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SVILUPPO E DELLA CELLULA**

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# *European Journal of Histochemistry*

## *a journal of functional cytology*

The *European Journal of Histochemistry* was founded in 1954 by Maffo Vialli and published until 1979 under the title of *Rivista di Istochimica Normale e Patologica*, from 1980 to 1990 as *Basic and Applied Histochemistry* and in 1991 as *European Journal of Basic and Applied Histochemistry*. It is published under the auspices of the University of Pavia, Italy.

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## CELL DEATH, DIFFERENTIATION AND CELL SIGNALING

### TRANSLATION INITIATION FACTOR EIF6 REGULATES APOPTOSIS AND ECDYSONE METABOLISM IN *D. MELANOGASTER*

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Eukaryotic initiation factor 6 (eIF6) regulates translation initiation by binding the 60S subunit<sup>1</sup> and it is upregulated in some cancers.<sup>2</sup> We demonstrated that eIF6 haploinsufficient mice show a reduction in lymphomagenesis.<sup>3</sup> These data indicate that cells need to tightly regulate eIF6 gene dosage. The eif6 gene is highly conserved from yeast to humans, leading us to use a *D. melanogaster* model to study the effects of eIF6 gene dosage. We focused on the eye, finding that eIF6 overexpression results in a rough eye phenotype, caused by an increased Programmed Cell Death (PCD). Moreover, we found a two-fold increase of general translation upon DeIF6 overexpression. We then analyzed gene expression by RNA-Seq analysis, which revealed alterations in genes specific for eye development and a tremendous decrease in ecdysone pathway related genes. We also found that during development there is a correlation between eIF6 and 20-HE levels, demonstrating that this initiation factor regulates this important metabolic process.

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### HSP90 C-TERMINAL DOMAIN INHIBITION INDUCES APOPTOSIS IN HUMAN CANCER CELL LINE

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Heat shock proteins, Hsp27, Hsp70 and Hsp90 are anti-apoptotic proteins with amplified expression in a wide range of tumor types portend a poor prognosis and increased resistance to therapies. They are involved in human cancer proliferation, differentiation, metastasis and invasiveness.<sup>1</sup> Hsp90 is a key protein in cancer cells that plays a central role in the correct folding of several oncoproteins and was overexpressed in lung, esophageal, melanoma, and leukemia. Hsp90 inhibition induced a blockade of multiple signaling pathways providing a combinatorial attack to cellular oncogenic processes.<sup>2</sup>

HSP90 targeting in cancer treatment via binding to the N-terminal ATP-binding site of HSP90 to prevent its function exhibits potent antiproliferative activity, but with adverse side effect such as hepatotoxicity and the strong induction of heat shock response (HSR), a compensatory mechanism that allows cell survival. In contrast the inhibition Hsp90 activity targeting C-terminus domain have been shown to induce apoptosis without the deleterious HSR.<sup>3</sup>

By a combined approach based on biophysical methods we identified the Hsp90 C-terminal pocket inhibitor that affected specifically leukemia cell viability in vitro and do not affect the prolif-

eration of human non-tumor cell line. Cell cycle distribution analysis showed a cytostatic effect, prevented the cycle progression, after 24h-exposure and cytostatic/cytotoxic effect with an increase of the apoptotic cells after a 48h cell treatment.

In addition, the Hsp90 activity inhibition induced a down-regulation of cell cycle cyclin-dependent kinases, Cyclin A, Cyclin D, CDK2, and CDK4 expression levels and a significant degradation of some representative oncogenic Hsp90-client proteins Raf-1, p-Akt, p-Erk and p53. Furthermore, the treatment with our compounds did not increase the Hsp90, Hsp70 and ER chaperone grp94 expression protein levels which, conversely is a hallmark resulting from Hsp-90 N-terminal inhibition.

The identification of new selective C-terminal modulators are greatly desired because they can represent a promising anti-cancer strategy.

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### NEW INSIGHTS INTO PLATELET DERIVED GROWTH FACTOR B SIGNALING DURING EMBRYONIC DEVELOPMENT

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The PDGF family consists of four ligands (PDGF-A to -D) and two tyrosine kinase receptors (PDGFR  $\alpha$  and  $\beta$ ).

In vertebrates, the outcomes of PDGF signaling activation include proliferation, survival, migration, matrix deposition and its up-regulation is implicated in the etiology of human gliomas.<sup>1</sup> Despite these evidences, the exact role of each family member during embryonic development is still incomplete and partially controversial. In *Xenopus* pdgf-a and pdgfr- $\alpha$  are essential for gastrulation and in Zebrafish and mouse the pdgfr signaling is required for the formation of craniofacial structures derived from neural crest cells (NCC).<sup>2,3</sup> Furthermore, evidence suggests that pdgf-a pathway is related to the control of NCC migration in *Xenopus* embryos.<sup>4</sup> On the contrary, nothing is known about the potential role of pdgf- $\beta$  in this process. We therefore investigated the possible involvement of this ligand in NCC development as we recently showed the presence of *pdgf-b* mRNA during embryonic development in territories adjacent to the NCC,<sup>5</sup> which express receptor *pdgfr- $\alpha$* .

We obtained *pdgf- $\beta$*  morphants by injecting a splice-blocking morpholino in *Xenopus* embryos, showing a significant inhibition of NCC migration.

Preliminary results suggested that *pdgf- $\beta$*  may control cranial NCC migration by regulating the expression of key molecules involved in this process, such as cadherin superfamily members, which mediate cell-cell adhesion promoting NCC directional migration.

These data proposed a new role for *pdgf- $\beta$*  during vertebrates development and *pdgf- $\beta$*  morphants as a new model to study the different functions of pdgf signaling *in vivo*.

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## ROLE OF GLIOBLASTOMA-DERIVED EXTRACELLULAR VESICLES IN CANCER THERAPY AND DIAGNOSIS

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The possibility to detect and monitor tumors in biofluids represents a fascinating field in cancer. Current clinical research concentrates on studying 3 distinct tumor-related biomarkers: extracellular macromolecules, extracellular vesicles, and circulating tumor cells.

The combination between them could significantly impact GBM management as it still represents one of the most malignant tumour and it is without exception lethal because of both diagnosis, treatment and monitoring are very difficult. Recently, it has been shown that several mechanisms of GBM pathobiology are mediated through extracellular vesicles (EVs), thus suggesting their use as diagnostic, prognostic and therapeutic targets. EVs, including microvesicles (MVs) and exosomes (EXOs), are important circulating "nanoconstructs" able to mediate intercellular communication regulating a broad range of processes. Plasma membrane-derived MVs by expressing surface antigens may dynamically reflect the disease status and constitute a source of circulating biomarkers. On the other hand, EXOs, by belonging to the endogenous intracellular communication system, act as shuttle of nucleic acids and proteins from the cell of origin to recipient cells, are very attractive in understanding the biology of cancer microenvironment that contribute to the aggressive nature of GBM.

The overall goal of this study is to investigate the release of MVs from GBM cells (U87MG, U373MG, U251MG and T98G) and study their molecular profile for a possible diagnostic tool and the release of EXOs and their involvement in activation of macrophages (differentiated THP-1 human monocytes). Our data indicate that 1) GBM cells shed a high number of EVs, displaying different molecules; 2) protein and lipid profile of EVs is different from that of the cell they are secreted from; 3) temozolomide treatment affect EVs release; 4) GBM-derived EVs modulate macrophages activation. These results indicate that MVs and EXOs could be considered pivotal in GBM management and corroborate the idea to manipulate EVs in designing new diagnostics (e.g., sensors) and new therapeutics (e.g., carriers for modulating intercellular communication).

## MORPHOLOGICAL AND FUNCTIONAL INTERACTION BETWEEN OREXIN AND ENDOCANNABINOIDS IN THE BRAIN OF ADULT ZEBRAFISH

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Orexins (OXs) neuropeptides are known to regulate numerous physiological functions, such as energy homeostasis, food intake, sleep/wake cycle, arousal and wakefulness, in vertebrates<sup>1,2</sup>. Previous studies in mice have revealed an intriguing OXs/endocannabinoids (ECs) signaling interaction at both structural and functional levels, with OX-A behaving as a strong enhancer of the

EC 2-arachidonol-glycerol (2-AG) biosynthesis<sup>3-7</sup>. In this study, we describe, for the first time in the brain of adult zebrafish, the anatomical distribution and co-expression of Orexin-2 receptor (OX-2R) and endocannabinoid receptor 1 (CB1), suggesting a functional crosstalk. The immunohistochemical colocalization of these receptors by confocal imaging in the dorsal and ventral telencephalon, suprachiasmatic nucleus, thalamus, hypothalamus, preoptic area and cerebellum, is reported. Moreover, biochemical quantification of 2-AG levels by LC-MS support the occurrence of OX-A-induced 2-AG biosynthesis in the adult zebrafish brain after 3hrs of OX-A i.p. injection (0.3pmol/g). This effect is likely mediated by OX-2R as it is counteracted by i.p. administration of OX-2R antagonist (SB334867, 10pmol/g). This study provides compelling morphological and functional evidence of an OX-2R/CB1 signaling interaction in the brain of adult zebrafish, suggesting the use of this well-established vertebrate animal model for the study of complex and phylogenetically conserved physiological functions regulating by orexinergic and endocannabinoid system.

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## TRKAIII COMMUNICATES ER-STRESS TO THE MITOCHONDRIA IN NEUROBLASTOMA CELLS, RESULTING IN GLYCOLYTIC METABOLIC ADAPTATION

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Stress-regulated alternative TrkAIII splicing of the neurotrophin receptor tropomyosin-related kinase TrkA represents a physiological mechanism in neural-related stem/progenitor cell populations that is conserved and subverted into an oncogenic mechanism in human neuroblastomas (NBs). TrkAIII is characterised by TrkA exons 6/7 skipping, resulting in deletion of the receptor extracellular D4 spontaneous activation-prevention Ig-like domain and N-glycosylation sites required for cell surface expression.<sup>1</sup> In contrast to fully spliced TrkA, TrkAIII is not expressed at the cell surface but accumulates within pre-Golgi membranes, within which it exhibits spontaneous ligand-independent activation. In support of an oncogenic function, TrkAIII induces malignant transformation of NIH3T3 cells, promotes primary and metastatic tumorigenicity in NB models and in human NBs associates with advanced-stage metastatic disease, post-therapeutic relapse and worse prognosis (1-3). Here, we report a novel function for TrkAIII in communicating ER stress to the mitochondria that results in "Warburg" glycolytic adaptation. ER stress, induced by DTT, A23187 and thapsigargin, causes full activation of the ER stress response in NB cells and promotes TrkAIII targeting to the mitochondria, TrkAIII internalisation into inner-mitochondrial membranes (IMMs) and Omi/HtrA2-dependent TrkAIII cleavage-activation to active fragments, in mitochondrial matrix orientation. Stress-induced activation of IMM-associated TrkAIII results in the tyrosine phosphorylation of mitochondrial pyruvate dehydrogenase kinase-1, associated with a metabolic switch to aerobic glycolysis (4). This novel role for TrkAIII in communicating ER stress to the mitochondria provides a potential druggable self-perpetuating mechanism through which ER-stress may help maintain the

metastasis promoting "Warburg" effect in TrkAIII expressing tumour cells.

