteins in rectal tumor and 300 in serum-derived sEV, 233 of which were common. We found a few differentially expressed proteins common for sEV and tumor, which could be potential candidates for rectal cancer-specific molecules present on tumor-derived EVs. GC–MS-based approach allowed the identification of 160 and 50 metabolites in tumor tissue and sEV, respectively. Multi-omic profiling showed common pathways connected with response to neo-RT in rectal cancer, including complement and coagulation cascade, platelet activation, immune response and energy metabolism.
Summary/Conclusion: This study revealed a specific pattern of proteins and metabolites in serum-derived sEV and tumor tissue, which could discriminate rectal cancer patients with different response to neo-RT.
Funding: This study was supported by the NCN, Poland, Grant 2017/26/D/NZ2/00964 (for US, LM, MP, AW).

PS06.10 | PD-L1-positive exosome-derived microRNAs in human hepatocellular carcinoma

Hagyeong Lee[1]; Yu Rim Lee[2]; Gyeonghwa Kim[1]; Se Young Jang[2]; Min Kyu Kang[3]; Jung Gil Park[3]; Hye Won Lee[4]; Ji-Min Kim[5]; Won Young Tak[2]; Soo Young Park[2]; Keun Hur[1]
[1]Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Daegu, Republic of Korea; [2]Department of Internal Medicine, Kyungpook National University Hospital, Daegu, Republic of Korea; [3]Department of Internal Medicine, Yeungnam University Medical Center, Daegu, Republic of Korea; [4]Department of Pathology, Dongsan Medical Center, School of Medicine, Keimyung University, Daegu, Republic of Korea; [5]Division of Rheumatology, Department of Internal Medicine, Keimyung University Dongsan Medical Center, Keimyung University College of Medicine, Daegu, Republic of Korea

Introduction: Hepatocellular carcinoma (HCC) is one of the most aggressive cancer and the third leading cause of cancer-related death worldwide. Recently, PD-1 and PD-L1 inhibitors, monoclonal antibodies that target either PD-1 or PD-L1, have been widely used in patients with advanced stage HCC. However, response rate of the PD-1/PD-L1 inhibitors is not certain in overall patients, which limits the application in clinical practice. Cancer cell derived exosomes have been evaluated as biomarkers for diagnosis

and prognosis in cancers. The aim of this study was to investigate exosomes from high PD-L1-expressing cancer cells and their containing microRNAs (miRNAs) in HCC.

Methods: Five HCC cell lines (HepG2, Hep3B, Huh7, SK-Hep-1, and SNU-398) and serum samples from HCC patients were analyzed. Exosomes were isolated from cell culture medium and serum samples using ExoQuick, and it was confirmed by expression of exosome markers (CD9, CD63, and ALIX) based on immunoblotting. PD-L1 protein expression was determined by western blot analysis using anti-PD-L1 antibody. Expression of PD-L1 mRNA and miRNAs were determined by quantitative real-time PCR (qRT-PCR).

Results: We found strong PD-L1 expressing cell lines group (Hep3B, SK-Hep-1, and SNU-398) and weak PD-L1 expressing cell lines group (HepG2 and Huh7). The two cell lines (SK-Hep-1 and HepG2) displayed consistent PD-L1 expression status in mRNA as well as protein. In further analysis of PD-L1 related miRNAs, 3 exosomal-miRNAs (miR-15a, -16, and -203) were positively correlated and 2 exosomal-miRNAs (miR-21 and -34a) were negatively correlated with PD-L1 expression. We also found significant correlations between PD-L1 expressing exosome numbers and clinicopathological features in serum samples of HCC patients.

Summary/Conclusion: We provided the evidence for the potential of exosomal-miRNAs as novel non-invasive circulating markers for prediction of treatment efficacy of anti-PD-1/anti-PD-L1 therapy in HCC patients.

PS06.11 | Profiling of exosomal lncRNAs from colorectal cancer patients by RNA sequencing

Marie Madrzyk[1]; Tina Catela Ivković[2]; Táňa Macháčková[1]; Karolína Trachtová[1]; Jan Kotouček[3]; Josef Mašek[3]; Milana Šachlová[4]; Tomáš Grolich[5]; Zdeněk Kala[5]; Ondřej Slabý[1]

[1]Central European Institute of Technology (CEITEC), Masaryk University, Brno, Czech Republic; [2]Department of Laboratory Medicine, Lund University, Lund, Sweden; [3]Department of Pharmacology and Toxicology, Veterinary Research Institute, Brno, Czech Republic; [4]Department of Gastroenterology, Masaryk Memorial Cancer Institute and Faculty of Medicine, Masaryk University, Brno, Czech Republic; [5]Department of Surgery, Faculty Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic

Introduction: Colorectal cancer (CRC) is the third most common cause of cancer-related deaths despite advancements in the disease detection. Late diagnosis is associated with poor prognosis therefore an early-stage CRC biomarkers are acutely needed. As a class of potential biomarkers, exosomal lncRNAs are often tissues specific and can serve as signalling molecules in intercellular communication. We aimed to optimise the RNAseq method of exosomal lncRNAs from the serum of CRC patients.

Methods: Exosomes were separated by size exclusion chromatography from 150 μl of serum of CRC patients and healthy donors. The particles were characterised by electron microscopy, dynamic light scattering and by vesicle-specific content analysis. Protein markers were detected by western blot. After RNA isolation, the samples were converted into libraries using NEBNext Ultra II Directional RNA Library Prep Kit (NEW England Biolabs). Libraries were pooled and sequenced on NextSeq 550 sequencer (Illumina).

Results: We successfully separated exosomes and created libraries from all samples despite very low volume of starting material. Sequencing results comparing exosomal RNA samples from CRC patients (n=20) and healthy volunteers (n=6) revealed significantly altered levels of protein coding and non-coding RNAs including 37 lncRNAs (p-value< 0.01, fold change>2). The analysis showed dysregulated levels of lncRNAs associated with CRC such as SNHG1 but also genes that were not previously reported (eg. FAM201A and AC105389.2). Moreover, the gene set enrichment analysis revealed significantly enriched gene sets (p-value < 0.001) linked to MYC targets or the G2/M checkpoint regulation.

Summary/Conclusion: Initial sequencing results suggest that lncRNAs have a great potential as biomarkers for non-invasive CRC detection, however it is necessary to validate lncRNAs of interest on the larger patient cohort. Also, some functional follow-up experiments are needed to determine their biological role in CRC pathogenesis.

Funding: This work was supported from the projects GA20-18889S, NU20-03-00127, BBMRI-CZ no. LM2018125, CZ.02.1.01/0.0/0.0/15_003/0000495, and MZE-RO0518.

PS06.15 | Development of exosome-based plasma RNA biomarkers for high-risk prostate cancer

Sudipto K. Chakrabortty[1]; Benjamin Spieler[2]; Yevgenia L. Khodor[1]; Christian Fischer[1]; Radka Stoyanova[2]; Matthew Abramowitz[2]; Sanoi Punnen[2]; Seth Yu[1]; Alan Pollack[2]; Alan Dal Pra[2]; Sandra Gaston[2]; Johan Skog[3]; Kailey Babcock[1]

[1]Exosome Diagnostics, a Bio-Techne brand, Waltham, USA; [2]University of Miami, Miami, USA; [3]Exosome Diagnostics, a Bio-techne brand, Waltham, MA, Waltham, USA

Introduction: Liquid biopsies using exosomes are emerging as clinically effective platforms for minimally invasive, highly sensitive diagnostics. There is an unmet need for liquid biopsy tests that can better stratify prostate cancer without the sampling errors inherent in tissue biopsy. In this study, we develop exosome-based liquid biopsy tests for use in managing patients with tissue biopsy-confirmed, NCCN low and high-risk prostate cancer.
Methods: Our pilot biomarker discovery study compared plasma exosomal RNA profiles from 11 high-risk and 9 low-risk, treatment-naïve, biopsy-confirmed patients with 4 healthy controls. The high-risk patients, as defined by NCCN guidelines, are enrolled in a radiotherapy trial (BLaStM NCT02307058) and the low-risk patients in an active surveillance trial (MAST NCT02242773). Exosomes were isolated from 1mL plasma, with RNA prepared using the ExosomeDx isolation platform ExoLution (PMID: 29051321). A hybrid-capture-based Next Generation RNA Sequencing (RNAseq) analysis was then performed to profile and identify differentially expressed genes.
Results: We detected over 10,000 protein coding genes and ~400 lncRNAs in plasma samples. 273 genes were differentially expressed between high-risk vs control but not in low-risk vs control. We identified four potential plasma RNA biomarkers of high-risk prostate cancer. These include three mRNAs with >20-fold lower expression in plasma from high-risk in comparison to low-risk patients. TCGA data show two of these mRNA markers are downregulated in prostate cancer tissue in patients with worse survival, confirming the role of the identified markers in aggressive prostate cancer. In addition, one lncRNA was identified that showed >20-fold higher expression in plasma from high-risk in comparison to low-risk patients.
Summary/Conclusion: Plasma exosomal long RNA provides a rich reservoir of currently untapped biomarkers for high-risk prostate cancer. Investigation in larger patient cohorts is warranted.

PS07: Physiology and pathology: EVs from microorganisms

Chair: Mariola J. Edelmann – University of Florida

Chair: Nicole Kruh-Garcia – Colorado State University

PS07.01 | Antigen discovery in circulating extracellular vesicles from Plasmodium vivax patients

Iris Aparici Herraiz[1]; Melisa Gualdrón López[2]; Carlos Castro-Cavadia[3]; Jaime Carmona-Fonseca[3]; María Fernanda Yasnot[4]; Carmen Fernandez Becerra[1]; Hernando A. del Portillo[5]
[1]ISGlobal IGTP, Barcelona, Spain; [2]ISGlobal, Hospital Clínic - Universitat de Barcelona, Barcelona, Spain, Barcelona, Spain; [3]Grupo de Salud y Comunidad Cesar Uribe Piedrahíta, Universidad de Antioquia, Medellín, Colombia, Medellín, Colombia; [4]Grupo de Investigaciones Microbiológicas y Biomédicas de Córdoba (GIMBIC), Universidad de Córdoba, Monteria, Colombia, Monteria, Colombia; [5]ISGlobal IGTP ICREA, Barcelona, Spain

Introduction: Plasmodium vivax is the most widely distributed human malaria parasite with 7 million annual clinical cases and 2.5 billion people living under risk of infection. There is an urgent need to discover new antigens for vaccination as only two vaccine candidates are currently in clinical trials. Extracellular vesicles (EVs) are small membrane-bound vesicles involved in intercellular communication and initially described in reticulocytes, the host cell of P. vivax, as a selective disposal mechanism of the transferrin receptor (CD71) in the maturation of reticulocytes to erythrocytes. We have recently reported the proteomics identification of P. vivax proteins associated to circulating EVs in P. vivax patients using size exclusion chromatography (SEC) followed by mass spectrometry (MS). Parasite proteins were detected in only 2 out of 10 patients.
Methods: We have implemented the direct immuno-affinity capture (DIC) technique to enrich in EVs derived from CD71-expressing cells.
Results: Parasite proteins were identified in all patients totaling 48 proteins and including several previously identified P. vivax vaccine candidate antigens (MSP1, MSP3, MSP7, MSP9, Serine-repeat antigen 1, and HSP70) as well as membrane, cytosolic and exported proteins. Notably, a member of the Plasmodium helical interspersed sub-telomeric (PHIST-c) family and a member of the Plasmodium exported proteins, were detected in 5 out 6 analyzed patients. Humoral immune response analysis using sera from vivax patients confirmed the antigenicity of the PHIST-c protein.
Summary/Conclusion: Enrichment of EVs by CD71-DIC from plasma of patients, allows a robust identification of P. vivax immunogenic proteins. This study represents a significant advance in identifying new antigens for vaccination against this human malaria parasite.
Funding: IAH is a predoctoral fellow supported by the Ministerio de Economia y Competitividad (FPI BES-2017081657). This project received support from Ministerio Español de Economía y Competitividad (SAF2016-80655-R).

PS07.02 | Bacterial extracellular vesicles from Porphyromonas gingivalis – isolation and characterization

Helene Rygvold Haugsten[1]; Hilde Kanli Galtung[2]; Trude Marie Haug[3]; Tine Merete Søland[3]; Morten Enersen[3]; Anne Karin Kristoffersen[3]; Hans Christian Dalsbotten Aass[4]

[1]University of Oslo, Institute of Oral Biology, Oslo, Norway; [2]Institute of Oral Biology, University of Oslo, Oslo, Norway; [3]University of Oslo, Oslo, Norway; [4]Oslo University Hospital, Oslo, Norway

Introduction: Periodontal disease (PD) is an inflammatory condition caused by bacteria that may lead to tooth loss by damaging the supporting tissues of the teeth. Here, fibroblasts are among the predominant cells. PD represents a serious health problem worldwide and may predispose to systemic diseases, such as Alzheimer's and cardiovascular disease. Porphyromonas gingivalis (P. gingivalis), a keystone pathogen in PD, releases bacterial extracellular vesicles (BEVs) that may contain virulence factors such as gingipains and play a role in the pathogenesis of PD.

This study aimed to isolate BEVs from three strains of P. gingivalis (clinical and laboratory strains), to characterize the BEVs and investigate BEV uptake into human oral fibroblasts.