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## EFFECTS OF NATURAL COMPOUNDS ON THE OXIDATIVE BALANCE IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

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Most of the recently developed anticancer drugs induce apoptotic cell death in tumor cells through up-regulating the intracellular ROS levels. New evidence suggests the promising role of curcumin, a yellow-gold color phytochemical turmeric, isolated from root of the *Curcuma longa*, and of graviola, (*Annona muricata*), a tropical plant belonging to family Annonaceae, known for its medicinal uses, in the treatment of cancer<sup>1-2</sup>. In our study we analyzed the effects on proliferation and apoptosis in ALL and Jurkat cell line of graviola and curcumin, alone and in combination with various chemotherapeutic agents (Daunorubicin, L-Asparaginase, Metotrexate, Vincristine and Desametazone). The proliferation, apoptosis, cell cycle and ROS production, before and after treatment with a ROS inhibitor, were investigated. Cell fragmentation was observed in Time lapse Imaging.

**Results:** Our preliminary data showed an inhibition of proliferation and an apoptosis induction after 20µg/mL both of curcumin and graviola treatment for 24h.

The combined treatment of curcumin respectively with Daunorubicin, L-ASPA, Vincristine and Desametazone showed a significant shift from early to late apoptosis after 24h, using the lowest effective concentration of drugs, compared to the higher dose of drugs alone: the average apoptotic increase was 49 ± 6.3% (p<0.05). Confocal analysis confirmed the internalization of curcumin in Jurkat cells, leading to cytoplasmic and partly nuclear fragmentation, especially when combined with vincristine. Curcumin treatment increased intracellular ROS levels, thus inducing apoptosis in leukemia cells. This selective activity could be attributed to the different redox states between healthy cells and leukemic cells. Curcumin has been described as an inducer of apoptosis and cell cycle arrest via regulating multiple cancer signaling pathways. The molecular insight onto curcumin-mediated anticancer property in leukemia suppression remains to be elucidated.

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## H<sub>2</sub>O<sub>2</sub> INDUCES NECROPTOSIS IN MESOANGIOBLAST STEM CELLS

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Stem cells are used in regenerative medicine, but their therapeutic efficacy is compromised by their huge death during the first

days post-transplantation. Indeed, the microenvironment within damaged tissues is hostile for stem cell survival mainly due to oxidative stress. H<sub>2</sub>O<sub>2</sub> may play a relevant role in inducing death of the injected cells. The aim of our study was to determine the mechanism of mesoangioblast (A6) cell death after an H<sub>2</sub>O<sub>2</sub> treatment.

FACS analysis with annV/PI showed that H<sub>2</sub>O<sub>2</sub> induced a dose and time-dependent decrement in A6 viability. We have also found an increase in caspases 8, 9 and 3 activity after the treatment. To assess their involvement in cell death, the pan caspase inhibitor Z-VAD was used. Neither early apoptosis, nor late apoptosis/necrosis, nor necrosis were reduced, suggesting that the cell death induced by H<sub>2</sub>O<sub>2</sub> was caspase-independent. Then, we tested whether H<sub>2</sub>O<sub>2</sub> is responsible for the autophagy activation. To study autophagy we evaluated the expression of specific markers. H<sub>2</sub>O<sub>2</sub> decreased beclin1, Atg5, Atg7 and the ratio LC3II/I, in a dose dependent way. At the same time it increased p62 protein expression indicating an impaired autophagic flux, also confirmed by the increase of pAKT, responsible for the activation of mTOR, a negative regulator of autophagy. According to these data A6 treatment with H<sub>2</sub>O<sub>2</sub> seems to not induce nor apoptosis or autophagy. For this reason we hypothesized the activation of necroptosis, a specific form of caspase-independent, non-apoptotic or necrotic cell death. To confirm whether the observed cell death was due to enhanced necroptosis, the proportion of necrotic cells was determined by annV/PI staining. FACS analysis showed an increase in percentage of both late apoptotic/necrotic and necrotic cells, which were further increased by pretreatment with Z-VAD. To investigate the relationship between physiological autophagy and necroptosis, cells were treated with H<sub>2</sub>O<sub>2</sub> in the presence of the autophagic inhibitor 3MA. AnnV/PI staining showed that the inhibition of autophagy by 3MA significantly enhanced necroptosis in A6 treated cells. Conversely, 3MA had no effect on apoptosis. In conclusion, our data indicate that the cytotoxicity of H<sub>2</sub>O<sub>2</sub> in A6 mainly occurred via the induction of necroptosis, enhanced by both apoptosis and autophagy inhibition.

## DO AGING-MEDIATED EPIGENETIC CHANGES CAUSE METABOLIC REMODELLING DURING AGING?

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The activity of the heart is highly dependent upon metabolism due to its high and constant energy requirements. To meet its energy needs, the heart is a metabolically dynamic organ able to use different substrates (e.g., fatty acid, glucose lactate and ketone bodies) as energy sources. This property allows the heart to choose the most efficient substrate for each physiological condition<sup>1</sup>. In normal heart, 95% of energy is obtained from mitochondrial oxidative phosphorylation, of which 50%-75% is fatty acid oxidation and the remaining (25%-50%) is glucose oxidation. During aging, similarly to what happens in heart failure, the heart loses this dynamicity and shifts from mitochondrial oxidation to glycolysis<sup>2</sup>. This process, known as metabolic remodelling, causes an "energy deficit" that contributes to impairment of cardiac function in the elderly<sup>3</sup>. The molecular mechanisms triggering this remodelling are not completely understood.

Histone marks, such as acetylation and methylation of histone H3, have an important role in defining the transcription program at the base of cardiomyocyte differentiation and heart homeostasis in the adult<sup>4</sup>. Our preliminary data support the hypothesis that changes in the genomic distribution of histone marks have a role in defining the transcription changes of genes encoding enzymes

and proteins involved in energy metabolism during cardiac aging.

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## MAGNESIUM DEPRIVATION AFFECTS DEVELOPMENT AND BIOMINERALIZATION IN THE SEA URCHIN *ARBACIA LIXULA*

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Skeletogenesis is a key morphogenetic event in the life of marine invertebrates. Marine calcifiers secrete their calcareous skeletons taking up ions from seawater.

Marine biominerals include aragonite and calcite, the latter of which in some taxa (e.g. echinoderms, coralline algae) can have a substantial magnesium (Mg) component.

Echinoderms have an extensive endoskeleton composed of high magnesium calcite and occluded matrix proteins<sup>1</sup>. As biomineralization in sea urchin larvae is sensitive to the Magnesium:Calcium ratio of sea water, we investigated the effects of magnesium deprivation on development and skeletogenesis in the Mediterranean sea urchin *Arbacia lixula*. Microscopic inspection revealed that embryos reared in Mg-free seawater exhibited developmental delay from 6 hours post-fertilization, complete lack of skeleton formation at 24 hours, and severe skeleton malformations in larvae (48-72 hours). We subsequently focused on the localization of the skeletogenic cells (primary mesenchyme cells) and the spatial expression of associated genes. Immunocytochemistry revealed abnormal ectopic location of the primary mesenchyme cells (PMCs) and of the developing skeleton of treated embryos. Expression of *msp130*, an important skeleton matrix protein gene expressed only in PMCs, detected by *in situ* hybridization, was normal at 24 hours, but this gene was not down-regulated at 48 hours, as in controls<sup>2</sup>. Strikingly, development of the pigment cells, immune cells that, like the skeleton, are mesodermal derivatives, was also impaired. These results suggest the essential role of Mg in skeleton formation in sea urchin embryos with an indication that this element is also generally important for development of mesoderm.

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## EXTRACELLULAR VESICLES AND MACROPHAGE POLARIZATION UPON HYPERGLYCAEMIC STRESS

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Immune system and metabolism are highly integrated. Among immune cells, macrophages are critical effectors in the initiation and progression of inflammation. Depending on the microenvironment and external *stimuli*, they can change their functional

state from a pro-inflammatory phenotype (classical activation called M1) to an anti-inflammatory one (alternative activation called M2). Thus, understanding the activation type of macrophages would be important for the characterization of ongoing pathogenic processes and for the knowledge of metabolic organ cross-talk. An important way of intercellular communication is the release of extracellular vesicles (EVs), including exosomes (EXOs) and microvesicles (MVs), as they can transport proteins and RNAs, both mRNAs and miRNAs, to adjacent cells or to distant organs. Recently, the use of EVs as diagnostic markers and pharmacological tools for several pathological conditions has been suggested but, to date, there is little information regarding the influence of metabolic stress (such as hyperglycaemia) or of dietary lifestyle on EVs secretion. In this regard, the aim of this work is to investigate the effect of hyperglycaemic stress on macrophage polarization and secretion of EVs. In particular, we set up a model of macrophage polarization that use THP-1 human monocytes differentiated into macrophages using Phorbol 12-myristate 13-acetate (PMA). Once differentiated (Mφ macrophages), the macrophages were incubated with IL-4 in order to obtain M2 macrophages or with IFN-γ and LPS to obtain M1 ones. Hyperglycaemic condition was obtained treating the Mφ macrophages with glucose 15 and 30 mM for 24h. EVs have been isolated by differential ultracentrifugation and molecular approach, as for gene expression and protein levels, has been used to detect the markers of polarization. The achieved data show that the excess of glucose is a strong inducer of the inflammatory markers and glucose overload (30 mM) causes an increase in the secretion of EVs, in particular of exosomes. This opens new perspectives in understanding the role of macrophages in diabetes.