Methods: BEVs from P. gingivalis were isolated through ultrafiltration and size-exclusion chromatography. To measure concentration and size distribution, we used Nano-Tracking Analysis.

Transmission electron microscopy was used to visualize the vesicles and flow cytometry was used to measure antibody captured gingipain.

Finally, fibroblast uptake of PKH67 labelled BEVs was visualized with confocal microscopy.

Results: We isolated BEVs using ultrafiltration and size-exclusion chromatography. Nano-Tracking Analysis showed concentrations of BEVs around 10^8 particles/ml and a mean diameter of 164 nm. TEM pictures showed vesicle-like structures and using flow cytometry we successfully demonstrated gingipain-labelled BEVs. Oral fibroblasts incubated with labelled BEVs from P. gingivalis displayed cytosolic labelling consistent with BEV internalization.

Summary/Conclusion: We managed to isolate and characterize BEVs from P. gingivalis. Additionally, we demonstrate uptake of BEVs in human oral fibroblasts. Further studies could contribute to a better understanding of the role of P. gingivalis and its BEVs in the pathogenesis of periodontal diseases and related systemic diseases.

PS07.03 | Characterization and proteomic analysis of EVs from dogs with canine leishmaniasis

Sofia S. Esteves[1]; Clara Lima[2]; Inês Costa[2]; Carmen Fernandez Becerra[3]; Nuno Santarém[2]; Anabela Cordeiro-da-Silva[2]

[1]Parasite Disease Group, Institute for Molecular and Cell Biology, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal & Serviço de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia da Universidade do Porto, Porto, Portugal; [2]Parasite Disease Group, Institute for Molecular and Cell Biology, Instituto de Investigação e Inovação em Saúde, Universidade do Porto & Serviço de Microbiologia, Departamento de Ciências Biológicas, Faculdade de Farmácia da Universidade do Porto, Portugal, Porto, Portugal; [3]ISGlobal IGTP, Barcelona, Spain

Introduction: Canine leishmaniasis (CanL), caused by Leishmania infantum parasites, is a disease with high mortality and morbidity. In endemic areas, dogs are considered the main reservoir, allowing the life cycle perpetuation associated with infected vectors. CanL is a risk factor for human zoonotic leishmaniasis, therefore CanL control is essential. The management of CanL requires new tools and biomarkers to address complex epidemiological scenarios. Due to their characteristics, EVs are a resource with great potential that might provide an alternative to conventional biomarkers.

Methods: EVs were separated from the plasma of healthy (n=8) and CanL (n=20) dogs by SEC. Fractions were characterized by protein quantification and bead-based flow cytometry assay, using EVs markers, such as CD5L and CD71. Selected fractions were submitted to proteomic analysis by LC-MS/MS.

Results: Proteomic analysis allowed the consistent identification of EVs markers. The number of identifications in healthy dogs was significantly higher ($p < 0.0001$) than in CanL dogs. To increase proteome robustness, a comparative analysis was performed using the merged data from each group. This originated a core proteome of 1786 proteins: 44% were detected in both groups, 37% were only detected in healthy dogs and 19% were only identified in CanL animals. Protein network visualization models and pathway analysis revealed a distinct profile between the 2 groups. Reactome pathways associated with central carbon metabolism; signaling by the B Cell Receptor; cytokine signaling in immune system and antigen processing - ubiquitination & proteasome degradation were enriched in healthy animals. No specific pathway enrichment was associated with CanL dogs.

Summary/Conclusion: EVs' characterization in CanL enabled the identification of distinct metabolic pathways that will contribute to a better understanding of disease pathophysiology and the discovery of disease-related biomarkers.

Funding: This work was financed by FCT in the framework of the project PTDC/CVT-CVT/6798/2020. FCT funded SE, IC and CL through SFRH/BD/140119/2018, SFRH/BD/140177/2018 and 2020.07306.BD, respectively. This study was approved by AWB on March 2021 (Despacho n° 2880/2015).

PS07.04 | Characterization of bacterial extracellular vesicles from a fish pathogen, their role in virulence and possible function as vaccine in aquaculture

Verena Mertes[1]; Athanasios Saragliadis[2]; Fabio Attenucci[3]; Agnieszka Wrobel[1]; Elia Ciani[4]; Dirk Linke[2]; Duncan Colquhoun[5]; Hanne Winther-Larsen[1]

[1]Department of Pharmacy, University of Oslo, Oslo, Norway, Oslo, Norway; [2]Department of Biosciences, University of Oslo, Oslo, Norway, Oslo, Norway; [3]Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark, Copenhagen, Denmark; [4]Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway, Ås, Norway; [5]Fish Health Research Group, Norwegian Veterinary Institute, Ås, Norway, Ås, Norway

Introduction: The Gram-negative bacterium Yersinia ruckeri is spread in water columns across the globe and is a threat for fish welfare as well as playing a negative financial factor for the fish farming industry. Infection with the pathogenic Y. ruckeri is causing the enteric redmouth disease (ERM), also called yersinosis and is mainly found in salmonids. Relatively little is known about the molecular and pathogenic mechanism of this bacterium.
The aim of the project is the further investigation of Y. ruckeri pathogenesis, where we will mainly focus on characterization of secreted extracellular vesicles (EV).
The secreted amount of bacterial EVs varies between bacteria species and they are shown to contain various components (protein, DNA, RNA, LPS, lipids) which resemble the mother bacterium from which they derive. Additionally, isolated EVs have been reported as an alternative vaccine candidate as their injection has been shown to elicit a protective immunity in a host.
Methods: For bacterial EVs to be economically beneficial as an immunogenic agent for aqua-cultural purposes, an applicable amount must be produced. In this project, knockout strains were generated, lacking a membrane protein. EVs were isolated from 2L of over-night culture, using tangential flow and ultra centrifugation.
Results: Mutant and wild type derived EVs were characterized by both electron microscopy and proteomics analysis. The isolated EVs will further be studied for their function in cellular uptake and cytotoxicity will be investigated in cell-based assays and initial results will be presented.
Summary/Conclusion: In the future, zebrafish (Danio rerio) will be used as infection and vaccine model where the fish will be immunized with isolated Y. ruckeri EVs. Injection of EVs may enable the host to evolve an immunity against the pathogen, resulting in a vaccinated state.

PS07.05 | Differential expression of gp82 and gp90 in EVs isolated from metacyclic trypomastigotes to distinct strains of T. cruzi

Paula Meneghetti[1]; João Paulo Ferreira Rodrigues[2]; Nobuko Yoshida[3]; Ana Claudia Torrecilhas[4]

[1]UNIFESP, Sao Paulo, Brazil; [2]UNIFESP, São Paulo, Brazil; [3]UNIFESP, SAo Paulo, Brazil; [4]Federal University of Sao Paulo, Sao Paulo, Brazil

Introduction: Trypanosoma cruzi, the etiologic agent of Chagas disease. The parasite spontaneously releases EVs into the culture medium. In previous studies of our group, we showed the importance of EVs release from parasite to communication between vertebrate and invertebrate host. The aim of our research is to characterize the EVs released from metacyclic trypomastigotes to distinct T. cruzi strains (Y, G and CL).
Methods: The EVs isolation and purification from metacyclic trypomastigotes (MT) of T.cruzi (Y,G and CL strains). We characterized size and concentration of the EVs from metacyclic trypomastigotes by NTA. We performed Immunoblotting and CL-ELISA used EVs isolated from MT to detected the expression of gp82, gp90, TS, alpha-GAL and total parasite membrane. In parallel, we performed functional assay (invasion assays) and NO production from monocytes supernatants.
Results: We observed the expression of gp82 only in extracts of parasites and EVs from G and CL strains. The EVs isolated from MT forms of CL strain have a higher number of particle concentration than EVs from Y and G strains. CL-ELISA showed that EVs from Y strain (TM) express more glycoconjugates, mucins and TS than G and CL strains. In the Functional assays, we showed that EVs isolated from Y, G and CL increase the number of intracellular parasites and the induction of NO production in human monocytes cultures.

Summary/Conclusion: The parasite development the mechanisms with host interaction and contribute to a better understanding of the diseases cause. In addition to opening perspectives for the identification of potential therapeutic targets in future research in Chagas Disease

Funding: FAPESP2020/07870-4 and CNPq Universal 408186/2018-6

PS07.06 | EVs populations from T. cruzi trypomastigotes are incorporated by human monocyte cells and induces differentiation into macrophages

Ana Claudia Torrecilhas[1]; Nicholy Lozano[2]; Gabrieli Camossa[2]; Milena Santana Costa[3]; Maiara Saboia Silva[2]; Or-yam Shoshana-Revach[4]; Ziv Porat[4]; Neta Regev-Rudzki[5]; Sergio Schenkman[2]

[1]Federal University of Sao Paulo, Sao Paulo, Brazil; [2]UNIFESP, Sao Paulo, Brazil; [3]UNIFESP, SAO PAULO, Brazil; [4]WIS, Rehovot, Israel; [5]Weizmann Institute of Science, Rehovot, Israel

Introduction: In our previous work, we have shown that vesicles released by infective forms of Trypanosoma cruzi, the agent of Chagas' disease, modulate the inflammatory response of macrophages, increase invasion of parasites, which could explain increased inflammation and the progression of disease in experimental models, and eventually in the progression of human Chagas' disease. We also found that this process varies among different parasite isolates that produce different patterns of infection. The EVs shedding from parasites are heterogeneous and contain glyconjugates that interact with host cells. To gain more information about the role of these different populations, we now characterized the populations released by trypomastigotes forms of (Y strain) and evaluate their interaction with THP-1 human monocytes into macrophages.

Methods: he isolated populations of EVs were labeled with RNA (TO) and DNA marker (Hoechst dye) and incubated with host human monocytes cells in RPMI 10% FBS and from 5 to 60 minutes by using an Image steam apparatus. The cells were further maintained for 24 to 48 hours and observed by Scanning Electronic microscopy to observe changes in the morphology and by Confocal microscopy after immunofluorescence labeling with markers of cell differentiation.

Results: Both populations of EVs released by T. cruzi could be labeled by RNA, but not by DNA markers. When incubated 10 minutes with cells, the RNA labeling was incorporated in 60% of the monocytes reaching maximal labeling withing 1 hour. The incorporation was higher when using EVs of larger sizes. The cells then start to undergo morphological changes, and after 24 hours became sprayed, with as typical macrophages. To confirm the transformation into macrophages, the cells were labeled with anti-actin and anti-vinculin, both expressed in differentiated macrophages.

Summary/Conclusion: All EVs of T. cruzi contains RNA that are rapidly transferred to monocytes. In parallel, these EVs can induce monocyte in vitro differentiation of a monocyte cell line to macrophages. Experiments are in progress to identify the components and the role of RNA present in the EVs involved in the differentiation process.

Funding: FAPESP2020/07870-4; FAPESP2019/15909-0 and CNPq Universal 408186/2018-6

PS07.07 | Extracellular vesicles from the liver flukes Fasciola hepatica and Dicrocoelium dendriticum trigger different responses in hepatic stellate cells

Christian M. Sánchez-López[1]; Aránzazu González-Arce[2]; Victor Ramírez-Toledo[3]; María Trelis[1]; Dolores Bernal[2]; ANTONIO MARCILLA[4]

[1]Universitat de Valencia, BURJASSOT, Spain; [2]UNIVERSITAT DE VALENCIA, BURJASSOT, Spain; [3]Centro de Salud Pública Manises, GVA, Manises, Spain; [4]Universitat de Valencia, Burjassot, Spain

Introduction: Dicrocoelium dendriticum and Fasciola hepatica are two worldwide-distributed trematodes than can coexist in the same host, complicating their specific diagnosis. They can infect the liver and bile ducts of many mammalian species, causing liver damage associated with parasite migration, the activity of fluke secreted products, and inflammation. The manipulation of the host response is mostly due to the release of molecules at the host-parasite interface (excretory/secretory product (ESP)), which include extracellular vesicles (EVs). Although there are a few reports on ESP interactions with liver cells, the effects elicited by EVs have not been described.

Methods: Parasites were obtained from local abbatoirs (Valencia, Spain) and cultured to obtain ESP. EVs were isolated by differential centrifugation and size-exclusion chromatography (SEC), and characterized by NTA, TEM and immunogold labeling for EV proteins (EVtrack ID EV220006). EVs were added to the LX-2 human hepatic stellate cell line (SCC064, Merck), cell pellets were obtained and analyzed through Label-free quantitative proteomics and Western Blotting. MTT assays were performed to assess EV cytotoxicity. All assays were performed using FBS-free culture media.

Results: EVs from F. hepatica (FhEVs) and D. dendriticum (DdEVs) were successfully isolated and characterized. 63 proteins were significantly up-regulated in LX-2 HSC cells upon exposure to DdEVs, including proteins involved in the ROS response and

NF-$\kappa\beta$ signaling, and 8 proteins were down-regulated. In cells treated with FhEVs, 14 proteins were up-regulated and 9 down-regulated, including fibrinogenic proteins involved in extracellular matrix remodeling. No common proteins were found up- or down-regulated in both conditions.

Summary/Conclusion: Our findings confirm the different modulatory effects exerted by EVs from F. hepatica and D. dendriticum on HSCs. FhEVs induce the production of fibrinogenic proteins by LX-2 HSC cells, suggesting their possible role in associated fibrosis, whereas DdEVs activate pathways related to inflammation, oxidative stress and apoptosis. These differences could correspond to differences in the migration of each fluke.

Funding: Supported by the Agencia Estatal de Investigación, Ministerio de Ciencia e Innovación, Spain (Grant PID2019-105713GB-I00/AEI /10.13039/501100011033), and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (Grant PROMETEO/2020/071). C.M.S-L is the recipient of a predoctoral fellowship (PRE2020-092458) funded by AEI /10.13039/501100011033. A. G-A is the recipient of a predoctoral fellowship (CPI-20-353) funded by Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (PROMETEO/2020/071). The research group is part of "Red Traslacional para la Aplicación Clínica de Vesículas Extracelulares, Tentacles", (RED2018-102411-T, Agencia Estatal de Investigación, Spain).

PS07.08 | Extracellular vesicles of Bacillus cereus: First insights into their potential role in pathogenicity

Astrid Digruber[1]; Markus Kranzler[1]; Tanja Buchacher[2]; Birgit Strobl[3]; Monika Ehling-Schulz[1]
[1]Institute of Microbiology, Department of Pathobiology, VetMed Uni Vienna, Vienna, Austria; [2]Turku Centre for Biotechnology, University of Turku and Åbo University, Turku, Finland; [3]Institute of Animal Breeding and Genetics, Unit of Molecular Genetics, VetMed Uni Vienna, Vienna, Austria

Introduction: Bacillus cereus is a Gram-positive spore forming bacterial pathogen, which is well-known for its food poisoning potential [1]. In recent years, this opportunistic pathogen is gaining increasing importance as a causative agent of non-GI related diseases, including systemic as well as local infections [2]. With our current work, we aim to gain the first insights into the role of extracellular vesicles (EVs) in pathogenicity of B. cereus.

Methods: In contrast to EVs of Gram-negative bacteria, EVs in Gram-positive bacteria are far less studied and their role in bacterial physiology and pathogenicity is largely unknown. Therefore B. cereus derived EVs were thoroughly analyzed by using multiple approaches: First EVs were isolated using a size-exclusion approach, which was further simplified to an ultracentrifugation only isolation protocol. The EV morphology was investigated using resin-embedded TEM, and NTA was used to enumerate the EVs and to estimate the sizes. To analyze the EV content a combinatory approach of proteomics and FTIR spectral fingerprinting was used. To assess the biological activity of the B. cereus EVs, in-vitro cell culture models using Caco2-Cells and bone-marrow-derived macrophages were used.

Results: Within our work, we could show that the morphology of B. cereus derived EVs is comparable to EVs secreted by other gram-positive bacteria. However, the EV secretion rate and FTIR spectral fingerprints varies significantly among different strains, although the EV sizes were very similar. Furthermore, we could detect cytotoxic effects in a dose and time-dependent manner in our in-vitro cell culture models.

Summary/Conclusion: In this study, we show for the first time that B. cereus secretes biologically active EVs in a dynamic manner. This work is expected to pave the way to decipher EV contribution to B. cereus pathogenicity and aid the development of novel strategies to combat pathogenic B. cereus strains.

PS07.09 | Fungal extracellular particles mediate adaptation to environmental stress

Teja Lavrin[1]; Samo Hudoklin[2]; Peter Veranič Veranič[2]; Ana Plemenitaš[1]; Metka Lenassi[3]
[1]Institute of Biochemistry and Molecular Genetics, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, Ljubljana, Slovenia; [2]Institute of Cell Biology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, Ljubljana, Slovenia; [3]Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia, Ljubljana, Slovenia

Introduction: Fungal extracellular vesicles (EVs) are important for intercellular communication in pathogenicity, but their role in response to environmental stress is lacking. Here we aimed to isolate, characterize and uncover the role of extracellular particles (EPs) released from Hortaea werneckii, an extremotolerant fungus known to adapt to high osmolarity conditions.

Methods: Hortaea was cultured in defined media with or w/o melanin biosynthesis inhibitor and 3M NaCl. EPs were isolated from conditioned media by sequential centrifugation followed by separation on sucrose density gradient or size exclusion chromatography. EPs morphology was characterized by TEM, concentration and size by Nanoparticle tracking analysis and molecular

content by immunoblotting and spectrophotometry. The role of EPs was evaluated by in vitro functional assay in different conditions. (EV-TRACK ID: EV210293)

Results: We successfully optimized isolation of EPs from non-stressed Hortaea cultures. TEM micrographs showed heterogeneous nature of EPs, which also included EVs with cupshaped morphology. Hortaea EPs had the average (±SE) mode diameter of 97(±3.7) nm and concentration of 1.7(±0.041) ×10^9 particles per mL of media. They carried typical EVs marker proteins α-tubulin and GAPDH, as shown by immunoblotting, but additionally packed Hog1, the main kinase in osmotic stress response. Further separation of EPs (density or size) showed heterogeneous population that differed depending on the external stimulus: depleted media, high osmolarity and/or absence of melanin. Generally, Hortea EPs can be stratified into 3 subgroups: EVs, EVs with bound melanin and melanin particles. Functional assays showed that the presence of melanin in EPs improved growth of Hortaea culture exposed to high osmolarity.

Summary/Conclusion: Extremotolerant Hortaea releases EPs enriched in melanin and protein Hog1, which contribute to osmotic stress adaptation.

Funding: The Slovenian Research Agency grants P1-0170, P3-0108.

PS07.10 | Lactobacillus-derived membrane vesicles modulate the production of pro-inflammatory cytokines in the Caco2 cell monolayer in context of IL-1beta stimulation

Kristýna Turková[1]; Miriam Sandanusová[2]; Eva Pecháčková[3]; Eva Vojtková[1]; Lukáš Kubala[2]

[1]Institute of Biophysics of the Czech Academy of Sciences, Kralovopolska 135, 61265 Brno, Czech Republic, Brno, Czech Republic; [2]Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic, Brno, Czech Republic; [3]Department of Biochemistry, Faculty of Science, Masaryk University, Kamenice 5, 62500 Brno, Czech Republic, Brno, Czech Republic

Introduction: Recently, membrane vesicles (MVs) produced by human gut microbiota were proposed to be the key player in the gut-microbiota interaction. At the same time, it was believed that gram-positive bacteria are not able to produce MVs due to their thick cell wall. Our aims were to characterize MVs from Lactobacillus rhamnosus CCM7091, originally isolated from human feces, and to monitor the influence of MVs on the production of pro-inflammatory cytokines in the context of IL-1beta stimulation.

Methods: Lactobacillus-derived MVs were isolated by ultracentrifugation-based floatation within sucrose cushion followed by the characterization using DLS, TEM, and WB (LTA marker). The protein cargo was determined by BCA, SDS-PAGE, and proteomics. Evaluation of MV-mediated bacteria-host intestine interaction was carried out using Caco2 cells and IL-1beta as a stimulator followed by detection of pro-inflammatory cytokines using ELISA.

Results: MVs were visualized by TEM and size range was established using DLS (100 nm being the most abundant). The purified MVs samples contain proteins in concentration 200-400 ug/ml per MVs pellet with similar protein profiles determined by SDS-PAGE. A total of 337 proteins were consistently identified in MVs prepared under the same standard conditions. In the absence of pro-inflammatory inducers, MVs had no effect on IL-6 and IL-8 release from Caco2 cells after 24-hour-incubation. IL-1beta-treated Caco2 cells displayed an increase in IL-6 and IL-8 release when compared to untreated cells, reflecting the pro-inflammatory effect. In the presence of MVs used as 24-hour pre-treatment of Caco2 cells, a decrease of IL-6 and IL-8 by IL-1beta–stimulated cells was observed.

Summary/Conclusion: Lactobacillus rhamnosus CCM7091 produces MVs with similar protein profiles under standardized cultivation conditions. Functional analysis suggests that MVs could be used as a pre-treatment tool to alleviate the response of the intestinal epithelium to IL-1beta stimulation.

Funding: Supported by the European Regional Development Fund - Project INBIO (No. CZ.02.1.01/0.0/0.0/16_026/0008451) and Internal grant of Institute of Biophysics of the Czech Academy of Sciences.

PS07.11 | Two cargo proteins of Fasciola hepatica EVs have anti-inflammatory properties on macrophages and show different effects on hepatic stellate cells

Christian M. Sánchez-López[1]; Aránzazu González-Arce[2]; Liz Fiorella Sánchez-Palencia[2]; María Trelis[1]; Dolores Bernal[2]; ANTONIO MARCILLA[3]

[1]Universitat de Valencia, BURJASSOT, Spain; [2]UNIVERSITAT DE VALENCIA, BURJASSOT, Spain; [3]Universitat de Valencia, Burjassot, Spain

Introduction: The trematode Fasciola hepatica is the causative agent of liver fluke disease (fasciolosis) in mammalian species. Modulation of host immunity is partly due to the release of extracellular vesicles (EVs) that can be internalized by host cells. We

have identified two proteins present in F. hepatica-derived EVs that have anti-inflammatory effects in macrophages and alter the proteomic profiles and EV production in hepatic stellate cells (HSCs).
Methods: Enolase and Fh16.5 proteins were produced in Escherichia coli M15, and purified with a Ni-NTA kit (Qiagen). Anti-inflammatory effects were assayed on THP-1-XBlue™-CD14 macrophages (InvivoGen), using the Quanti-blueTM reagent (Invivogen). Proteins were added to the LX-2 HSC (SCC064, Merck), and cell pellets were analyzed by semi-quantitative proteomics and Western Blotting. EVs from treated LX-2 cells were purified using Size Exclusion Chromatography (SEC), and characterized by NTA, TEM and proteomic profiling (EVtrack ID EV220011). MTT assays were used to assess cytotoxicity. All assays were performed on FBS-free culture media.
Results: Enolase, but not Fh16.5 protein, showed anti-inflammatory properties in LPS-stimulated macrophages and neither protein produced cytotoxicity. 38 proteins were significantly up-regulated in LX-2 cells upon exposure to the Fh16.5 protein and 80 were down-regulated. Regarding the enolase treatment, 33 proteins were up-regulated and 44 down-regulated. After the treatment with each protein, EVs from LX-2 HSC cells were successfully isolated and characterized, showing a higher concentration after treatment with Fh16.5, but not enolase. The proteomic analysis of the EVs from HSC cells showed that a total of 11 proteins were significantly up-regulated and 14 proteins down-regulated after Fh16.5 treatment, while 2 proteins were up-regulated and 5 down-regulated after enolase treatment. For both cells and their EVs, most of the proteins affected by these treatments are involved in fibrosis.
Summary/Conclusion: Enolase and the Fh16.5 tegumentary protein from F. hepatica EVs show anti-inflammatory effects in vitro and alter the proteomic profile of HSCs, suggesting their role in vivo modulating the fibrosis produced by the parasite.
Funding: Supported by the Agencia Estatal de Investigación, Ministerio de Ciencia e Innovación, Spain (Grant number PID2019-105713GB-I00/AEI/10.13039/501100011033), and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (Grant PROMETEO/2020/071). C.M.S-L is the recipient of a predoctoral fellowship (PRE2020-092458) funded by AEI /10.13039/501100011033. A. G-A is the recipient of a predoctoral fellowship (CPI-20-353) funded by Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (PROMETEO/2020/071). Part of "Red Traslacional para la Aplicación Clínica de Vesículas Extracelulares, Tentacles", (RED2018-102411-T, Agencia Estatal de Investigación, Spain).

PS08: Technologies and methods: Characterization of EV-molecules

Chair: Alice Gualerzi, Laboratory of Nanomedicine and Clinical Biophonics, IRCCS Fondazione Don Carlo Gnocchi, Milan, Italy

PS08.01 | Characterization and analysis of aqueous humor derived extracellular vesicles

Roberta Verta[1]; Gabriele Saccu[1]; Lola Buono[1]; Adele Tanzi[1]; Cristina Grange[2]; Sharmila Fagoonee[1]; Simona Scalabrin[1]; Raffaele Nuzzi[1]; Benedetta Bussolati[3]
[1]University of Turin, Torino, Italy; [2]Department of Medical Sciences, University of Turin, Turin, Italy; [3]Department of Medical Sciences, University of Turin, 10126 Turin, Italy, Torino, Italy

Introduction: Extracellular vesicles (EVs) are abundant in all biological fluids, however the EV characterization in the aqueous humor (AH) is still undetermined. The aim of this work was to characterize EVs from AH (AH-EVs) in term of surface markers of cellular origin and function.
Methods: We obtained AHs from patients with cataract undergoing surgical phacoemulsification and insertion of intraocular lenses (n=10 patients), after approval by the ethical committee and patients' written consent. AH-EVs were isolated by ultracentrifugation and size and quantification performed using nanoparticle tracking analysis. The AH-EVs were subjected to TEM, MACSPlex and Super Resolution microscope analysis. Subsequently, we investigated their in vitro effects on proliferation and wound heling of human immortalized keratinocyte cells (HaCaT) compared to PBS, mesenchymal stem cell-EVs (MSC-EVs, positive control) or serum-EVs (negative control).
Results: EVs were characterized by TEM and MACSPlex for size (around 100 nm), cup-shaped morphology and expression of tetraspanins. Super resolution microscopy confirmed co-expression of CD9, CD63 and CD81. Moreover, MACSPlex analysis showed expression of mesenchymal/stem (CD105, CD133) and epithelial (EPCAM) markers. The in vitro evaluation showed that HA-EVs but not serum-EVs induced a significantly higher proliferation rate of HaCaT cells and a faster wound repair, comparable to the effect of MSC-EVs.
Summary/Conclusion: We here provided evidence of the presence of EVs in the AH and characterized them for exosomal, mesenchymal and epithelial cell marker expression, highlighting a possible origin from limbal stem cells and corneal epithelium. Moreover, AH-EVs displayed in vitro proliferative and regenerative capacity, potential beneficial effects in eye pathophysiology.