## ROLE OF E3 UBIQUITIN LIGASE TRIM3 IN THE REGULATION OF AUTOPHAGY AND INFLAMMATORY RESPONSE IN GLIOBLASTOMA MULTIFORME CELLS

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Glioblastoma multiforme (GBM) is a fatal disease of the central nervous system with a 5-year survival rate of less than 22%. The deadly nature of GBMs are due to the inflammatory microenvironment. Inflammatory cytokines greatly enhance the proliferation, invasiveness, stemness and resistance to current therapies of GBM cells<sup>1</sup>. The chemotherapy employed in GBM therapy is based on temozolomide, a drug known to induce cell death in an autophagy-dependent manner. Autophagy is a major intracellular catabolic process that plays both anti-tumoral and pro-tumoral roles depending on the stages of tumor development. Targeting this process thus represents a promising strategy to develop alternative anti-glioblastoma therapies.

Recent reports have shown that the members of the TRIM E3 ubiquitin ligase family have an important role in the regulation of inflammation and autophagy<sup>2</sup>. In fact, numerous TRIM proteins are overexpressed or down regulated in different cancers, and some TRIM proteins have been postulated to be prognostic factors for the outcome of anti-cancer treatments.

The gene encoding for TRIM3, located on chromosome 11p15.5, is frequently deleted in GBM<sup>3</sup>. The aim of this work is to investigate the role of TRIM3 in the regulation of autophagy and the inflammatory micro-environment in glioblastoma cells. Our data show that the overexpression of TRIM3 inhibits the proliferation



of GBM cells, which correlates with a modulation of the levels of the chronic pro-inflammatory state of these cells, while no major effects were observed in the induction of autophagy. These data suggest that TRIM3 may represent a therapeutic target for development of anti-inflammatory drugs for the treatment of glioblastoma.

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### PROBIOTIC DSF COUNTERACTS CHEMOTHERAPY INDUCED NEUROPATHIC PAIN

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Chemotherapy-induced peripheral neuropathy (CIPN) is a widespread and potentially disabling side effect of various anticancer drugs.<sup>1</sup> In spite of the intensive research focused on obtaining therapies capable to treat or prevent CIPN, the medical demand remains very high. Microtubule-stabilizing agents, among which taxanes, are effective chemotherapeutic agents for the therapy of several oncologic diseases. The inflammatory process activated by chemotherapeutic agents has been interpreted as a potential trigger of the nociceptive process in CIPN. Several reports have indicated that probiotics are capable to regulate the balance of anti-inflammatory and pro-inflammatory cytokines.<sup>2,3</sup> Accordingly, it has been suggested that some probiotic formulations, may have an effective role in the management of inflammatory pain symptoms. We tested the hypothesis that paclitaxel-induced neuropathic pain can be counteracted by the probiotic DSF by using an *in vitro* model of sensitive neuron, the F11 cells. On this model, the biomolecular pathways involved in chemotherapy induced peripheral neuropathy depending on inflammatory cytokines were investigated by Real-time PCR, Western blotting and confocal microscopy. The results obtained, i.e. the increase of acetylated tubulin, the increase of the active forms of proteins involved in the establishment of neuropathic pain, point towards the use of this probiotic formulation as a possible adjuvant agent for counteracting CIPN symptoms.

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### THE DOUBLE ROLE OF RACK1 IN THE TRANSLATIONAL CONTROL

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The translation is constituted by four phases: initiation, elongation, termination and re-cycling. The initiation phase, considered

the limiting step of translation, is regulated by different eukaryotic initiation factors, which, consequently, represent the main targets to investigate the protein synthesis. Among proteins participating to eIF activities, the *Receptor Active C Kinase* (RACK1) protein is still poor investigated. RACK1 has been initially identified as scaffold protein for the active PKC II<sup>1</sup>, and, next, as element of 40S ribosomal subunits, to promote the joining between 40S and 60S in the 80S formation<sup>2</sup>. Although it has been proposed as a regulator of global translation regulated by PKC<sup>3</sup>, the role of ribosomal RACK1 in protein synthesis is not clear yet. For this aim, we have compared in SH-SY5Y neuroblastoma cells the effects of overexpression of a mutated RACK1 (RACK1DE) enable to associate with translational machinery with those induced by up or down regulation of RACK1WT. The results indicate that ribosomal RACK1 leads to a decreased proliferation rate. Such effect is obtained by altering the progression through the G0/G1 phase and by specifically affecting the expression of cyclin D1, D2 and B1. Furthermore, we determined that ribosomal RACK1 is not involved in general protein synthesis regulation, which is instead dependent on overall levels of total RACK1 and on PKC, but independent of mTOR and eIF4E phosphorylation. All together, these results suggest that cell cycle, can be regulated at translational level by RACK1 with two different mechanisms: modulating the global translation and regulating the translation of specific mRNAs<sup>4</sup>

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### OLEA EUROPAEA COMPOUNDS IN TUMOUR INITIATION AND PROGRESSION OF BREAST CANCER CELLS

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It has been showed that modifications in dietary intake and the benefits of the Mediterranean diet can importantly increase life expectancy, reduce the risk of developing cancer and other major chronic diseases and improve quality of life and well-being. Several studies assigned a highest reduction in tumour incidence to monounsaturated and saturated vegetable lipids, such olive oil. On these bases, we focused on the effects of *Olea europaea* compounds on the initiation and progression phases in breast cancer. Breast cancer is the most frequently diagnosed cancer (23% of the total) and the main reason of tumour death among females (14%). *Olea europaea* leaves, oil and fruits have a potential effect to inhibit proliferation and to induce apoptosis in different cancer cell lines. The main mechanisms contributing to these properties entail anti-inflammatory and antioxidant actions, related to their ability to scavenge free radicals and prevent cellular injury. Among *Olea europaea* compounds, olive polyphenols received great attention, particularly the major one called Oleuropein (OL) as well as its antioxidant metabolite,

Hydroxytyrosol.

In this respect, our research focuses in the analysis of Olive leaf extracts rich in OL (~50%) as a potential cell viability reducing agent on a malignant TNBC, *MDA-MB-231*. This model represents the claudin-low/mesenchymal subtype, that overexpresses stem cell-enriched genes and has a natural tendency to metastasize to brain and lungs.

Cell viability was measured by MTS after 24,48 and 72h of treatment followed by cell cycle analysis by Flow Cytometry at 24 and 72h. Preliminary results seem to indicate that Olive extract at high concentrations (200-400 µg/mL) can reduce *MDA-MB-231* cell viability and induces a block of the cycle in the S/G2 phase.

### **hAFSCs-CONDITIONAL MEDIUM RESCUES NEURONS FROM ISCHEMIA/REPERFUSION INJURY**

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Stroke remains a leading cause of death and disability in the world. The neurological functional disruption caused by stroke is often severe.<sup>1</sup> Stem cell-derived paracrine effects have emerged as promising strategy for the reactivation of endogenous mechanisms of repair and regeneration in several disease models. Emerging evidences have shown that transplanted stem cells can release trophic signals that influence the microenvironment. Recent studies have shown that the beneficial effects observed following stem cell transplantation in several preclinical models of experimental ischemic disease and injury could be mediated by stem cell *secretoma*. In particular, many studies have reported the potential efficacy of secreted vesicles in stimulating neural plasticity following stroke. In this study *in vitro* model of ischemia/reperfusion was used as stroke model. Injured cells were treated with human amniotic fluid stem cells (hAFSCs) conditional media obtained from two different donors and cells were assayed for viability and for the expression of proteins responsible for neuronal survival such as BDNF, TrkB, ERK5 and neuronal death such as pro-BDNF, p75, JNK. The results obtained indicated a strong neuroprotective activity of hAFSCs conditional media by activating neuronal survival pathways, decreasing neuronal death and increasing the number and length of neurites. Interestingly a significant difference in counteracting neuronal death was observed among the different donors.

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### **SIRT-1 ACTIVATOR RESVERATROL PROTECTS HUVECS FROM HIGH GLUCOSE-DEPENDENT REDOX AND GLYCATIVE IMBALANCES**

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**Objective:** To investigate whether the sirtuin 1 (SIRT1) activator resveratrol (RSV) may protect human umbilical vein endothelial cells (HUVECs) from redox impairment and glycation-related processes that are induced by high glucose.

**Methods:** HUVECs growth was assessed by Trypan blue-exclud-

ing staining, after either 24 or 48 hours of high glucose (HG; 25 mM) treatment. The corresponding normoglycemic conditions were used as controls. In order to find the lowest effective concentration (LEC) of RSV in terms of SIRT1 overexpression, HUVECs were treated with different concentration of RSV (0 - 20 µM) for 24 hours in normoglycemia, then SIRT1 protein levels were detected by immunoblotting and viability was assessed by Trypan blue-based cell counting. Finally, HUVECs were treated with LEC of RSV (5 µM) to reveal whether RSV may modify HG-induced changes in the protein expression of SIRT1, SIRT3, GL01, GL02, CAT, NRF2, which are all thought to mediate hyperglycemia-dependent impairment of endothelial dysfunctions.

**Results:** 24h HG treatment showed less cytotoxicity, with respect to the 48-hour incubation. In addition, 5 µM RSV increased SIRT1 protein expression in normal glucose condition, with respect to controls treated with vehicle only, and this was confirmed by all subsequent experiments. The protein levels of SIRT1, SIRT3, CAT, and NRF2 were significantly down-regulated by HG. Interestingly, RSV completely abolished such a negative effect of HG, thus restoring the normal protein levels of SIRT1, SIRT3, CAT and NRF2. Conversely, GL01 was found to be greatly increased in cells treated with HG, and this effect was totally reverted by RSV treatment. However, RSV significantly increased GL02 level in HG condition compared to HG+DMSO.

**Conclusions:** SIRT1 may be crucial in regulating the response of human endothelial cells to resveratrol upon hyperglycemia. In fact, RSV may ameliorate the hyperglycemia-induced pro-oxidant and pro-glycation stress on HUVECs possibly by regulating SIRT1-dependent pathway. Nevertheless, further studies are needed to elucidate the underlying mechanisms.