PS08.02 | Characterization of endothelial and plasma derived extracellular vesicles

Johannes Oesterreicher[1]; Johannes Grillari[2]; Wolfgang Holnthoner[1]
[1]*Ludwig Boltzmann Institute for Traumatology, Vienna, Austria;* [2]*Ludwig Boltzmann Institute of Traumatology in Cooperation With AUVA, Vienna, Austria., Vienna, Austria*

Introduction: Endothelial derived extracellular vesicles (EndoEVs) have been found to influence processes such as inflammation, coagulation, or the specific progression of atherosclerosis. Nevertheless, their small subfractions (< 200 nm) in general as well as possible differences between different vascular origins remain poorly understood. Hence, to enable the use of EndoEVs to their full potential in context of various pathologies regarding not only the blood but also the lymphatic vasculature further research is key.
Methods: We used nanoparticle tracking analysis (NTA) and fluorescence triggered -flow cytometry (FT-FC) to study EndoEVs derived from different vascular beds including blood, human umbilical vein and lymphatic endothelial cells as well as plasma-derived EVs in coherence with the MISEV guidelines. Cell type specific markers such as CD31, CD41/61, CD34, Podoplanin and others alongside the EV marker CD81 were used to evaluate expression on EVs and their presence in both in vitro and in vivo samples. Furthermore, protein concentrations were approximated using spectrophotometry to assess influences on assays using lipid membrane specific dyes.
Results: The analysis of samples with different protein concentrations resulted in significant decrease in detection rates of stained vesicles showing important indications for sample preparation and data evaluation. Both NTA and FT-FC resulted in the detection of both cell type specific CD31 as well as the EV marker CD81. NTA analysis of plasma EVs showed signals significantly higher than the background whereas CD31 did not exceed the isotype control.
Summary/Conclusion: We conclude that our work provides a basis for further characterization and evaluation of EndoEVs using sophisticated fluorescence-based methods. To further add to the current understanding of physiological EndoEV levels additional markers will be included from here on to set necessary parameters for the assessment of pathological deviations.
Funding: Conducted work was partially funded by Particle Metrix.

PS08.03 | Comparison of mineralization properties of matrix vesicles and Na,K-ATPase-proteoliposomes

Heitor G. Sebinelli[1]; Bruno Z. Favarin[2]; Marcos A. E. Cruz[3]; Maytê Bolean[4]; Massimo Bottini[5]; José Luis Millán[6]; Saida Mebarek[7]; René Buchet[7]; Ana Paula Ramos[8]; Pietro Ciancaglini[9]
[1]*Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) - Universidade de São Paulo (USP), Ribeirão Preto, Brazil;* [2 1]*Molecular Biophysics Laboratory, Department of Physics, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo* [2]*Dept. Chemistry, FFCLRP-USP, SP, Brazil* [3] *Sorbonne Université, CNRS, Collège de France, Laboratoire de Chimie de la Matière Condensée de Paris (LCMCP), F-75005 Paris, France, paris, France;* [3]*University of São Paulo, Ribeirão Preto, Brazil;* [4]*Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) - Universidade de São Paulo (USP), ribeirão preto, Brazil;* [5]*University of Rome Tor Vergata, Roma, Italy;* [6]*Sanford Burnham Prebys, La Jolla, CA, USA, La Jolla, USA;* [7]*Université de Lyon-1, Lyon, France;* [8]*Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) da Univeridade de São Paulo (USP), Av. Bandeirantes, 3900, Brazil;* [9]*Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP) da Univeridade de São Paulo (USP), Ribeirão Preto, Brazil*

Introduction: Matrix vesicles (MVs) are extracellular nanostructures, released by mineral competent cells, initiating the nucleation of apatite inside MVs and bind to collagen fibrils, where apatite is released and continues to grow sustained by Pi and Ca2+ in the extracellular media (ECM). Tissue-nonspecific alkaline phosphatase (TNAP), hydrolyses extracellular PPi, which inhibits apatite formation, forming extracellular Pi, while ectonucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) produces extracellular PPi from extracellular ATP. Na,K-ATPase (NKA), a plasma-membrane ATPase, can hydrolyse intracellular ATP. The three enzymes are present in MVs. However, the role of NKA during mineralization, and the orientation of NKA in the MVs' membrane remain unclear.
Methods: Mineralization and phosphatase activity of purified NKA from rabbit kidneys and in proteoliposomes containing dipalmitoylphosphatidylcholine (PC) and dipalmitoylphosphatidylethanolamine (PE) 1:1 (w/w) was assessed with ATP and compared to isolated MVs from femurs of chicken embryos. The minerals composition was studied by ATR-FTIR. Lipidomics of MVs highlighted presence of phosphatidylethanolamine.
Results: The mineralization ability of PC:PE-NKA induced by the addition of 3.0 mM ATP and 2.0 mM Ca2+ was higher compared to that of pure PC-NKA, suggesting the preference of the active site of NKA toward ECM of PC:PE-NKA. The addition of 3 mM ouabain, which inhibits ~90% of NKA, in the presence of ATP and Ca2 did not hinder nucleation nor mineral propagation.

ATPase activity of solubilized MVs determined by a colorimetric assay and by 31P NMR in the presence of specific inhibitors showed decrease in ATP hydrolysis by the addition of ouabain of ~7%, which suggested that ATP was mostly hydrolyzed by other phosphatases. Similar findings were found using non-solubilized MVs. We observed an increased inhibition of MVs in the presence of 10 mM Levamisole (72.2%) and 10 uM SBI-425 (35.4%) which are TNAP inhibitors.

Summary/Conclusion: Our findings confirmed that TNAP is the main enzyme responsible for promoting MVs-driven mineralization. The active site of NKA is located toward the lumen of the MVs and can contribute for a small part to the mineralization in the lumen of MVs.

Funding: Brazil-France USP-COFECUB Uc Sv 184/20; FAPESP (2019/08568-2); CNPq (304021/2017-2); CAPES-PRINT 88887.368020/2019-00

PS08.04 | Comprehensive proteomic profiling of extracellular vesicles in plasma and serum reveals the presence of tissue-specific proteins in healthy individuals

Satoshi Muraoka[1]; Masayo Hirano[2]; Junko Isoyama[2]; Satoshi Nagayama[3]; Takeshi Tomonaga[4]; Jun Adachi[2]
[1]National Institutes of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan; [2]National Institute of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan; [3]The Cancer Institute Hospital of the Japanese Foundation for Cancer Research, Tokyo, Japan; [4]National Institute of Biomedical Innovation, Health and Nutrition, ibaraki, Japan

Introduction: Extracellular vesicles (EVs) are ubiquitously secreted by almost all tissues and cells and carry many cargoes, including proteins, RNAs and lipids, which are related to various biological processes. EVs are shed from tissues into the blood and expected to be used as biomarkers for diseases. We introduced a novel approach to identify tissue-specific proteins suitable for tissue-derived EV enrichment in plasma and serum.

Methods: EVs were separated from both EDTA plasma and serum of six healthy subjects (n = 3) by an affinity capture isolation method. We applied them in Mass spectrometry, data-independent acquisition. For tissue-derived EV enrichment analysis, we searched tissue-specific proteins within the plasma and serum EV proteomics data using a combination of RNA sequencing analysis with Human protein atlas (HPA) as a reference and coregulation analysis across individuals.

Results: A total of 4079 proteins were successfully identified by comprehensive EV proteomics. The 321 tissue-specific proteins, including liver, brain and skeletal muscle were found by the HPA database, and 123 and 39 proteins were annotated as liver and brain tissue-specific proteins in healthy blood EVs, respectively.

Summary/Conclusion: These EV proteins are expected to be used for more specific and sensitive enrichment of tissue-specific EVs and for screening and monitoring of disease without diagnostic imaging in patient blood in the future.

PS08.05 | Evaluation of culture conditions on the presence of Ago2 and miRNAs in small extracellular vesicles

Alissa M. Weaver; Lizandra Jimenez; Cherie Saffold; Bahnisikha Barman
Vanderbilt University School of Medicine, Nashville, USA

Introduction: Extracellular vesicle (EV)-carried miRNAs can influence gene expression and phenotypes of recipient cells. Ago2 is a critical RNA silencing protein and can transport miRNAs into small EVs (SEVs). However, detection of Ago2 and RNA inside of EVs has been variable. Here, we evaluate the effects of serum and growth factors on the detection of Ago2 and miRNAs in SEVs.

Methods: Colorectal cancer cells were conditioned with 4 different culture media (Serum-free DMEM, EV-depleted FBS in DMEM, DMEM+EGF, and Opti-MEM). SEVs were purified from conditioned media by cushion-density gradient ultracentrifugation and analyzed by nanoparticle tracking, Western, and dot blot analyses, and QRT-PCR in the presence or absence of detergent and RNase.

Results: For all media conditions, we find both Ago2 and EV markers in gradient fractions 6 and 7. Ago2 is also present in the non-vesicular fractions for the media containing EV-depleted serum. Western blots of equal vesicle numbers revealed that Ago2 levels are reduced in SEVs purified from Opti-MEM and EV-depleted FBS conditions, compared to the serum-free DMEM condition. A detergent sensitivity analysis further showed Ago2 primarily on the inside of SEVs for all conditions. QPCR revealed that miRNAs are primarily on the inside of SEVs purified under Serum-free DMEM and Opti-MEM conditions, as they only were sensitive to RNase A treatment in the presence of Triton X-100. In contrast, the same miRNAs are primarily on the outside of SEVs purified from conditioned media containing EV-depleted serum. We also tested the effect of supplementing DMEM with EGF and found no change in Ago2 SEV levels compared to Serum-free DMEM.

Summary/Conclusion: Media conditions strongly affect the presence of intravesicular and extravesicular Ago2 and miRNA. The use of serum in conditioned media is a major source of contaminating extravesicular Ago2 and miRNA and should be avoided for downstream detection of RNA.
Funding: P01CA229123

PS08.06 | Fibroblast reprograming by extracellular vesicles from pancreatic tumors harboring mutant p53

Etti Azulay[1]; Kristopher Raghavan[2]; Edna Eti Cukierman[2]; Tomer Cooks[3]
[1]The Shraga Segal Department of Microbiology and Immunology, Ben Gurion University of the Negev, Beer Sheva, Israel; [2]Lewis Katz School of Medicine at Temple University, FOX CHASE cancer center, Philadelphia, USA; [3]The Shraga Segal Department of Microbiology and Immunology, Ben Gurion University of the Negev, beer sheva, Israel

Introduction: Pancreatic Ductal Adeno carcinoma (PDAC) has a poor survival rate due to late stage diagnosis. Usually, upon discovery, this cancer type will mostly be resistant to chemotherapy and radiation, characterize with fibrotic tissue and highly metastatic. Also, 80-85% of advanced cases are found to be p53 mutated. As previously described, tumors expressing p53 gain of function mutations (GOF) are known to induce pro-invasive alterations to the extra cellular matrix (ECM) secreted by fibroblasts. Notably, over 70% of the tumor mass are cancer associated fibroblasts (CAFs). CAFs can be found as activated and naïve cells in the tumor tissue and presents heterogeneous phenotypes. In PDAC, fibroblasts activation leads to the secretion of tumorigenic factors, cytoskeleton rearrangements and remodeling of tumor-associated ECM. Hence, changes in the ECM were reported to modulate cancer cell phenotypes and found to be dependent on CAFs infiltration. Such rearrangements in the PDAC tissue might form dense ECM produced by CAFs also known as desmoplasia or fibrosis. Importantly, desmoplasia is detected in ~85% of PDAC patients. ECM-secreting CAFs promote cancer progression fueled by a cross talk with PDAC. We hypothesized that such interaction mediated by extracellular vesicles (EVs). EVs are small nanoparticles (40-150 nm) which can be secreted from all cells. The molecular cargo shipped by EVs (typically nucleic acids and proteins) has the capacity to modulate cellular phenotypes at the receiving end. Cancer cells can secret EVs carrying oncogenes and regulatory factors to neighboring and distant cells. The uptake of this cancer EVs can potentially lead to changes in cells faith.
In this study we examined whether EVs shed by PDAC harboring mutant p53 can facilitate CAFs activation in a 3D in vitro system.
Methods: In this study, we based our method on a 3D system for ECM deposition. Additionally, EVs isolation was done according to MISEV 2018 guidelines.
Results: Our analysis suggests that EVs taken up by Fibroblasts results in changes of the fibers, orientation, and ECM thickness.
Summary/Conclusion: TP53 mutated PDAC EVs uptake leads to changes in ECM orientation. Further experiments will be dedicated to study the molecular mechanism through which TP53 mutations are leading to changes in ECM deposition. We believe that EVs play a crucial role in fibrosis creation. These findings might lead to better understanding of this aggressive cancer and open a door for a new treatment approach.