**Key words:** Resveratrol, hyperglycemia, Endothelial dysfunction, Oxidative stress, dicarbonyl stress.

### **C5a PATHWAYS IN CHEMOTHERAPY-INDUCED NEUROPATHIC PAIN**

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Chemotherapy induced peripheral neuropathy (CIPN) is a potentially disabling side effect of many anticancer drugs such as taxanes (e.g., Paclitaxel).<sup>1</sup> It has been previously demonstrated that Paclitaxel treatment increased the expression of acetylated  $\alpha$ -tubulin, pFAK, pJAK2, pSTAT3, PI3K and p-Cortactin proteins involved in neuropathic pain induction.<sup>1</sup> C5a, an anaphylatoxin, is an important effector of the complement cascade that upon binding to C5aR1 receptor on neutrophils, becomes a potent neutrophil attractant. It has been reported that complement components have a direct effect on nociceptors. Application of C5a or C3a to peripheral nerves *ex vivo* sensitizes nociceptor C fiber. This effect may be mediated by a direct binding to the C5a receptor, since the C5a receptor is expressed in DRG.<sup>2,3</sup> Since, the inflammatory process activated by chemotherapeutic agents has been considered as a potential trigger of nociceptive process, in this study we investigated the effect of C5a in comparison to paclitaxel in F11 cells, an *in vitro* model of DRG. In addition, the effects of DF2593A, a specific inhibitor of C5aR, in suppressing the neurotoxic effects induced by paclitaxel or C5a in F11 cells were also investigated. The data obtained demonstrate that C5a mimic the neuropathic pain induced by Paclitaxel, i.e increase of pFAK, pJAK2, pSTAT3, PI3K and p-Cortactin proteins. Moreover, the specific antagonist of C5aR, mediating the effect of C5a, is able to counteract the effects of paclitaxel. Further experiments are needed to better understand the mechanism of

action of the C5a and its receptors, particularly by the use of a specific antibody against C5aR1 in combination with Paclitaxel in order to observe whether it will induce the same effects of C5a antagonist in counteracting C1NP pathways. Since the fundamental questions of how and whether C5a may be implicated in sensory neuronal function have remained unanswered, this study elucidate the mechanism/s of action of C5a and C5aR in the onset of C1NP, thus giving some insight for the development of novel treatments.

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#### TYPE 4 PHOSPHODIESTERASES: A POSSIBLE ROLE IN HEPATOCYTE TRANSFORMATION

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Type 4 phosphodiesterases (PDE4) constitute a major class of hydrolases involved in modulation of intracellular signaling pathways mediated by cAMP, an important intracellular second messenger with key cellular functions, including cell proliferation, differentiation and survival. Expressed in most human tissues and abundant in liver, PDE4 has been proposed as a therapeutic target for a variety of human tumors, while the role in liver tumorigenesis remains to be defined.<sup>1</sup> With the aim of shedding light on the role of PDE4 in hepatocyte transformation and survival, as well as on liver tumor aggressiveness, cAMP levels and cAMP-PDE activity were measured in hepatocellular carcinoma cell lines (HCC) of different origin (HepG2, Hep3B and Huh7.5) and in the terminally differentiated cell line HepaRG. Rapidly proliferating HCC cells (Hep3B and Huh7.5) exhibit significantly decreased levels of total cAMP and elevated PDE activity, PDE4 in particular. Western blot analysis using antibodies specific for the different PDE4 isoforms (A, B, C and D) showed highly increased levels of PDE4A and PDE4D proteins in Hep3B and Huh7.5 cells, compared to the less tumorigenic HepG2 and HepaRG, with major changes found in the higher MW splicing variants of both isoforms. These data indicated a connection between expression of PDE4A and PDE4D isoforms and the degree of tumor aggressiveness.

siRNA-mediated silencing of PDE4D expression appreciably slowed HCC growth through differential modulation of molecules with a key role in cell cycle progression and survival. RNAi experiments for silencing of the PDE4A gene are currently underway to investigate in addition the role of PDE4A overexpression in hepatocyte growth, thus providing a more inclusive depiction of type 4 phosphodiesterase role in hepatocyte transformation and tumorigenesis.

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#### TIGHT TRANSLATIONAL CONTROL OF METABOLISM ORCHESTRATES CD4<sup>+</sup> T LYMPHOCYTES LINEAGE DEVELOPMENT AND FUNCTION

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Upon antigen encounter, quiescent naïve T lymphocytes proliferate and differentiate towards effector cell subsets. In humans, the lifespan of naïve cells has been estimated to be years,<sup>1</sup> implying a tight control of cell growth and metabolism. Upon activation, these cells undergo a precise and fast metabolic reprogramming<sup>2,3</sup> that supports growth and imprints distinct functional fate, but the molecular basis for this is unclear. We performed the first multiple "omics" analysis of human resting and naïve T cells following activation *in vitro* and discovered that T cells exert the transitional process through translational control. We unveil that naïve cells are poised at the preinitiation step of translation, accumulate untranslated mRNAs encoding for glycolysis and fatty acid synthesis factors, and present a unique metabolomic profile. Upon TcR engagement, activation of the translational machinery leads to synthesis of GLUT1 protein that steers glucose entry. Next, translation of ACC1 mRNA, via eIF4E, completes metabolic reprogramming toward an effector phenotype. Notably, inhibition of eIF4E almost completely constrains Th17 cell polarization towards anti-inflammatory Foxp3<sup>+</sup> regulatory T (Treg) cells, defining ACC1 as a key regulatory node. Our data demonstrate that translation is the mediator of T cell metabolism and function and suggest that manipulation of translation factors is a possible avenue for immunotherapy.

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#### ADAPTIVE CONDITIONING ELICITED BY 1950 MHZ ELECTROMAGNETIC FIELD ESPOSURE IN SH-SY5Y CELLS CHALLENGED WITH MENADIIONE

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In previous works, our research group observed that pre-exposure to radiofrequency electromagnetic field (RF-EMF) was able to reduce DNA damage induced by a subsequent treatment with mitomycin-C or X-rays *in vitro*.<sup>1,2</sup> Similar finding was reported by other research groups in mammalian cells<sup>3</sup>. To provide more evidence of a possible RF-induced adaptive response, in this study SH-SY5Y human neuroblastoma cells were pre-exposed for 20 h to 1950 MHz, UMTS signal, and 3 h after the end of exposure they were treated for 1 h with 10 µM menadione (MD), a semi-quinone largely used to induce oxidative stress. No differences were observed between sham- and RF-exposed samples.

MD-dependent DNA damage was significantly decreased by the pre-exposure to RF, at 1.25 W/kg ( $P < 0.01$ ) and 0.3 W/kg ( $P < 0.01$ ) SAR values. Moreover, pre-exposure to RF abolished the down-regulation induced by MD of glutathione peroxidase and oxoguanine DNA glycosylase, whereas catalase gene was up-regulated only when both RF treatment and menadione challenge were applied. RF pre-exposure reverted the up-regulation of superoxide dismutase 2 gene expression induced by MD alone. Overall, our findings suggested that RF pre-exposure reduced menadione-dependent DNA oxidative damage, probably by enhancing antioxidant scavenging efficiency and restoring DNA repair capability. Our results provided some insights into the molecular mechanisms underlying the RF-induced adaptive response in human neuroblastoma cells challenged with menadione.

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## NEURONAL DEVELOPMENT: ORDER AND DISORDER

### CRISPR/CAS9-INDUCED INACTIVATION OF THE AUTISM RISK GENE SETD5 LEADS TO SOCIAL IMPAIRMENTS IN ZEBRAFISH

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The SETD5 gene encodes for a putative histone H3 methyltransferase whose loss-of-function (LoF) mutations in humans have been recently associated to intellectual disability (ID) and autistic spectrum disorders (ASD). The aim of this study is to generate and characterize zebrafish models in which *setd5* has been knocked down or knocked out. *setd5* is expressed at early developmental stages while at later stages its expression is localized to the developing central nervous system (CNS) of zebrafish larvae. *setd5* morphant embryos are characterized by microcephaly, cardiac edema and reduced locomotor behavior. *setd5* knock-down determined a reduction of the expression domain of CNS specification markers paralleled by a reduced brain size compared to control embryos, associated to increased apoptosis. Furthermore, we generated stable *setd5* mutant zebrafish lines through Crispr/Cas9 strategy: *setd5* LoF causes microcephaly, a significant reduction of body length and locomotor activity. Moreover, we characterized the behavioral features of heterozygous *setd5* LoF adults, focusing on social interaction. In particular, in a social preference test, *setd5* heterozygous adults showed reduced sociality when compared to wild type siblings and these altered behavioral traits triggered by *setd5* LoF are ameliorated by risperidone, an antipsychotic drug commonly used to treat behavioral traits in ASD patients.

These zebrafish models will be extremely useful to identify the molecular mechanisms underlying SETD5 LoF phenotype. The future perspective is to screen for targeted compounds able to rescue the developmental and behavioral defects, to identify novel promising therapeutic compounds for individuals affected by ASD and ID due to SETD5 haploinsufficiency.

### NEURITIN IN MOUSE NEURONAL DEVELOPMENT: A NEW THERAPEUTIC TARGET FOR RETT SYNDROME?

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Rett syndrome (RTT) is a genetic X-linked, progressive neurodevelopmental disorder mainly caused by sporadic mutations in the MECP2 gene. In RTT, neuronal development and synaptic coupling are incomplete. In mice, MeCP2 ablation causes neuronal dysfunctions and morphological aberrations, including brain atrophy. The current hypothesis is that neuronal atrophy in RTT is caused by reduced synaptic activity leading to poor trophic support of the neuronal dendritic arborization and spines. Among the potential neurotrophic factors candidates to rescue



neuronal atrophy, Neuritin has recently emerged for its ability to promote growth and stabilization of axonal and dendritic arbors, synapse formation and maturation during development. Aim of this study is to characterize the expression of Neuritin in WT and MecP2 KO or heterozygous mice brains in order to ascertain a possible dysregulation in the animal model of the pathology and investigate its effects on RTT neuronal atrophy.

During development, neuronal processes explore their environment to identify appropriate partners before establishing presynaptic and postsynaptic contacts leading to formation of stable synapses. We previously showed that this process can be reproduced in vitro, in primary mouse hippocampal neuronal cultures that achieve a mature state through 6 developmental stages. Using this in vitro model, we previously identified the developmental stages at which neuronal atrophy (days in vitro, DIV, 9-12) and synaptic uncoupling (DIV 15) occur in RTT neurons. In this study, we are investigating the expression of Neuritin in WT and KO brains and testing its ability to counteract neuronal atrophy in MecP2 KO neurons in vitro. Preliminary results show that levels of Neuritin in mouse whole brains at post natal day 42 are unchanged while data from primary hippocampal cultures at 12 DIV show an mRNA reduction in these neurons. These preliminary results, suggest that Neuritin is dysregulated in RTT during development but not in the adult brains.

#### DEVELOPMENT OF SEROTONERGIC FIBERS IN THE POST-NATAL MOUSE BRAIN

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Raphe nuclei serotonergic neurons provide a diffuse innervation of the central nervous system and are involved in the modulation of several functions in both developing and adult brain.<sup>1,2</sup> Classical studies have described the post-natal development of serotonergic axons as a linear process of terminal field innervation.<sup>3,4</sup> However, technical limitations have hampered a fine morphological characterization. We used the Tph2GFP knock-in mouse line, in which GFP expression allows a specific labelling of serotonergic neurons and axons,<sup>5</sup> for confocal microscope imaging, and we performed 3-dimensional reconstruction to morphologically describe the development of serotonergic fibers in specified brain targets from birth to adulthood. Our analysis highlighted region-specific developmental patterns of serotonergic fiber density ranging from a linear and progressive colonization of the target to a transient increase in fiber density occurring with a region-specific timing. Despite a common pattern of early post-natal morphological maturation in which a progressive rearrangement from a dot-shaped to a regular and smooth fiber morphology was observed, starting from post-natal day 28 serotonergic fibers acquire the regional morphological features observed in the adult. In conclusion, we provided novel, target-specific insights on the morphology and temporal dynamics of the developing serotonergic fibers.

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#### THE INTERPHOTORECEPTOR MATRIX: ROLE OF IMPG2 IN RETINAL DEVELOPMENT AND FUNCTION

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Retinitis pigmentosa (RP) is one of the most commonly inherited retinal dystrophies. It leads to progressive degeneration of rods, followed by loss of cones and retinal pigmented epithelium. The genetic background of RP is heterogeneous, as are inheritance modes. Recent studies have reported that mutations in the interphotoreceptor matrix proteoglycan 2 (IMPG2) gene, responsible for the introduction of premature stop codons and the production of a truncated protein, are associated with autosomal recessive RP (arRP) in humans.<sup>1</sup> This gene encodes the proteoglycan IMPG2, expressed in the interphotoreceptor matrix (IPM), in which photoreceptors are embedded.

We chose zebrafish to investigate IMPG2 function and expression, as its retinal structure and organization is very similar to the human situation.<sup>2</sup> In zebrafish, IMPG2 is present in two isoforms, IMPG2a and IMPG2b. Their expression as well as their role and possible differences are not yet known. RT-qPCR experiments performed on zebrafish embryos at different developmental stages revealed that both IMPG2a and IMPG2b are transcribed from 3 days post fertilization (dpf) in whole fish. In adults, both isoforms have an eye-specific expression. Western blot analyses showed a similar expression pattern for the proteins. Furthermore, immunohistochemistry experiments performed on eye sections showed that the expression of IMPG2 is specifically found in the outer segment of photoreceptors starting from 4 dpf. Microinjection of antisense morpholino oligonucleotides (MOs), specific for each of the two isoforms provided preliminary evidence that IMPG2 is involved in eye and head development and RPE pigmentation in zebrafish. Generation of a zebrafish line carrying the human IMPG2 protein truncations, by using CRISPR/Cas9 technology, will allow us to study the adult phenotype and perform large-scale testing of therapeutic compounds.

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#### FUNCTIONAL ANALYSIS OF AGE-REGULATED GENES DURING EMBRYONIC DEVELOPMENT

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The major risk factor for neurodegeneration and cognitive decline is the aging of the brain. This decline has been attributed both to a reduction of adult neural stem cells (aNSCs) pool and to an increased state of quiescence of the remaining stem cells. Assessing precisely how factors and signals affect stem cell behavior is of vital importance on the understanding of their long-term effects on adult neurogenesis. Recently, we identified a short list of brain age-regulated genes of possible regulatory function specifically associated with aNSCs in *Nothobranchius furzeri*, an innovative animal model system in aging studies, by

means of next-generation sequencing.<sup>1</sup> These potential neurogenic regulators are down-regulated with age in an evolutionarily conserved manner and are expressed in at least one neurogenic region of the Zebrafish embryo. Among them, we analysed the expression profile and the function of *Znf367* and *Mex3A* genes, codifying respectively for a RNA binding protein and a transcription factor, in embryonic neurogenesis. Functional studies suggested that these genes could be involved in the regulation of embryonic neurogenesis, both in *Xenopus* and Zebrafish embryos. *Znf367* emerged as a new player in primary neurogenesis regulating the neuroblast cell-cycle progression<sup>2</sup>, while *mex3A* seems to be necessary to keep neural precursor cells in a proliferative state (data unpublished). Recently, we also started to generate CRISPR-CAS9 zebrafish lines to knock out these genes to define their role also in adult neurogenesis. The identification of molecular mechanisms involved in embryonic and adult neurogenesis could represent the first step on defining interventions that could increase neurogenesis in the aged brain that could lead to improved maintenance and even repair of neuronal function.

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### GENETIC DISSECTION OF HOXB1 FUNCTION IN THE DEVELOPING MOUSE AUDITORY SYSTEM

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The central auditory pathway consists of sensory nuclei that transmit the ascending acoustic information and efferent motoneurons that modulate primary afferent responses. It is known that rhombomere 4 (r4) and *Hoxb1* are involved in the development of the central circuit that allows the perception and amplification of sound. Moreover, patients with mutations in the *HOXB1* locus do suffer of auditory deficiencies<sup>1</sup>. The sensory area of the Corti organ consists of two cell types: the inner hair cells (IHC) and the outer hair cells (OHC). IHCs are the major detectors of auditory stimuli and are innervated by lateral olivocochlear motoneurons (LOC), whereas the medial olivocochlear motoneurons (MOCs) synapse with OHCs involved in the cochlear amplification process. Both LOC and MOC are under the control of *Hoxb1* to develop. We previously showed that MOC and LOC are absent in *Hoxb1<sup>null</sup>* mutants; mice have hearing impairments and a degeneration of OHCs. Scansion electron microscopy (SEM) investigations show a considerable disorganization of OHC stereocilia and cell loss at the apical level where low frequency sounds are normally perceived<sup>2</sup>. Degeneration of OHCs may be caused by the absence of synaptic/trophic stimulation of OHCs from the MOCs during a postnatal critical period<sup>3</sup>. To test this hypothesis and exclude a possible contribution of central auditory nuclei in this phenotype, we analyzed by SEM conditional *Hoxb1* mutants (*Ptf1acre Hoxb1 Flox/Flox; Atoh1cre Hoxb1 Flox/Flox*) in which *Hoxb1* is eliminated in dor-

sal/sensory structures involved in the acoustic pathway. Our preliminary data show that in the presence of *Hoxb1* in the ventral/motor domain, where MOC and LOC motoneurons develop, but in the absence of *Hoxb1* in sensory central nuclei, OHCs show a regular morphology and fail to reproduce the severe phenotype observed in *Hoxb1<sup>null</sup>* mutants. To ultimately confirm a role of MOCs as major effectors, *Hoxb1* function will be abolished in the ventral domain of r4 using a motoneuron-specific *Cre-recombinase* line.

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### EPIGENETIC REGULATION OF THE ENDOCANNABINOID SYSTEM IN ACTIVITY-BASED MODEL OF ANOREXIA NERVOSA

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Anorexia nervosa (AN) is a psychiatric disorder characterized by dramatic reduction in caloric intake by excessive dieting and irrational fears of gaining weight, often accompanied by over-exercise. Numerous studies have proven the role of endocannabinoid (EC) system in the regulation of feeding behaviour and its impaired signaling in AN.<sup>1</sup> Activity-based anorexia (ABA) is a bio-behavioural phenomenon that mimics key symptoms of AN in rodents, where animals housed with running wheels and subjected to daily food restriction show paradoxical reductions in food intake and increases in running wheel activity.<sup>2</sup> We investigated the implication of endocannabinoid system in AN in ABA rat model, with the two critical time points for short and long ABA induction period in order to allow for anorexic-like phenotype development and potential rescue from the disease. Obtained results have shown downregulation of cannabinoid type-1 receptor (*Cnr1*) gene after 6, but not 3 day ABA induction period in nucleus accumbens of ABA rats. Consistently, pyrosequencing revealed increased DNA methylation levels in the promoter region of the same gene. In the genetic animal model the only relevant change detected was downregulation of CB1 gene in prefrontal cortex. Our findings demonstrate selective and time-dependent epigenetic modulation of CB1 in ABA rats in relevant brain regions and support the central role played by CB1 in food intake.

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## NEURODEGENERATIONS

### A BEHAVIOURAL ZEBRAFISH MODEL OF METHYL-CYCLOPENTADIENYL MANGANESE TRYCARBONIL (MMT) ENVIRONMENTAL TOXICITY

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Manganese (Mn), one of the most abundant elements on the earth,<sup>1</sup> is essential to human health<sup>2</sup> and its over-exposure is considered a risk factor for neurodegenerative disorders such as Parkinson's disease<sup>3</sup>. High Mn concentration in water and food represents contamination sources for the population.<sup>4</sup> Among additional source is gasoline combustion that releases Mn particles within the respirable size range<sup>5</sup> for the presence of MMT, an organic Mn-containing fuel additive.

Despite evidence of structural and functional damage of the CNS induced by Mn-based compounds, behavioural effects of chronic exposure to sub-lethal MMT are still limited. We used the zebrafish (*Danio rerio*), a toxicology and behavioral study model,<sup>6</sup> and the Y-Maze task<sup>7</sup> to test the hypothesis that embryo exposure to 100 µM MMT (corresponding to 20 µM Mn<sup>2+</sup>, that reflects the exposure of individuals in highly polluted cities<sup>8</sup>) may affect fish behaviour. Results show alterations in detectable behavioral traits in larvae and cognitive impairments in exploration, orientation and spatial memory in 5 month old specimens.