PS08.07 | Monitoring distribution dynamics of EV RNA cargo within recipient monocytes and macrophages

Daniel Alfandari[1]; Hila Ben Ami Pilo[2]; Paula Abou Karam[3]; Osnat Dagan[2]; Carine Joubran[2]; Ron Rotkopf[3]; Neta Regev-Rudzki[3]; Ziv Porat[4]
[1]Faculty of Biochemistry, Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot 7610001, Israel, Rishon Lezion, Israel; [2]Faculty of Biochemistry, Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot 7610001, Israel, Rehovot, Israel; [3]Weizmann Institute of Science, Rehovot, Israel; [4]WIS, Rehovot, Israel

Introduction: We have previously developed a method that allows us to label EVs without the need for antibodies but by labeling other cellular components. By doing so, we could monitor different EV populations according to their distinct cargo components, and the cargo distribution inside recipient cells post uptake. Here, we took this approach one step further, and monitored by live uptake assay the kinetics distribution of the signal of RNA cargo of Pf-derived EVs within two different recipient cells of the human host immune system, monocytes vs macrophages. By staining the RNA cargo of the vesicles, we were able to directly track the cargo's internalization over time and measure the dynamics of the RNA distribution inside human monocytes and macrophages. We examined three different parameters post uptake of the vesicles to the recipient cells over the course of 1 hour. Surprisingly, we observed significant differences in the dynamics of the RNA cargo distribution within monocytes vs macrophages, which might suggest a distinct roles for the RNA cargo post uptake to the target cells. These data demonstrate

that IFC can serve as a powerful approach to directly follow the distribution dynamics of EV cargo components post uptake into different target cells. This could pave the way to a not just better understanding of the EV's mediated communication, but also may provide new insights on the functions of distinct cargo component.
Methods: We obtained EVs from malaria-infected red blood cells, stained their RNA cargo using thiazole orange and followed the distribution dynamics of the RNA cargo following uptake to different recipient cells-monocytes and macrophages.
Results: The distribution of the RNA cargo inside recipient monocytes and macrophages is different.
Summary/Conclusion: Our measurements may hint different properties of the cargo distribution dynamics inside these recipient host cells. Interestingly, while the kinetic of the uptake was similar, we observed a significant difference in the signal distribution pattern, showing an opposite trend in its pixel intensity distribution and the dynamic of cellular localization over time.

PS08.08 | Proteomic diversity and ethanol effects on the fetal neural stem cell and their extracellular vesicles

Dae Chung[1]; Khang Le[1]; Michael Lai[1]; Natalie Collins[1]; Elizabeth Payne[1]; Nihal Salem[1]; Susan Weintraub[2]; Rajesh Miranda[1]
[1]Texas A&M HSC, Bryan, USA; [2]The University of Texas Health Science Center at San Antonio, san antonio, USA

Introduction: Prenatal alcohol exposure can alter the growth and maturation of neural stem cells (NSCs), leading to diminished brain growth. NSCs reside in a complex microenvironment rich in sub-200 nanometer-sized extracellular vesicles (EVs), which may traffic protein, lipid, and RNA cargo between cells, as a mode of intercellular communication.
Methods: Using fetal mouse derived cortical neuroepithelium, cultured ex-vivo as non-adherent neurosphere cultures, we investigated the impact of ethanol on the proteome of NSC-EVs by employing quantitative proteomics to profile the protein expression across treatment groups of 18 EV and its 18 parent NSCs.
Results: We hypothesized that the proteome of EVs differs from the proteome of their parent cells, and that alcohol alters the EV proteome differently from the cell proteome. For subcellular location categorization, we found significantly different protein expression in EV vs Cell and in Sex. In all 6 locations, there were a group of proteins that were significantly enriched in EV samples compared to cell samples, proposing a possible specific loading of proteins into EVs at the expense of cells. This broad categorization of proteins by subcellular location gives insight into the diversity and complexity of EV proteome.
Furthermore, ethanol exposure of NSCs significantly altered the profile of proteins packaged within EVs. Statistical analyses showed ethanol exposure differentially regulated EV proteins, with majority of them being upregulated, while majority of differentially regulated proteins in cells were downregulated. Due to this contrast, we hypothesized that ethanol increases loading of specific proteins into EVs. For this, expression of proteins that were significantly altered in EVs by ethanol exposure were compared to the same proteins in cells. For both moderate and heavy ethanol exposures, the majority of these proteins were upregulated in EVs but downregulated in cells, meaning that increased level of certain proteins in EVs is not due to increased expression of same proteins in cells. Therefore, at the expense of their intracellular levels in NSCs, ethanol exposure results in increased loading of specific ethanol-sensitive proteins into EVs.
Summary/Conclusion: In summary, our proteomic data supports that ethanol alters the proteins of EVs differently from their parent NSCs, and that EV proteome differs from their parent cell proteome. Due to the heterogeneity of NSC population, where only a subpopulation of cells expresses specific RNAs and proteins at a given time, EVs may be carrying ethanol-sensitive proteins as intercellular mediators within the NSC niche. By better understanding the effects of alcohol on EV proteins, we hope to elucidate the mechanisms that mediate PAE's effects on NSC growth and maturation.
Funding: NIAAA- F31AA028446, NIAAA- R01AA024659

PS08.09 | SPRi platform implemented with gold nanoparticles as an amplification strategy for EV characterization

Silvia Picciolini; Alice Gualerzi; Francesca Rodà; Valentina Mangolini; Marzia Bedoni
Laboraotry of Nanomedicine and Clinical Biophotonics, IRCCS Fondazione Don Carlo Gnocchi, Milan, Italy, Milan, Italy

Introduction: The use of Surface Plasmon Resonance imaging (SPRi) for EV analysis has attracted much attention due to its ability to monitor in real time the interaction between ligands and targets. As the sensitive area of SPRi detection is within 200 nm on the chip surface, EVs are ideal targets that perfectly meet the requirement of the technology allowing to detect EV populations according to the presence of surface markers. Once detected, EVs could be further characterized analyzing the expression of other molecules; to do this the sensitivity of the SPRi method needs to be improved. Gold nanoparticles (AuNPs) are surface plasmon-assisted field amplifiers and intrinsic refractive index sensors representing a powerful tool thanks to their rich surface chemistry, low toxicity and high electron density.
In this work we used spherical AuNPs for the amplification of SPRi signal intensity, in order to investigate the expression levels of antigens on the surface of multiple EV populations.

Methods: AuNPs of 14 nm have been synthetized following Frens method and functionalized with antibody anti-VEGFR-2. EVs, isolated from human serum of healthy subjects and stroke patients, have been separated on a SPRi biochip according to their interactions with ligands specific for EVs derived from endothelium and neural cells. Then, AuNPs were injected and SPRi data were collected and analyzed.
Results: We optimized the effective conjugation of antibody on AuNPs and we verified the absence of aspecific binding on the chip. Once the EVs have been detected on each ligand, the injection of conjugated AuNPs showed the different expression of VEGFR-2 on specific EV populations. The analysis revealed that VEGFR-2 is less present on endothelial EVs isolated from stroke samples compared to control samples, suggesting that the physiological levels of angiogenesis are compromised after stroke onset.
Summary/Conclusion: The SPRi platform implemented with the use of AuNPs as a signal amplification strategy could be used for the multiplexing characterization of EVs.
Funding: The project was supported by the Italian Ministry of Health.

PS08.10 | The effect of starting volume of cerebrospinal fluid for extracellular vesicle proteomics profile

Petra Kangas[1]; Jenni Karttunen[1]; Tuula Nyman[2]; Tim Williams[3]; Liisa Metsähonkala[4]; Tarja S. Jokinen[1]
[1]Department of Equine and Small Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland, Helsinki, Finland; [2]Department of Immunology, University of Oslo and Oslo University Hospital, Norway, Oslo, Norway; [3]Department of Veterinary Medicine, University of Cambridge, UK, Cambridge, United Kingdom; [4]Epilepsy Unit, Hospital for Children and Adolescents, Helsinki, Finland, Helsinki, Finland

Introduction: Cerebrospinal fluid (CSF) flushes the brain, making it a promising biofluid for isolating brain-derived extracellular vesicles (EVs). CSF EV proteome reflects the composition of the cells of origin and can reveal novel biomarkers for diseases of the brain. In many clinical studies only a limited volume of CSF is available. Here, we explored the relationship between CSF volume and the proteomic profile of the EV preparations.
Methods: CSF was collected from euthanized dogs donated for research. EVs were isolated from four volumes (6 ml, 3 ml, 1 ml, and 0.5 ml) using ultrafiltration combined with size-exclusion chromatography. In addition, the CSF with or without depletion of most common plasma proteins was analysed. Three independent replica samples were analysed. Particle concentration of the EV preparations was determined with nanoparticle tracking analysis (NTA). Proteomics analysis was performed with high-resolution liquid chromatography-tandem mass spectrometry. Protein data are presented as mean ± standard deviation.
Results: NTA found a mean of 1.1E+10, 3.7E+9, 1.2E+9 and 4.7E+8 particles from the 6 ml, 3 ml, 1 ml, and 0.5 ml EV preparations, respectively. From the same samples, 1287 ± 309, 1210 ± 283, 873 ± 171, and 687 ± 116 proteins were identified. Of these proteins, 623 ± 107 were shared between all volume groups with 403 ± 72 matching to Vesiclepedia. The number of proteins identified from CSF with and without the protein depletion was 1104 ± 312 and 999 ± 289 proteins, respectively. Of these, 912 ± 262 were shared, and of these 544 ± 166 were found in Vesiclepedia.
Summary/Conclusion: These results confirmed that the particle concentration and the number of proteins identified from the samples correlate with the starting volume. The largest decrease in the number of proteins detected was between 3 ml and 1 ml starting volume. This information is valuable when deciding on CSF starting volume for EV isolations.
Funding: The study was funded by Academy of Finland and Finnish Veterinary Foundation.

PS08.12 | Platelets in serum and stored plasma samples release small extracellular vesicles ex vivo

Małgorzata S. Malys[1]; Maximilian Köller[1]; Christof Aigner[2]; Helga Schachner[1]; Andrew J. Rees[1]; Renate Kain[1]
[1]Department of Pathology at Medical University of Vienna, Wien, Austria; [2]Department of Nephrology and Dialysis at Medical University of Vienna, Wien, Austria

Introduction: Small extracellular vesicles (sEV) are membrane bound particles released from cells that act like messengers between them. Isolating pure sEV from human blood is challenging due to co-purification of plasma proteins and lipoproteins (Malys et al). We characterised proteins expressed on the surface of sEV in human blood samples with the objective of identifying their origin.
Methods: Serum, plasma, and platelet poor plasma (PPP) samples were used to purify sEV by differential ultracentrifugation (DUC), either immediately or after storage for 14 days at either 4°C or - 80°C. Concentration and size were measured by nanoparticle tracking analysis (NTA) while vesicle morphology, size and CD9 positivity were evaluated by transmission electron microscopy (TEM). SEV were further analysed by western blotting and MACSPlex – bead-based antigen binding array – for protein profiling.

Results: There was no difference in the number or diameter of sEV isolated from fresh serum, plasma, and PPP, or from samples stored at -80°C. By contrast, MACSPlex analysis showed that molecules associated with platelets (including CD9, CD63, CD41b, CD42a and CD62P) were more abundant in sEV preparations purified from fresh serum than fresh plasma. Platelet marker abundance also increased in sEV from stored plasma, while these changes were greatly attenuated in sEV from PPP. CD81 - the exosome marker that is not present on platelets - was not affected. It suggested that a substantial proportion of sEV detected in serum and stored plasma are generated ex vivo from platelets. We confirmed it by activating platelets with addition of Ca2+. TEM also identified vesicles with diameters < 70 nm, which is below the detection limit of NTA.
Summary/Conclusion: We have shown that serum and stored plasma contain similar populations of sEV, substantial portion of which were not present in the circulation but were released from platelets ex vivo; this phenomenon can be attenuated by purifying sEV from fresh plasma or from PPP.
Funding: The authors declare no conflict of interest. This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 813545.

PS08.13 | Multiparametric characterization of the secretome produced by mesenchymal stromal cells towards regulatory compliance

Elise Madec[1]; Jeanne Volatron[2]; Julien Branchu[2]; Amanda Silva[3]; Max Piffoux[2]; Shony Lemieux[2]
[1]University of Paris / EVerZom / MSC-Med laboratory UMR7057, Paris, France; [2]EVerZom, Paris, France; [3]Laboratoire Matière et Systèmes Complexes, Paris, France

Introduction: Extracellular vesicles (EVs) are nanometer-sized subcellular particles naturally produced by cells. As EVs can replicate the properties of their parent cells, mesenchymal stromal cell (MSC) derived EVs represent a promising alternative to regenerative cell therapy. However, the ability to manufacture EVs meeting regulatory requirements is a major bottleneck hampering the progress to the clinic. According to the guidelines on investigational biologicals, active substance and drug product specifications are mandatory for the following quality attributes (QA): quantity, identity, purity, and microbiological quality. Upper limits for impurities should be defined. It is also mandatory to include a test for biological activity. The aim of this investigation is to perform a multiparametric study on MSC EVs to create a standardised characterization toolbox complying with regulatory requirements.
Methods: The first step was to test and select relevant methods to characterize the quantity and identity of EVs. Several methods were investigated: two nanoparticle tracking analysis (NTA) devices (NanoSight, ZetaView), a nanoflow cytometer, a Videodrop and an ExoView. For all devices, the linearity range was evaluated over several decades of EV concentration. Then, the relevant methods were validated analytically according to ICH Q2. To assess EVs biological activity, scratch tests and immunomodulation tests were assessed.
Results: For quantity determination, the ZetaView demonstrated its superiority due to its large range and high linearity. The Exoview was selected for the identity analysis. For both, the linearity, specificity, accuracy, range, precision, quantification and detection limit were determined. Scratch tests and cytokine quantification assays were combined to confirm EVs in vitro biological activity.
Summary/Conclusion: The ZetaView and Exoview were selected for the characterization of EV quantity and identity QA and combined with biological tests. Protocols were standardised and the analytical validation was performed according to regulatory requirements. The same approach for purity and impurity QA represents the next step. This work is of outmost importance for the release of EV batches intended for clinical use to make the EV field's promises come closer to patients while maintaining quality, safety, and efficacy.