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### MOTOR AND STRUCTURAL ASYMMETRIES: BIOMARKERS IN EARLY-PHASE PARKINSON'S DISEASE CHARACTERIZATION

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A wide time window precedes clinical motor manifestation of Parkinson's Disease (PD), when a substantial cell loss has already occurred. The therapeutic intervention is currently based on dopamine (DA) precursor L-Dopa substitutive therapy. Unfortunately, side effects of L-Dopa treatment worsen motor impairments in late PD. The characterization of the amotoric stage is of particular interest for earlier diagnosis and intervention to delay L-Dopa-based treatment and develop new therapeutic interventions.<sup>1,2</sup>

To define the early phase of the neurodegeneration progression, the comparative analysis of behavioral, immunohistological and structural MRI data from unilateral nigral 6-OHDA-induced lesion rat model of PD was used. The time course of motor asymmetry, inter hemispheric changes in Tyrosine hydroxylase (TH<sup>+</sup>) immunostaining and T<sub>1</sub> relaxation maps were compared in a time window of 3 weeks following 6-OHDA lesion. The sham-lesioned brains did not show inter-hemispheric differentiation in T1 maps and T2\*-W structural images. On the contrary, we observed, starting in the first week post lesion, a significant increase of the T1 values (up to about 8%) in the ipsilateral striatum with respect to the contralateral one. The above finding nicely correlated with TH<sup>+</sup> IHC immunohistochemistry.

Temporal evolution in motor asymmetry and interhemispheric DA changes allowed us to characterise motor asymmetry during early development of neurodegeneration. We believe that the time course of motor behavioral and striatal T1 asymmetry may be effective biomarkers useful for early diagnosis and preclinical neuromodulation of PD.<sup>3</sup>

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### IMAGING OF NEURONAL AND METABOLIC ACTIVITY IN ZEBRAFISH LARVAE

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We have developed imaging methods for probing neuronal and metabolic activity in zebrafish larvae (3-5 days post fertilization) and correlate it with different types of stimuli inducing larval behaviors in normal or pathological conditions (for example epilepsy or mitochondrial diseases). We employ wide field fluorescence applied to larvae expressing the genetically encoded calcium indicator GCaMP6s in all neurons for real time measurements in the whole brain during spontaneous activity and PTZ-induced convulsions (Turrini et al. 2017). Wide field microscopy provides a two-dimensional mapping that allows correlating neuronal activity with locomotor behaviors of the larva. For gaining three-dimensional measurements we apply Selective Plane Illumination Microscopy (SPIM) and map the patterns of spontaneous activity in resting larvae, with cellular resolution in the whole encephalon. Finally, two-photon microscopy is used for mapping neuronal responses induced by visual stimuli and for measuring metabolic activity based on NADH and FAD autofluorescence. These methods can be applied during development to assess metabolism at different stages and in different organs and tissues with subcellular resolution.

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## IMBALANCE OF ANTI-GLYCATIVE DEFENCE IN RETT SYNDROME

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Rett syndrome (RTT) is a rare neurodevelopmental disorder, resulting from mutations in the X-linked methyl-CpG-binding protein 2 (MECP2) gene. Recent studies have shown that oxidative stress (OS) and subclinical inflammatory status play a key role in RTT pathogenesis.<sup>1,2</sup>

Methylglyoxal (MG), an endogenous cytotoxic  $\alpha$ -oxoaldehyde, is the main precursor of advanced glycation end products (AGEs), by inducing OS in several pathological conditions.

On this basis, a possible involvement of the MG-targeting defense system in the RTT-related OxInflammation processes may be hypothesized.

Two enzymes, GLO-1 and GLO-2, along with glutathione (GSH), are the main responsible for MG scavenging, with GLO-2 catalyzing the rate limiting step of this system. In the present study, we have evaluated the levels of GLOs (mRNA, protein, activity), the level of MG-dependent protein damage and the cellular response to exogenous MG in fibroblasts from RTT patients and healthy volunteers (N=6 per group).

Our results revealed that in Rett syndrome, while there were no significant changes in the levels of GLO-1 transcript, protein or specific activity, the GLO-2 enzymatic activity was significantly increased. Nevertheless, Rett syndrome fibroblasts did not show alterations in the levels of MG-induced damage, but at the same time, they seemed to be more prone to exogenous MG-induced stress. Our findings suggest that patients with RTT possibly show altered dicarbonyl stress defense system that may render cells more susceptible to further glycation insults due to the already challenged GLOs machinery.

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## ZEBRAFISH AS AN *IN VIVO* MODEL TO STUDY ALEXANDER DISEASE PATHOGENESIS

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Alexander disease (AxD) is a rare form of leucodystrophy that predominantly affects the white matter of the central nervous system (CNS), caused by a heterozygous mutation in the gene encoding the glial fibrillary acidic protein (GFAP).<sup>1</sup> GFAP is an intermediate filament primarily expressed in astrocytes and ependymal cells. Mutation in the *GFAP* gene has been observed to result in a toxic effect, disrupting the formation of the normal network and resulting in Rosenthal fiber formation. Rosenthal fibers are homogeneous eosinophilic inclusions in the cytoplasm of astrocytes and they are the pathognomonic feature for the AxD. *In vitro* functional studies have confirmed that mutant GFAP proteins do aggregate within cells, instead of producing filamentous structures and that stimulation of both the heat

shock response and autophagy by drugs can induce mutant GFAP to correctly assemble.<sup>2</sup>

Zebrafish has become a powerful tool in the developmental neurobiology research and neurodegenerative diseases due to its genetic tractability, small body size, ease of *in vivo* experimental manipulations and fast development. In our laboratory we started to use zebrafish as *in vivo* model to study AxD. The *Gfap* gene of zebrafish shares 67% identity and 77% similarity with human *Gfap* sequence.<sup>3</sup> In fact, the mutation affects preferentially amino acid residues of GFAP that are evolutionarily conserved<sup>4</sup>. By using microinjection techniques and Tol2 transgenesis we are demonstrating the utility of zebrafish as vertebrate model for AxD. In particular, transgenic zebrafish embryos expressing the mutated R239C GFAP protein, associated with a very severe disease phenotype, present the characteristic aggregates, thus suggesting that zebrafish could be a suitable model to study this disease.

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## DISPOSABLE ELECTRODES FOR DIRECT ENZYME-FREE H<sub>2</sub>O<sub>2</sub> SENSING IN A PARKINSON'S DISEASE *IN VITRO* MODEL

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Reactive Oxygen Species (ROS) are reduced forms of oxygen such as superoxide anion, hydroxyl radical or hydrogen peroxide. These molecules have a critical role in physiological processes like cellular signalling, immunological activity. However, an overproduction may cause the so-called oxidative stress (OS) which is able to cause damage to lipids, proteins or DNA. These alterations promote pathophysiological conditions such as diabetes, cancer, Alzheimer's and Parkinson's disease. In this work, we present the combination of Carbon Black (CB) and electrodeposited Prussian Blue (PB) covered with a Nafion layer on disposable Screen-Printed electrodes (CB/PB-SPE) used for non-enzymatic H<sub>2</sub>O<sub>2</sub> sensing in Neuroblastoma cell line SH-SY5Y. These cells were challenged with 6-hydroxidopamine (6-OHDA) for modelling Parkinson's disease. The electrode's surface was investigated using Scanning Electron Microscopy (SEM) and electrochemically characterized, in terms of electroactivity and stability. Electrochemical sensing of H<sub>2</sub>O<sub>2</sub> was carried out at very low potentials (-50mV), allowing interference-free detection of H<sub>2</sub>O<sub>2</sub> in the selected cell culture. The H<sub>2</sub>O<sub>2</sub> concentration was successfully monitored in an experimental model of Parkinson's disease at different times. These results could pave the way to a method for the monitoring of H<sub>2</sub>O<sub>2</sub> in culture medium for future studies of the role of H<sub>2</sub>O<sub>2</sub> and oxidative stress in Parkinson's disease.

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## EVALUATION OF FUNCTIONAL ASYMMETRY IN HEMI-PARKINSONIAN RATS USING A MODIFIED TAIL SUSPENSION TEST

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The postural instability and motor asymmetry are two widely recognized cardinal motor features of Parkinson's Disease (PD).<sup>1</sup> The mechanisms underlying such lateralization are poorly known, although this condition is diagnostically important. Interestingly, the side initially affected in PD has been recently associated with symptom trajectories.<sup>2</sup> Unilaterally injected 6-hydroxydopamine (6-OHDA) rats provide a useful model of hemiparkinson. Apomorphine-induced rotation counting in the open field test (OFT) is a reliable method for the quantification of motor asymmetries.<sup>3</sup> To characterise the time-course of postural and motor asymmetries, we carried out an analysis of the relationship between tail suspension swing test (TST),<sup>4</sup> OFT and striatal dopamine (DA) immunostaining, in 6-OHDA-lesioned rats. We performed a 10-trials/session sampling of TST behaviour. A swing was counted whenever the animal moved its head out of the vertical axis to either side. We investigated the correlation between the apomorphine-induced TST and motor asymmetry in the OFT. The TST showed a strong differentiation between spontaneous and apomorphine-induced behaviour. Furthermore, we studied the correlation among the striatal degeneration and TST-measured postural bias. Since our modified TST method is fast and simple, thus it seems appropriate to be used for the accurate behavioural assessment of the neurodegenerative progression in toxin-induced animal models.

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## EVOLUTION AND DEVELOPMENT

### NITRIC OXIDE REGULATES MOUTH DEVELOPMENT IN AMPHIOXUS

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Nitric Oxide is a gaseous molecule, enzymatically produced by Nitric Oxide Synthases (NOS), which is involved in a wide range of physiological processes. Most of the studies regarding NO and NOS physiology were carried out in mammals but the interest about NO role in non-mammalian organisms have steadily increased. Therefore, in recent years the spectrum of NO functions has expanded enormously. Investigating the ancestral role of animal NOS and their acquired functions during evolution is an issue of broad interest to understand the importance of NO system in animals. For this purpose, the use of amphioxus is very promising. Amphioxus is an invertebrate chordate deuterostome, member of the subphylum *Cephalochordata*. The morphological and genomic simplicity of amphioxus and its key phylogenetic position make it an invaluable model organism for both evolutionary and developmental studies (EvoDevo). The objectives of the present study are mainly focused on clarify the genetic pathway downstream NO signaling during amphioxus embryonic development. We interfered with the normal amphioxus development by inhibiting the production of NO and recently demonstrated its importance for the pharyngeal region formation. We performed a differential NOS-inhibited RNAseq experiment that, among others, highlighted a massive deregulation of the Retinoic Acid pathway. We are now working to demonstrate which kind of interaction between those two ancestral signaling pathways occurs in animals.

### UPDATE AVAILABLE: COMPREHENSIVE TOOLS FOR COMPARATIVE ANATOMY VER.2.0

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Treatises on comparative anatomy<sup>1,2</sup> are the evidence of how investigating anatomical structures have been crucial to better know living organisms and their interplay with the surrounding environment which could induce significant morphological variation. Traditional anatomical dissections for exploring internal morphology were not suitable to maintain integrity of samples. In the last few years, bio-imaging techniques paired with geometric morphometrics (GM), and applied to comparative anatomy studies, overcame these limits becoming at the same time widely non-invasive and highly descriptive. In fact, they preserved samples in their whole integrity, unfolding new descriptors of form variation previously unknown. The application field of these techniques ranged from 2- to 3-dimensional GM studies, covering both invertebrates and vertebrates. Radiological instruments belonging to the family of Computed Tomography (CT) scanners definitively allowed to advance in the knowledge of either known or neglected biological structures. Our implementations covered several animal orders (e.g. Decapoda, Ephemeroptera Mytiloida, Macroscelidea, Primates). Here we focused on the feasibility of

the cone-beam CT for 3D surface scanning of the mussel *Mytilus galloprovincialis* (Lamarck, 1819) valves,<sup>3,4</sup> and the potential of micro-CT scanner in detecting and 3D characterizing virtual volumes of genital bones in primates (*i.e.*, *baculum* in males and *baubellum* in females).<sup>5</sup> These innovative practices help to engage and deepen the meaning of shape in animal biology, from both structural and evolutionary views. They allow to describe the relation between phylogeny and morphogenesis, identifying all possible links between structure, function and fitness, and mechanical and ontogenetic modifications due to environmental pressures, including anthropogenic alterations.

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### EFFECTS OF RETINOIC ACID SIGNALING ALTERATIONS ON DEVELOPMENT OF PERIPHERAL NERVOUS SYSTEM OF CEPHALOCHORDATE AMPHIOXUS

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The retinoic acid (RA) signaling pathway plays an essential role in the developing central nervous system of chordates<sup>1,2</sup>. However, little is known about how RA signaling is involved in the formation of the peripheral nervous system (PNS). Then, the functions of RA signaling during the development of epidermal sensory neurons (ESNs) have been investigated in the invertebrate chordate amphioxus.

The neurogenesis of amphioxus PNS was studied by immunohistochemistry and gene expression studies, in order to identify two distinct populations of early and late ESN progenitors in the ectoderm of amphioxus embryos. Furthermore, manipulation of RA signaling, achieved using pharmacological treatments with either the RAR antagonist BMS493 or with exogenous all-trans RA, reveals that manipulation of RA signaling influence the ability of amphioxus larvae to respond to sensory stimulation likely altering the distribution of a neurogenic niche containing ESN progenitors and then the production of ESNs.

From a comparative point of view, a similar RA signaling has been reported for neurogenic niches of CNS and placodes of vertebrates, suggesting that this system is evolutionary conserved among chordates.

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### CYCLICAL NEUROGENESIS AND NEURODEGENERATION IN THE COLONIAL TUNICATE *BOTRYLLUS SCHLOSSERI*

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In *Botryllus schlosseri*, a colonial tunicate, neurogenesis occurs simultaneously to neuro-degeneration. During its asexual weekly cycle, three different generations of zooid coexist: adult individuals, their buds, and budlets produced by the latter<sup>1</sup>. At change of generation, or takeover, adult zooids regress and are substituted in filtration by their buds that become the new adults; in the meantime, budlets become buds and produce a new generation of budlets. This process is well synchronized and necessary for colony survival. While in developing budlets and buds brain development takes place, brain degeneration occurs in reabsorbing adults. In *B. schlosseri*, therefore, this neurodegenerative process is genetically controlled and not pathological. Taking advantage on this weekly zooid turnover, we studied brain differentiation and degeneration combining morphological data (ultrastructural and histological, 3D reconstructions, confocal microscopy) and behavioral tests. We found that, in adult filtering zooids, the brain neuron number is not constant. At the beginning of adult life, an initial increase of neuron number is recognizable; this is followed by a decrease, in which apoptosis is involved. Eventually, the brain is completely resorbed during the take-over. This trend of neuron number reflects the zooid behavior to respond to both oral siphon and oral tentacles stimulations. These two stimulations evoke two different behavioral answers (the direct response and crossed response), which are reflexes mediated by different nerve circuits. Experiments showed a greater sensibility to stimuli when the neuron number is maximum, followed by a decrease in zooid sensitivity during the last period of the adult life. Since *B. schlosseri* is a tunicate, the taxon considered the closest one to vertebrates<sup>2</sup>, we propose this species as a useful model to provide new insights into compensatory mechanism protecting neurons from diseases associated with neuron loss and aging.

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### EXPRESSION OF SYNAPSINS AT NON-NEURONAL LOCATIONS IN *OCTOPUS VULGARIS*

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Synapsins are a family of highly conserved phosphoproteins mostly located in the nervous system and involved in the regulation of neurotransmitter release. Interestingly, in the last years, several lines of evidence point toward the expression of synapsins also in non-neural tissue where they probably exert a role in exocytosis processes and vesicle trafficking.<sup>1</sup> In this study we aimed at identify the presence of synapsin in the octopus transcriptome and inquire about its phylogenetic position. We then investigated the presence of synapsin in reproductive organs and their expression during sexual maturation.<sup>2</sup> Three isoforms were identified in octopus brain and gonads, they probably derive from alternative splicing of the same gene and are correlated to that of other

invertebrates. All the isoforms contain domains for ATP binding, actin filaments and secretory vesicles interaction. Their potential role in secretory function was supported by molecular modelling predicting a structure functionally relevant to these features. Furthermore, in order to evaluate the expression and possible role of synapsin at extrasynaptic locations we performed *in situ* hybridization and immunohistochemistry on ovary and testis at various stages of maturation. We found that synapsin mRNA and protein were mainly expressed in oocyte cytoplasm and in follicular cells at all stages of maturation. In male testis synapsin transcript and protein were mostly located in the head of maturing spermatids and spermatozoa. Moreover, the protein level of expression positively correlated with the overall degree of gonadic maturation. These results points toward a functional role of synapsins in non-neuronal tissues and to their potential implication in secretory mechanisms occurring during sexual maturation.

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### COMBINED METHODS TO INVESTIGATE AT HIGH RESOLUTION THE COMPLEXITY OF HEMATOPOIESIS IN A MOLLUSCAN RESEARCH ORGANISM

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Increasing evidence indicates that the human immune system, beside discriminating between self and nonself, also contributes in controlling numerous metabolic and neural functions in physiological conditions. This is associated with the observation that each immune cell could present different roles on the basis of the molecular milieu and local cytokine equilibrium. Simpler models of immune system development could help to understand the influence of environmental cues on the maturation of innate components. Molluscs are one of the most studied long-living invertebrates in terms of adult hematopoiesis, and they display an immune system devoted to control the microbiome rather than to attack the non-self. In the research organism *Pomacea canaliculata* (Mollusca, Gastropoda), we already described the circulating hemocytes, the hematopoietic tissue (HPT) in adults and possible hemocyte maturational sites, such as the ampulla. In order to better characterize the hemocyte maturational process in adult hematopoiesis, previous morphological data have been correlated with those collected with ImageStream® X Mark II Imaging Flow Cytometer. By integrating flow cytometry and microscopy technologies, ImageStream® allows a highthroughput analysis of cell morphologies and their frequency in different samples, such as HPT, dissociated ampulla and circulating hemocytes. At the same time, in order to identify cell markers useful to follow the different maturational stages of hemocytes, previous morphological data have been integrated with organspecific gene expression analysis and fluorescence *in situ* hybridization, performed on the same tissues. Altogether our data reveal that *P. canaliculata* adult hematopoiesis is a complex and dynamic process. The number of cell populations potentially involved is significantly higher than the one suggested by previous studies. The multi-technique approach we propose may represent a great help in investigating at high resolution the complexity of immune system maturation in invertebrate and vertebrate models.

### EXPRESSION OF NEUROGLIA-SPECIFIC MARKERS IN THE AMPHIOXUS EMBRYO AND LARVA: A PRELIMINARY STUDY

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Neuroglia, together with neurons, is one of the two cell types that form the nervous tissue of the bilaterians and it has been proposed that changes in its organization could have driven the evolution of the nervous system.<sup>1</sup> Despite its importance, neuroglia has so far been neglected by scientists, with a single study published on amphioxus.<sup>2</sup> To investigate the structure and development of neuroglia in amphioxus (a cephalochordate used as proxy for the ancestral chordate condition), we identified and cloned the orthologs of the astrocyte marker *GFAP* as well as other glial markers and studied their expression by *whole mount in situ* hybridisation and immunocytochemistry.

The glial fibrillary acidic protein (GFAP) gene encodes a type III intermediate filament (T3IF) protein that is present only in astrocytes and ependymal cells in the vertebrate nervous system. In amphioxus, AmphiGFAP-like transcripts were first detected at late neurula stage in dorsolateral cells of the cerebral vesicle and of the caudal half of the neural tube, a position that has been previously ascribed to glial cells<sup>1</sup>. In the larva, the expression in the neural tube is reduced to scattered cells in its anterior- and posterior-most portions while a strong signal appears in some cells of the lateral epidermis. This expression pattern seems to suggest that AmphiGFAP-like is functionally halfway between vertebrate GFAP and the T3IF protein vimentin, being expressed in both radial glial cells and differentiated astrocyte-like cells. The difficulty to identify a bona fide vimentin sequence in the amphioxus genome also supports this hypothesis.