PS09: Milk and plant EVs

Chair: Martinjn van Herwijnen

Chair: Mauro Manno – Cell-Tech HUB at Institute of Biophysics (IBF), National Research Council of Italy (CNR), Palermo, Italy

PS09.01 | An enzyme-based system for the extraction of small extracellular vesicles from plants

Kewei Zhao[1]; qing zhao[1]; guilong liu[1]; manlin xie[1]; yanfang zou[1]; zhaodi guo[1]; fubin liu[1]; jiaming dong[1]; jiali ye[1]; yue cao[1]; ge sun[1]; Lei zheng[2]

[1]*Guangzhou University of Chinese Medicine, guangzhou, China (People's Republic);* [2]*Nanfang hospital, Southern medical university, guangzhou, China (People's Republic)*

Introduction: Plant-derived nanovesicles (NVs) and extracellular vesicles (EVs) are considered to be the next generation of nanocarrier platforms for biotherapeutics and drug delivery. However, EVs exist not only in the extracellular space, but also within the cell wall. Due to the limitation of isolation methods, the extraction efficiency is low, resulting in the waste of a large number of plants, especially rare and expensive medicinal plants.There are few studies comparing EVs and NVs.
Methods: To overcome these challenges, we proposed and validated a novel method for the isolation of plant EVs by degrading the plant cell wall with enzymes to release the EVs in the cell wall, making it easier for EVs to break the cell wall barrier and be collected. We extracted EVs from the roots of Morinda officinalis by enzymatic degradation(MOEVs) and nanoparticles by grinding method (MONVs) as a comparison group.
Results: The results showed smaller diameter and higher yield of MOEVs.Both MOEVs and MONVs were readily absorbed by endothelial cells without cytotoxicity and promoted the expression of miR-155. The difference is that the promotion of miR-155 by MOEVs is dose-effective. More importantly, MOEVs and MONVs are naturally characterized by bone enrichment.
Summary/Conclusion: These results support that EVs in plants can be efficiently extracted by enzymatic cell wall digestion and also confirm the potential of MOEVs as therapeutic agents and drug carriers.
Funding: This work was supported by the National Natural Science Foundation of China [grant numbers 81973633], National Natural Science Foundation of China [grant numbers 82174119],Key Laboratory Construction Project of Guangzhou Science and Technology Bureau[grant numbers 202102100007], Collaborative innovation team project of double first-class and high-level universities in Guangzhou University of traditional Chinese Medicine[grant numbers 2021xk62], Youth Innovative Talents Project of Guangdong Province(grant numbers 2020KQNCX015),and Medical Science and Technology Research Fund project of Guangdong Province(grant numbers A2021339).

PS09.02 | Phloem sap EVs from melon plants carry active proteasomes against aphid infestation

Christian M. Sánchez-López[1]; Carla Soler[2]; Elisa Garzo[3]; Alberto Fereres[3]; Pedro Pérez-Bermúdez[4]; ANTONIO MARCILLA[5]
[1]*Universitat de Valencia, BURJASSOT, Spain;* [2]*Universitat de València, BURJASSOT, Spain;* [3]*Inst. de Ciencias Agrarias, ICA-CSIC, Madrid, Spain, Spain;* [4]*Universitat de València, BURJASSOT (VALENCIA), Spain;* [5]*Universitat de Valencia, Burjassot, Spain*

Introduction: The morphogenesis of higher plants requires communication among distant organs throughout vascular tissues (xylem and phloem). Numerous investigations have demonstrated that phloem also act as a distribution route for signalling molecules since different macromolecules, including nucleic acids and proteins, change under stress situations. The participation of extracellular vesicles (EVs) in this communication has been suggested, although little is known about their role. In fact, the presence of EVs in plants has originated a great controversy in the last decade, where major concerns arose from their origin, isolation, and even nomenclature.
Methods: Phloem exudates from 5-week-old melon plants, either uninfested or infested with Aphis gossypii (n=15, 4 replicates each), were collected by cutting the tip of the stem with a sterile blade. EVs were isolated from phloem sap by differential centrifugation followed by Size Exclusion Chromatography. EVs were characterized using NTA and TEM (EVtrack ID EV220007). EVs protein content was analyzed by LC-MS/MS. Confirmation of proteasome proteins in sap EVs was achieved by TEM-immunogold and activity was measured as described for total leaf extracts (1) with several modifications. The proteasome inhibitor MG132 was used as a control.
(1) Üstün S & Börnke F (2017). Ubiquitin Proteasome Activity Measurement in Total Plant Extracts, Bio-protocol 7 (17): e2532.
Results: Here we confirm the presence of EVs in phloem sap in vivo and characterize them by NTA and TEM. Phloem sap was obtained from melon plants, either uninfested or infested by aphids. Changes in number and composition of those EVs were observed in response to insect feeding, revealing the presence of typical defence proteins in EVs as well as more components of the proteasome complex. EVs from infested plants showed two times more proteasome activity than those from healthy plants. In both cases, such activity was inhibited in a dose-dependent manner by MG132.
Summary/Conclusion: Our findings confirm the presence of EVs in phloem sap and explain the high abundance of proteasome complex proteins usually found in proteomic studies, and open up the possibility of an evolutionary conserved mechanism of defence against pathogens/stresses by higher eukaryotic organisms.
Funding: Supported by AEI-Ministerio de Ciencia e Innovación, Spain (Grant PID2019-105713GB-I00/AEI /10.13039/501100011033), and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Spain (Grant PROMETEO/2020/071). C.M.S-L is the recipient of a predoctoral fellowship (PRE2020-092458) funded by AEI/10.13039/501100011033. Part of "Red Traslacional para la Aplicación Clínica de Vesículas Extracelulares, Tentacles", (RED2018-102411-T, Agencia Estatal de Investigación, Spain).

PS09.03 | The anti-inflammatory and anti-oxidant properties of lemon-derived extracellular vesicles are achieved through the modulation of ERK/NF-κB and AhR/Nrf2 signaling pathways

STEFANIA RAIMONDO[1]; Ornella Urzì[2]; Serena Meraviglia[3]; Marta Di Simone[3]; Anna Maria Corsale[4]; Nima Rabienezhad Ganji[4]; Roberta Gasparro[4]; Antonio Palumbo Piccionello[5]; Giulia Polito[5]; Elena Lo Presti[6]; Francesco Dieli[4]; Francesco Mercati[7]; Guglielmo Puccio[8]; Alice Conigliaro[2]; Riccardo Alessandro[2]

[1]University of Palermo, Dep. of Biomedicine, Neurosciences and advanced Diagnostics, PALERMO, Italy; [2]University of Palermo, Dep. of Biomedicine, Neuroscience and Advanced Diagnostics (Bi.N.D.), Palermo, Italy; [3]University of Palermo, Dep. of Biomedicine, Neurosciences and advanced Diagnostics, Palermo, Italy; [4]Dipartimento di Biomedicina, Neuroscienze e Diagnostica Avanzata, Università degli Studi di Palermo, palermo, Italy; [5]Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Università degli Studi di Palermo, palermo, Italy; [6]Institute for Biomedical Research and Innovation (IRIB), National Research Council (CNR), 90146 Palermo, palermo, Italy; [7]5Institute of Biosciences and Bioresources (IBBR), National Research Council, Palermo, Italy, palermo, Italy; [8]Dipartimento di Scienze Agrarie, Alimentari e Forestali, Università degli Studi di Palermo, palermo, Italy

Introduction: Plant-derived extracellular vesicles (PDEVs) are gaining increasing interest in the scientific community for their biological properties, in particular for their role in the cross-kingdom interaction. In this study, we have in-depth characterized the content of EVs from Citrus limon L. (LEVs) and we explored their possible protective effect against inflammatory and oxidative processes both in vitro and ex vivo models.

Methods: We isolated PDEVs from the juice of Citrus limon L. through sequential centrifugation and ultracentrifugation. LEVs were characterized at dimensional (DLS, NTA), morphological (TEM), and biochemical (western blot) levels. We also identified flavonoids, limonoids, and lipid contents through liquid chromatography coupled to mass spectrometry. Finally, we carried out a Next-generation sequencing analysis to identify the RNA profile of LEVs. To investigate whether LEVs have a protective role on the inflammatory and oxidant processes, murine and primary human macrophages, as well as skin fibroblasts were pre-treated with LEVs and then stimulated with LPS and UV radiation.

Results: LEVs possess a variety of flavonoids and limonoids that exert beneficial roles, such as anti-inflammatory, anti-oxidant, and anti-cancer. The identification of flavonoids in LEVs prompted us to investigate their role in inflammatory and oxidative stress models. We found that pre-treatment with LEVs decreased gene and protein expression of pro-inflammatory cytokines and reduced the nuclear translocation and phosphorylation of NF-κB in LPS-stimulated murine macrophages. The inhibition of NF-κB activation was associated with the reduction of ERK1-2 phosphorylation. Furthermore, the anti-inflammatory properties of LEVs were confirmed ex vivo, in human primary T lymphocytes. LEVs were also able to exert anti-oxidant effects on human skin fibroblasts, modulating the AhR/Nrf2 pathways.

Summary/Conclusion: In conclusion, we demonstrated that LEVs exert anti-inflammatory and anti-oxidant effects by modulating the ERK1-2/NF-κB and the AhR/Nrf2 signaling pathways. Ongoing studies are aimed at correlating the observed biological effects with the Inc-RNAs and microRNAs identified in LEVs.

Funding: Stefania Raimondo is supported by PON "Ricerca e Innovazione" 2014–2020- AIM "Attraction and International Mobility".

PS09.04 | Microalgal extracellular vesicles as nature designed delivery platforms for therapeutic and cosmetic applications

Giorgia Adamo[1]; Sabrina Picciotto[2]; Daniele Romancino[3]; Angela Paterna[4]; Estella Rao[4]; Aldo Nicosia[5]; Samuele Raccosta[4]; Antonella Cusimano[5]; Rosina Noto[6]; Nicolas Touzet[7]; Mauro Manno[4]; Antonella Bongiovanni[3]

[1]Cell-Tech HUB at Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo 90146, Italy, Palermo, Italy; [2]Cell-Tech HUB at Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo, Italy; Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy, Palermo, Italy; [3]Cell-Tech HUB at Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy; [4]Cell-Tech HUB at Institute of Biophysics (IBF), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy; [5]Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo 90146, Italy, Palermo, Italy; [6]Cell-Tech HUB at Institute of Biophysics (IBF), National Research Council of Italy (CNR), Palermo 90146, Italy, Palermo, Italy; [7]Institute of Technology Sligo, Centre for Environmental Research Innovation and Sustainability, Sligo, Ireland, Sligo, Ireland

Introduction: Nanoalgosomes are microalgae-derived extracellular vesicles (EVs) that can be used as nanocarriers for precision medicine. Our previous works have thoroughly characterized these nanovesicles, eliciting their EV identity. Here, we exploit the natural properties of nanoalgosomes as effective carriers of bioactive ingredients for the development of novel pharmaceutical and

cosmetic formulations. Specifically, we focused on their endogenous cargo bioactivities and then we evaluated nanoalgosomes loaded with different bioactive molecules to develop natural drug delivery systems.
Methods: Nanoalgosomes are separated at pilot-scale from a suspension of Tetraselmis chuii cells using tangential flow filtration (TFF) and the physicochemical characterization was carried out according to our quality control check, based on MISEV2018 guidelines. Next, nanoalgosome bioactivity is evaluated using different approaches (e.g., antioxidant assay). different molecules (e.g., doxorubicin as chemotherapeutic drug model and RNAs) and protocols were used to efficiently perform nanoalgosome-loading. After the nanoalgosome engineering, the loaded nanoalgosome functionality is analysed in normal and tumour cells.
Results: Our experiments show that nanoalgosomes are able to decrease free radical damage in vitro, probably due to the presence of microalgal anti-oxidant compounds. Moreover, they can be efficiently loaded, improving the cellular uptake of small drug and nucleic acids.
Summary/Conclusion: These studies demonstrate how nanoalgosomes represent an efficient natural delivery system of high-value microalgal substances and exogenous bioactive biomolecules and/or synthetic drugs, hence fostering the exploitation of such sustainable EVs in a scalable manner.
Funding: VES4US and BOW projects funded by the EU-H2020 programmes under grant agreements N. 801338 and N. 952183.

PS09.05 | Broccoli-derived extracellular vesicles as nanocarriers of exogenous microRNAs for RNA-based therapy

Lorena del Pozo-Acebo[1]; María- Carmen López de las Hazas[1]; Judit Gil-Zamorano[1]; João Tomé-Carneiro[1]; Andrea del Saz[1]; Luis A. Chapado[1]; Francisco Marín[2]; Alberto Dávalos[1]
[1]Imdea Food, Madrid, Spain; [2]Universidad Autónoma de Madrid, Madrid, Spain

Introduction: Cell communication is essential for organism development. To facilitate communication, both plant and animal cells can release nanoparticles, known as extracellular vesicles (EVs), that transport bioactive molecules including miRNAs. Plant-derived EVs protect miRNAs against RNase digestion and increase miRNA stability. Characterization methods have shown that plant EVs have similar structures to mammalian EVs which may facilitate their uptake, tissue distribution and ability to produce biological effects in animal cells by a cross-kingdom regulation. These observations suggest the potential use of plant-derived EVs from natural sources as delivery vehicles for oral miRNA-based therapy.
Methods: In this study, we evaluated the use of plant EVs as potential vehicles for RNA drug delivery. EVs were isolated from broccoli combining ultracentrifugation and size exclusion chromatography. Isolated EVs were then loaded with exogenous ath-miR159a, a highly expressed miRNA in plant EVs. miRNA stability was assessed by RNase A protection assay and in-vitro digestion in GI simulated conditions. The suitability of EVs as delivery vehicles for extracellular RNAs was tested by evaluating the absorption of ath-miR159a by Caco-2 cells. The potential gene targets were predicted by bioinformatic tools.
Results: Broccoli-derived EV isolation was confirmed by Transmission Electron microscopy and EV concentration was measured by Nanoparticle Tracking Analysis. In-vitro digestion revealed the protector effect of the EVs and the effective miRNA incorporation. In-vitro exposure analysis of exogenous ath-miR159a (loaded into broccoli EVs) revealed significant uptake in Caco-2 cells. Furthermore, the miRNA transported within plant EVs could exert a biological effect through the cross-kingdom modulation of gene expression.
Summary/Conclusion: Results support that plants are a cost-effective natural source of extracellular vesicles which can be used as nanocarriers of bioactive miRNAs with a potential use in RNA-based therapy.
Funding: Spanish Agencia Estatal de Investigación and European Feder Funds (PID2019-109369RB-I00, RTI2018-093873-A-I00), by the Spanish Foundation of Arteriosclerosis ("Manuel de Oya" Nutrition Grant 2021).

PS09.06 | Nanoalgosomes from marine diatom Phaeodactylum tricornutum

Veronika Kralj-Iglič[1]; Darja Bozic[2]; Matej Hočevar[3]; Marko Jeran[2]; Matic Kisovec[4]; Apolonija Bedina-Zavec[4]; Marjetka Podobnik[4]; Tadeja Matos[5]; Rok Tomažin[5]; David Škufca[6]; Ester Heath[7]; Nicolas Touzet[8]; Antonella Bongiovanni[9]; Gabriella Pocsfalvi[10]; Gitta Schlosser[11]; Tjaša Griessler-Bulc[12]; Ksenija Kogej[13]; Aleš Iglič[14]
[1]University of Ljubljana, Faculty of Health Sciences, Laboratory of Clinical Biophysics, Ljubljana, Slovenia; National Research Council of Italy, Institute of Biosciences and BioResources, Extracellular Vesicles and Mass Spectrometry Laboratory, Naples, Italy, Ljubljana, Slovenia; [2]University of Ljubljana, Faculty of Health Sciences, Laboratory of Clinical Biophysics; University of Ljubljana, Faculty of Electrical Engineering, Laboratory of Physics, Ljubljana, Slovenia, Ljubljana, Slovenia; [3]Institute of Metals and Technology, Department of Physics and Chemistry of Materials, Ljubljana, Slovenia, Ljubljana, Slovenia; [4]National Institute of Chemistry, Department of Molecular Biology and Nanobiotechnology, Ljubljana, Slovenia, Ljubljana, Slovenia; [5]University of Ljubljana, Medical Faculty, Institute of Microbiology And Immunology, Ljubljana, Slovenia, Ljubljana, Slovenia; [6]University of Ljubljana, Faculty

of Health Sciences, Laboratory of Clinical Biophysics, Ljubljana, Slovenia, Ljubljana, Slovenia; [7]Jožef Stefan Institute, Department of Environmental Sciences, Ljubljana, Slovenia, Ljubljana, Slovenia; [8]Institute of Technology Sligo, Centre for Environmental Research Innovation and Sustainability, Sligo, Ireland, Sligo, Ireland; [9]Cell-Tech HUB at Institute for Research and Biomedical Innovation (IRIB), National Research Council of Italy (CNR), Palermo, Italy, Palermo, Italy; [10]National Research Council of Italy, Institute of Biosciences and BioResources, Extracellular Vesicles and Mass Spectrometry Laboratory, Naples, Italy, Naples, Italy; [11]Eötvös Loránd University, Institute of Chemistry, Department of Analytical Chemistry, Budapest, Hungary, Budapest, Hungary; [12]University of Ljubljana, Faculty of Health Sciences, Department of Sanitary engineering; University of Ljubljana, Faculty of Civil and Geodetic Engineering, Ljubljana, Slovenia, Ljubljana, Slovenia; [13]University of Ljubljana, Faculty of Chemistry and Chemical Technology, Chair of Physical Chemistry, Ljubljana, Slovenia, Ljubljana, Slovenia; [14]University of Ljubljana, Faculty of Electrical Engineering, Laboratory of Physics; University of Ljubljana, Faculty of Medicine, Laboratory of Clinical Biophysics, Ljubljana, Slovenia, Ljubljana, Slovenia

Introduction: Nanoalgosomes (NAs) have recently become a subject of growing interest due to their pharmaceutical and biotechnological potential. NAs have previously been studied in detail in the marine microalgae Tetraselmis Chuii. In this work, we harvested and characterized extracellular material of Phaeodactylum tricornutum. The production of NAs was studied in cultures grown at varying conditions and at different growth phases.
Methods: P. tricornutum (CCAP 1052) was grown in artificial marine water enriched with f/2-Guillard's solution. Cultures grown at different conditions (varying culture medium, ventilation, illumination and temperature) were observed. The cell density was quantified by flow cytometry. NAs were isolated by differential centrifugation from selected cultures. The cells and NAs isolates were visualized by light (LM), scanning electron (SEM), and cryogenic transmission electron microscopy (cryo-TEM). Isolates of NAs were also characterized by dynamic light scattering (DLS), and SDS-PAGE electrophoresis. NAs were lysed in 0.2% RapiGest detergent using 5 freeze–thaw cycles in liquid nitrogen and sonication; digested with trypsin, and analysed by LC-ESI-MS/MS-based proteomic workflow.
Results: Cells in different liquid and solid cultures exhibited different morphologies and organizations. The largest amount of colloidal nanoparticles was produced in cultures of fusiform cells in the exponential growth phase. Various roughly round particles ranging from 20 nm up to several micrometers were observed by SEM in the culture samples, while only the small ones (about 50-300 nm in size) were observed in the NAs isolates. Cryo-TEM of the NAs isolates revealed membrane-enclosed vesicles with a radially-oriented fibrous decoration. The isolates also contained a significant amount of long nanofilaments. In the shaken cultures, a large number of NAs was observed by SEM at the epiteca of a subset of the cells, which may represent the budding mechanism or an important NAs uptake site in this alga. SDS-PAGE profile of NAs showed numerous protein bands.
Summary/Conclusion: We isolated NAs from cultures of P. tricornutum. Microscopic images showed globular particles enclosed by a membrane decorated by a dense mucilage. It is indicated that NAs formation is increased when the microalgae are in the exponential growth phase. Nanofilaments found in the samples affect the flotation and three-dimensional organization of the cells in the cultures, and may impact the isolation by differential centrifugation.
Funding: This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 801338 and ARRS project P3-0388.

PS09.07 | Cow and goat milk extracellular vesicle effects on two in vitro models of human intestinal inflammation

Samanta Mecocci[1]; Alessio Ottaviani[2]; Elisabetta Razzuoli[3]; Livia De Paolis[3]; Floriana Fruscione[3]; Paola Fiorani[2]; Giovanni Chillemi[4]; Katia Cappelli[1]
[1]University of Perugia, Perugia, Italy; [2]University of Tor Vergata, Rome, Italy; [3]Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle D'Aosta, Genova, Italy; [4]University of Tuscia, Viterbo, Italy

Introduction: Among their known functions, Extracellular Vesicles (EVs) can modulate immunity and inflammation. An EV role in the pathogenesis of chronic inflammatory diseases such as inflammatory bowel disease (IBD) has been found. IBD, mainly composed of Crohn's Disease and Ulcerative Colitis, have a dramatically rising incidence in western countries and lacks a resolutive therapy. Several factors seem to contribute to its onset, but IBD are certainly characterized by immune dysregulation and barrier function disruption. Milk-derived EVs (mEVs) can have immunomodulatory and anti-inflammatory effects and milk is one of the most promising food sources of EVs. In this context, the aim of this study was to evaluate bovine and goat mEV anti-inflammatory and immunomodulating effects on two in vitro models of human bowel disease: a co-culture of Caco-2 and THP-1 cells, and IPEC-J2 cells, an accepted swine model.
Methods: To this porpoise, mEVs were isolated and characterized following the methods used in our previous study (https://doi.org/10.3390/ijms222312759), a proinflammatory environment was induced through IFN-g and LPS stimuli, and cell gene expression was evaluated through RT-qPCR in SYBR Green chemistry.

Results: The pro-inflammatory environment instauration was assessed through the significant up-regulation of the proinflammatory cytokines CXCL8, IL1B, TNFA, IL12A, IL23A, TGFB1, and the cellular damage indicators NOS2 and MMP9 in inflamed cells compared to those in basal culture conditions. The administration of mEVs led to a partial restoration of initial homeostatic conditions highlighted by a statistically significant decrease of most of the tested cytokines together with a significant down-regulation of MMP9 and the up-regulation of MUC2 and TJP1.
Summary/Conclusion: These results indicate, in addition to the clear effect on inflammation which is reduced following the mEV administration, beneficial effects of mEVs on the intestinal barrier in an attempt to restore cellular homeostasis and mucosal functions.

PS09.08 | Protective Effects of Bovine Milk-Derived Extracellular Vesicles on Cartilage

Onno Arntz[1]; Bartijn Pieters[1]; Anouk Feitsma[2]; Joost van Neerven[2]; Fons van de Loo[1]
[1]RadboudUMC, Nijmegen, Netherlands; [2]FrieslandCampina, Amersfoort, The Netherlands, Wageningen, Netherlands

Introduction: Previously, we have shown that treatment of arthritic mice with BMEVs ameliorated cartilage damage but a direct effect on cartilage cells (chondrocytes) was not studied (1). In this study, we evaluated the direct effect of BMEVs on cartilage proteoglycan depletion and chondrocyte gene expression. Furthermore, their effect to IL-1 induced catabolic effects in chondrocytes was studied.
1. Arntz O.J. Mol Nutr Food Res. 2015
Methods: Isolated BMEVs were characterized by NTA, microBCA, transmission electron microscopy, sucrose density gradient, and western blot (WB). Human cartilage explants, their enzymatically isolated chondrocytes, and immortalized chondrocytes were exposed ex vivo, to BMEVs. BMEVs were PKH-67 labelled and uptake in chondrocytes was determined by fluorescence microscopy. By RT-qPCR, mRNA expression levels of cartilage destructive enzymes (ADAMTS5, MMP1, MMP3, MMP13) and the enzyme inhibitor (TIMP-3) were measured in chondrocytes after BMEVs exposure. The effect on glycosaminoglycan (GAG) release from cartilage explants into the culture medium was studied by the 1,9-dimethylmethylene blue (DMMB) assay. The effects of BMEVs on IL-1-induced gene expression was determined by RT-qPCR.
Results: Based on size, protein content, density and presence of exosome markers CD63, CD81, HSP-70, and Alix, we confirmed that BMEVs were pure and express markers of exosomes. Chondrocytes showed uptake of BMEVs. BMEVs exposure to chondrocytes resulted in >50% reduced expression of ADAMTS5, MMP1, MMP3 and MMP13, while TIMP-3 was enhanced (>300%). Cartilage explants exposed to BMEVs, showed reduced GAG release (>33%) and inhibition of MMP1 gene expression (>70%). IL-1 treated chondrocytes showed enhanced MMP1, 3 and 13 mRNA levels which were counteracted by BMEVs.
Summary/Conclusion: This study showed a direct protective effects of BMEVs on human chondrocytes and cartilage, and suggest that the observed cartilage protection in murine arthritis models could be due to a direct effect of BMEVs on chondrocytes.
Funding: This study was powered by Health-Holland, Top Sector Life Sciences & Health (grant 441 LSHM19108 SGF, REFIT project) allocated to Dr. Fons AJ van de Loo.

PS09.09 | Bovine milk exosomes for oral delivery of siRNA

Yunyue Zhang; Driton Vllasaliu
King's College London, London, United Kingdom

Introduction: RNA interference, which is mediated by small interfering RNA (siRNA), is a powerful therapeutic approach. However, oral administration of siRNA is currently not possible due to extremely poor delivery efficacy. Poor delivery efficacy in turn is a result of nucleic acid instability in the gastrointestinal (GI) tract and highly inefficient transfection due to poor transport across the biological barriers of the intestinal mucosa. Bovine milk exosomes have been demonstrated to be capable of crossing the intestinal mucosa and hence possess potential as delivery vehicles for facilitating intestinal (i.e. oral) delivery of siRNA. This work explored this potential.
Methods: Exosomes were isolated from bovine milk by ultracentrifugation and purified by size exclusion chromatography. Exosomes were characterised for size, zeta-potential and expression of exosomal protein markers. GI stability of exosomes was measured by exposure exosomes to fasted-state and fed-state simulated small intestinal fluids (FaSSIF and FeSSIF, respectively). The ability of exosomes to transport across an in vitro intestinal model (Caco-2 monolayers) was tested before and after exposure of exosomes to simulated small intestinal fluids. siRNA was incorporated into exosomes via transfection and the effect of exosome-mediated delivery on cell uptake (Caco-2 cells) of siRNA established.
Results: Exosomes were characterized with the size around 130 nm and slightly negative charge. Isolated exosomes were identified with confirmed expression of typical protein markers. Both of FaSSIF and FeSSIF demonstrated no effect on exosomes size and

membrane stability, while the permeability of exosomes through Caco-2 monolayers was not negatively affected by exposure to simulated small intestinal fluids. siRNA could be encapsulated into exosomes with loading efficiency of 27.5%, and the uptake of exosome-incorporated siRNA into intestinal Caco-2 cells was significantly higher than that of naked siRNA.
Summary/Conclusion: Bovine milk exosomes were stable in simulated small intestinal fluids and possess ability to transport through intestinal epithelium. siRNA uptake into intestinal epithelial cells is increased via incorporation into bovine milk exosomes.

PS09.10 | The lipidomic profile of human milk (HM) extracellular vesicles (EVs) from mothers of term and preterm infants

Isabel Ten-Doménech[1]; Victoria Ramos-Garcia[1]; Alba Moreno-Giménez[1]; María Gormaz[2]; Alex P. Shephard[3]; Marta Gómez-Ferrer[1]; Pilar Sepúlveda[1]; Guillermo Quintás[4]; Julia Kuligowski[1]
[1]Health Research Institute La Fe, Valencia, Spain; [2]University and Polytechnic Hospital La Fe, Valencia, Spain; [3]NanoView Biosciences, Malvern, United Kingdom; [4]Leitat Technological Center, Valencia, Spain

Introduction: HM EVs encase specific molecules for delivery from mother to infant. The objective of this study was to compare the lipid composition of EVs isolated from HM of mothers of term (TI) and preterm infants (PI).
Methods: HM samples were collected from five mothers of PI and five of TI after establishing full enteral nutrition. The study was approved by the Ethics Committee for Biomedical Research of the Health Research Institute La Fe (#2019-289-1) and written informed consents were obtained. EVs were isolated employing a multi-stage ultracentrifugation procedure. Size distribution and quantification were analyzed using the ExoView platform (NanoView Biosciences, MA, USA) and total protein amount with the BCA assay. After single-phase extraction, lipidomic fingerprinting was carried out using liquid chromatography-mass spectrometry. Automated MS/MS-based annotation was carried out using HMDB, METLIN, LipidBlast, and MSDIAL databases. Lipid ontology (LION) enrichment analysis was performed.
Results: A median size of 61 (IQR=3) nm and a median number of 8x1014 particles mL-1 (3x1015 IQR) with protein concentrations ranging from 2 to 6 g L-1 were obtained. A total of 439 LC-MS features in HM EVs were annotated. The classes with highest relative abundance in HM EVs were phosphosphingolipids (26%), triradylglycerols (26%), glycerophosphocholines (19%), glycerophosphoethanolamines (7%), glycerophosphoserines (3%), and ceramides (3%). LION enrichment analysis found main differences between HM EVs from PI vs. TI in cellular components (endosome/lysosome) and in physical and chemical properties of lipids, and to a lesser extent in functions (membrane component).
Summary/Conclusion: Subtle differences between HM EVs from PI and TI were identified. Studies related with the impact of lipid composition of HM EVs on infant health and growth are encouraged.
Funding: This project received support from Instituto de Salud Carlos III (Spain) [CP16/00034, CD19/00176, IFEQ21/00170].

PS09.11 | Study of the correlation between somatic cell count variation and extracellular vesicles miRNA cargo in milk from healthy quarters

Mara D. Saenz-de-Juano; Giulia Silvestrelli; Susanne E. Ulbrich
ETH Zurich, Zurich, Switzerland

Introduction: Subclinical mastitis, the inflammation of the mammary gland without clinical signs, is one of the most prevalent and costly diseases in dairy farming worldwide. It usually derives from an intramammary infection (IMI) that induces a rise in the somatic cell count (SCC) in the milk. For that reason, the SCC is a common detection method with 200'000 cells/ml as the threshold. However, the SCC is highly variable and apart from an IMI, other factors are of influence. Extracellular vesicles (EVs) and their miRNA cargo have been suggested as potential biomarkers for mammary gland health. The current study aimed to evaluate whether changes in SCC are reflected in changes in the miRNA cargo of milk EVs.
Methods: Milk from four dairy cows was collected individually from one quarter during four consecutive days (2 milking sessions/day). The SCC was determined and the milk EVs were isolated from skimmed milk using acid precipitation, 0.22 μm filtration and ultracentrifugation. The presence of EVs was confirmed by transmission electron microscopy (TEM) and western blot (WB). Small RNA libraries were produced from 10 ng of extracted RNA and sequenced.
Results: The milk SCC of the consecutive milking sessions was highly variable and three cows showed a high SCC value above 200'000 cells/ml despite showing no signs of clinical illness. The heterogeneity and integrity of EVs and the protein EV markers CD9 and TSG101 were confirmed by TEM and WB. The sequencing revealed that despite the SCC changes during milking sessions, the miRNA cargo abundance in milk EVs stayed constant. Moreover, there was no difference between morning and afternoon milking sessions, and we could not detect the presence of the mastitis related miRNA bta-miR-223 in any sample.

Summary/Conclusion: Our results show that miRNA cargo of milk EVs is very stable regardless of the variation in the SCC, supporting their potential use as diagnostic markers for subclinical mastitis.
Funding: ETH Research Grant ETH-53 16-1

PS09.12 | Fate of milk extracellular vesicles during infant gastro-intestinal digestion

Charlotte Oliver[1]; Lorraine O'Driscoll[2]; Andre Brodkorb[3]; Linda Giblin[3]
[1]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland, P61 C996 and School of Pharmacy and Pharmaceutical Sciences, Trinity Biomedical Sciences Institute, Dublin 2, Ireland, Fermoy, Ireland; [2]School of Pharmacy and Pharmaceutical Sciences, Trinity Biomedical Sciences Institute, and Trinity St. James's Cancer Institute, Trinity College Dublin, Dublin 2, Ireland., Dublin, Ireland; [3]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland, P61 C996, Fermoy, Ireland

Introduction: The aim of this study was to track the fate of bovine milk extracellular vesicles (EV) during human infant gastrointestinal digestion.
Methods: Bovine milk was digested according to the INFOGEST digestion protocol modified for the infant gut (no oral phase; gastric phase 1h, pH 5.3, pepsin 286 U/mL; intestinal phase 1h, pH 6.6, trypsin 16 U/mL, bile 3.1 mmol/L) (Ménard et al. 2018. Food Chem). Samples were taken after gastric and intestinal phase and for indepth studies, bile or enzymes were excluded or diluted. For EV separation fat was removed from test samples by centrifugation (3000g, 15 min) and caseins were removed by acidification (pH 4.6). Samples (6mL) were then centrifuged (12,000g 1h; supernatant 35,000g 1h; and supernatant 70,000g 1h). The pellet was collected after the final centrifugation at 110,000g for 90 min. EVs presence was evaluated by western blot (TSG101, CD9, xanthine dehydrogenase (XDH), contamination marker: β-lactoglobulin), nanoparticle tracking analysis and atomic force microscopy.
Results: Milk EVs survived gastric phase conditions, but failure to detect markers TSG101, XDH and CD9 indicated that EVs were disrupted and digested during the intestinal phase. This was not due to bile concentration, but rather to intestinal enzymes. In addition, intact EVs were not detected after intestinal phase by atomic force microscopy. The particle concentration of milk EVs before digestion was 1.82x1010±1.34x1010 particles/mL of milk and it significantly ($P < 0.05$) decreased after intestinal digestion to 5.97x108±6.25x107 particles/mL of milk.
Summary/Conclusion: Intact milk EVs were present at the end of the gastric digestion, but intestinal digestive enzymes negatively impacted on EV structure and yield.
Funding: Irish Department of Agriculture food and fisheries, Grant No. 17F234-MilkEVs

PS09.13 | The cell study and stability of human milk exosome

Xiang Luo[1]; Driton Vllasaliu[2]
[1]King's College London, London, United Kingdom; [2]King's College London, London, United Kingdom

Introduction: Human breast milk is the best food for most nutrition before the infant's digestive system is mature. Exosomes in human milk attract our attention since these membrane-bound spheres contain proteins and some specific RNAs. Compelling evidence indicates exosomes have potential in drug delivery and as biomarkers for diagnosis. In our study, the cell study for human milk exosome (HME) was carried out to determine the biological activity in vitro. We also measure the stability of HME in the intestinal digestion solutions.
Methods: HME isolation: The pH of human milk was adjusted to 4.6. Then a series of gradient ultracentrifuge and filtration were carried out. The exosome pellet was washed by PBS solution.
Characterization of HME: a) Nanoparticle tracking analysis for size, b) Transmission electron microscopy for imaging, c) Exocheck exosome antibody arrays kit for protein determination
Cell transport study for permeability of HME, the Caco-2 cell was incubated on a transwell.
Cell uptake: The cell uptake ability was measured after cell transport studies.
Cell proliferation study: 4-day Caco-2 cell proliferation study was conducted. The cell viability was measured by MTS every 24h after sampling.
Stability of HME in intestinal digestion solution:
Fasted State Simulated Intestinal Fluid (FaSSIF) and Fed State Simulated Intestinal Fluid (FeSSIF) were used to simulate intestinal fluid.
The stability of HME membrane was measured by DPH assay. DPH is a common fluorescent probe in the study of the structural properties of phospholipid membranes.
Biocinchonic Acid (BCA) protein assay and NTA were used to measure the protein concentration and the size after digestion.

Results: HME size is around 123 nm. TEM images were got.
Antibody kit: The signals were detected on TSG101, Alix, CD81 and ICAM but no signal on GM130.
Cell permeability: around 30% of HME was transported after 3h incubation.
Cell uptake: around 15% of HME was uptake after 3h incubation.
Cell proliferation: HME increases the cell viability especially at 48h and 72h and the peak is at 72h.
Stability of HME:
DPH: HME was damaged in FaSSIF and FeSSIF and 1% SDS compared with that in PBS. The FaSSIF and FedSSIF groups have similar intensity.
The size of HME in FedSSIF mostly decrease when Fastsif and PBS batches also have a decrease compared with stock exosome. There is no significant difference amount these digested exosomes.
Summary/Conclusion: From the results so far, this isolation method can prepare exosomes from human milk. The characterization of HME was measured and determined. The cell permeability, cell uptake ability and the stability of HME show the potential use in oral drug delivery. The cell proliferation of HME shows the potential in the treatment of intestinal damage.

PS09.14 | EVs from Citrus clementina isolated by scalable methodology show potential as immunomodulatory ingredients for different industrial sectors

Laura Soriano-Romaní; Elías Hurtado; Begoña Ruiz
AINIA, Parque Tecnológico de Valencia. C/ Benjamín Franklin 5-11, E46980 Paterna (Valencia). Spain, Valencia, Spain

Introduction: EVs from fruit juices raise as new potential sources to obtain plant-derived functional ingredients for both food and topical use products. It is known that the isolation technology modifies the cargo of the EVs and, consequently, their functional effect. The objective was to characterize Citrus clementina derived EVs, isolated by scalable methodology, in terms of intestinal and dermal absorption and possible immunomodulatory role using different cell models
Methods: To meet our goal, the scalable technology of ion exchange chromatography was used to isolate EVs from Citrus clementina juice. EVs were characterized by NTA and electronic microscopy. Then, EVs were fluorescent labeled with CFSE, and intestinal epithelial cells and keratinocytes were seeded in permeable filter supports for transport assays. In addition, the immunomodulatory role of EVs was evaluated in macrophages, analyzing changes in ARN expression of cytokines. High quality protein from EVs was isolated and characterized by LC-HRMS
Results: EVs showed normal morphology and a mean size of 194±21 nm. The in vitro cell assays with labeled EVs showed a transport of 39.8% after 4h in contact with intestinal cells and 29.2% after 1h in contact with keratinocytes. EVs increased the expression of IL-8 and IL-10 but not IL-1b in macrophages, showing a clear immunomodulatory effect. Preliminary proteomic results showed several protein clusters, including different cysteine peptidases that may explain the biological effect in macrophages
Summary/Conclusion: EVs from Citrus clementina isolated by scalable methodology for its industrial application, were transported by intestinal and dermal cells and showed potential as functional immunomodulatory ingredients. A more extensive omic study on its biocargo could identify and explain new potential effects and the mechanism of action of them
Funding: Project financed by IVACE and the European Regional Development Fund, ERDF (Ref. IMDEEA/2020/97)