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### RELATIONSHIP BETWEEN PROTEIN POST TRANSLATIONAL MODIFICATIONS AND VALVE SHAPE VARIABILITY IN *MYTILUS GALLOPROVINCIALIS* HELPS TO REVEAL DETRIMENTAL ECOSYSTEM CONDITIONS

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Several attempts have been engaged to prevent, ameliorate or remedy the effects caused by human impacts, and using living organisms as 'early warning systems' (EWSs) may be as important as the development of environmental monitoring tools describing the environmental health.<sup>1</sup> Specifically, we propose an integrated approach exploiting sessile bivalves as biological EWSs to contribute in monitoring and managing the coastal marine habitats. Integrating diverse WSs at different biological levels increases detection probabilities of threats, and provide helpful elements to identify solutions mitigating their impact as well.<sup>2,3,4</sup> Here, we used both protein post translational modifications in two different tissues (*i.e.*, muscle adductor and digestive gland) and body shape features (*i.e.*, valve geometric morphometric - GM - descriptors) in the sessile bivalve *Mytilus galloprovincialis*. Mussels were collected in north Italy, translocated, and released within small inox steel, flow through holding cages in undisturbed and disturbed sites of both eastern Tyrrhenian and

western Ionian Sea. All cages were deployed and suspended by scuba divers in two different periods: 1) Autumn for 60 days (from October to December 2013); 2) Spring for 48 days (from April to May 2014). The significant covariation of nitration and GM descriptors were evidenced, thereby highlighting how different biological levels may act as early warning systems for the same stressors, depending on their intensity and seasonal occurrence. The exploitation of quality biotic elements provides an objective environmental monitoring integrated tool to facilitate the development of sanitary, economic, and social strategies related to sustainable exploitation.

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## GAMETOGENESIS AND REPRODUCTION

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### MAJOR ENDOCANNABINOID-BINDING RECEPTORS ARE DIFFERENTIALLY MODULATED DURING OOCYTE MEIOTIC MATURATION

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Endocannabinoids (eCBs) are recognized as key-players of female fertility<sup>1</sup> and potential biomarkers of reproductive dysfunctions in mammals.<sup>2</sup> The present study investigated the localization and expression of CB1R, CB2R, GPR55 and TRPV1 in mouse oocytes collected at different stages of in vivo meiotic maturation (GV, MI and MII), as previously described<sup>3</sup>, through quantitative PCR, confocal imaging and Western blot analysis. Despite the significant decrease of receptor mRNAs from GV to MII stage, CB2R and GPR55 protein contents significantly increased during meiotic maturation. Conversely, CB1R content was drastically reduced and disappeared from oocyte plasma membrane during the transition from GV to MI stage. Instead, mRNA and protein content of TRPV1 remained always very low. Oocytes matured with CB antagonists SR1 and SR2 (both at 0.5  $\mu$ M) had a significant delay in germinal vesicle breakdown compared with controls, that was sustained by higher intraoocyte cAMP concentration. Both antagonists did not affect polar body I emission nor meiotic spindle morphology. These findings support that activation of CB1R and CB2R, and inhibition thereof of adenylyl cyclase, are engaged in the control of meiotic resumption of mouse oocytes. These findings open a new avenue to interrogate oocyte pathophysiology and offer potentially new targets for therapy of reproductive alterations, as well as novel biomarkers for fertility problems.

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### PUBERTY ONSET AND OOCYTE MATURATION ON SWORDFISH *XIPHIAS GLADIUS*: NEW INSIGHT FROM DE NOVO TRANSCRIPTOME ASSEMBLY AND FOURIER TRANSFORM INFRARED MICROSCOPY (FTIRM) ASSAY

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The swordfish (*Xiphias gladius*) is an important commercial species with an extensive seasonal migration and a circumglobal distribution. It is a gonochoristic species and females are multiple spawners with asynchronous ovaries. To date, information on swordfish reproduction is lacking and does not provide compre-



hensive insights into gonadal development. In the present study a *de novo* assembly and annotation of the transcriptome of *X. gladius* was performed and a total of 100.869 sequences were assembled from cDNA libraries of ovaries from immature and mature females. To provide knowledge about the biological pathways involved in *X. gladius* puberty onset, a differential expression analysis was performed comparing mature and immature ovaries and a total of 6.501 transcripts were found differentially expressed. GOEA and KEGG pathway analysis showed that differentially expressed transcripts were involved in key pathways driving oocyte maturation as ovarian steroidogenesis, progesterone-mediated oocyte maturation and endosome lysosome formation. Concomitantly, the macromolecular chemistry of oocytes at different developmental stages was analysed by FT-IRM a powerful technique to describe the macromolecular building of single cell. Coupling imaging and semiquantitative analyses, we provided macromolecular characterization of vitellogenin vesicles, their formation from the plasma membrane and their fusion with bigger vitellogenin vesicles, beyond macromolecular changes of cortical alveoli and oil droplets during maturation phase.

In addition, macromolecular properties of zona radiata in oocytes at previtellogenic stages were compared to that of late vitellogenic and mature oocytes. An increase in width and changing in its protein, carbohydrates and lipids composition were observed along the oocyte maturation process. In conclusion, advances in comprehensive understanding of oogenesis process of *X. gladius* obtained by *de novo* transcriptome assembly and FTIR will undoubtedly contribute to improve basic knowledge on the reproduction process as well as on the environmental effects (overfishing, pollutants, food availability ...) on egg quality in an endangered species such as swordfish.

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### **PACAP (PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE) AND ITS RECEPTORS IN *Mus musculus* TESTIS**

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Spermatogenesis is a process finely regulated by both systemic and local testicular factors, as PACAP (Pituitary Adenylate Cyclase-Activating Polypeptide), a neuropeptide of the secretin-glucagon family more abundant in the testis than in the whole brain;<sup>1</sup> it was hypothesized that PACAP is involved in spermatogenesis, steroidogenesis, as well as sperm motility.<sup>2,3</sup>

We have previously investigated the PACAP/receptors localization/role in non-mammalian vertebrate testis<sup>4-7</sup>; now we study the PACAP/receptors system in *Mus musculus* testis and we highlighted that: 1) spermatogonia synthesize PACAP; 2) PACAP and its receptors are widely represented in testicular germ cells, particularly in spermatocytes, spermatids, and spermatozoa, with a distribution partially comparable to that reported in rat and human testis;<sup>2,8</sup> PACAP and its receptors are represented also within Leydig cells: this is the first evidence in mammal testis. The obtained results strongly suggest an involvement of PACAP in the control of spermatogenesis and in steroidogenesis in *M. musculus* testis.

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### **THE SPERM ASTER NUCLEATION AND MICROTUBULE ELONGATION IN *IN VITRO* FERTILIZED SHEEP ZYGOTES**

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Successful fertilization process and embryo development relies on functional centrioles/centrosomes which have been confirmed to be paternally inherited in most farm animals, including sheep.<sup>2,3,4</sup> Shortly after fertilization, the sperm proximal centriole typically nucleates microtubular aster that function as microtubule organizing center and ensure paternal and maternal genomes merging.<sup>5,6,7</sup> At the moment, there are no data on the timing and dynamics of sperm aster organization in sheep. In this study, we have traced the fate of sperm centriole after fertilization to evaluate the timing of the sperm microtubular aster nucleation in early sheep zygotes. To this extend, we have imaged sperm aster nucleation at different post-fertilization moments throughout  $\alpha$ -tubulin immunofluorescence in early *in vitro* fertilized sheep oocytes. To visualize the process of sperm aster nucleation as well as microtubules elongation, sheep oocytes were subjected to *in vitro* maturation (IVM) for 24 h followed by *in vitro* fertilization (IVF). IVF was performed in 50  $\mu$ L drops of *synthetic oviductal fluid* (SOF) with estrus sheep serum and 16  $\mu$ M isoproterenol, covered by mineral oil. In a preliminary experiment, we have established that spermatozoon takes almost 3 hours to complete the fertilization and to enter the oocyte. Fertilization has been arrested at different timing after sperm-egg co-culture (from 4 up to 7 hours) and then presumptive zygotes have been removed from *zona pellucida*, fixed and examined with anti- $\alpha$ -tubulin immunofluorescence under confocal microscopy. We have observed that the sperm centriole initiates sperm aster nucleation within 1 hour post-fertilization (4h from sperm-egg co-culture) and that microtubules elongation takes place approximately 3 hours post-insemination. Future investigations will aim to identify which sperm centriole contributes to embryonic inheritance.

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## THE MUSSEL *MYTILUS GALLOPROVINCIALIS* IN THE NAPLES BAY: NEW INSIGHTS ON OOGENIC CYCLE AND ITS HORMONAL CONTROL

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Mussels were utilized by scientists for many reasons, partly because they are suspension feeders, and need to filter large volumes of water to collect huge amounts of particulate to be used for growth and reproduction.<sup>1,2,3</sup> In *Mytilus galloprovincialis* more recently, we investigated the most relevant aspect of the oogenic cycle, the vitellogenesis, showing the role of germ and somatic cells in such a process,<sup>4,5</sup> as well as the spermatogenic<sup>6</sup> cycle together an investigation on *M. galloprovincialis* as sentinel organism for Naples Bay.

Now, the aim of the present work was to investigate the oogenic cycle of *Mytilus galloprovincialis* sampled in the Naples Bay, and to immunolocalize the  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ -HSD),  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ -HSD) and P450 aromatase, enzymes involved in the synthesis of two sex hormones: testosterone and  $17\beta$ -estradiol. We demonstrate that oogenic cycle starts in late summer-early fall and continues in early winter when the first event of spawning occurs; other spawnings take place until June, when the ovary is spent and constituted by a few empty ovarian follicles and numerous somatic cells, i.e. adipogranular cells (ADGs) and vesicular connective tissue cells (VTCs). During the oogenic cycle, apoptotic events occur at the level of oogonia, previtellogenic oocytes, as well as follicle cells; by contrast, necrosis events probably take place in vitellogenic oocytes, which, once degenerated, transfer their content to healthy oocytes. Finally, the present data demonstrate that  $3\beta$ -HSD,  $17\beta$ -HSD and P450 aromatase are present in the ovary both during the reproductive and non-reproductive phases. The present data suggested that, in *M. galloprovincialis*, sex steroids could have a potential role in the control of reproduction cycle.

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## P450 AROMATASE EXPRESSION IN TESTIS AND BRAIN OF SEASONALLY-BREEDING SPECIES

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P450 aromatase (P450 aro), member of the P450 cytochrome family encoded by the gene *cyp19*, is the key enzyme in the estrogen synthetic pathway. It irreversibly converts testosterone into  $17\beta$ -estradiol (E2). In seasonally-breeding species, E2 modulates the cyclic activity of the spermatogenesis and in the brain has functional properties of neuromodulators, coordinating a

variety of morphological, physiological and behavioral traits needed for successful reproduction. In this study we report testis and brain P450 aro expressions during the reproductive cycle of seasonally-breeding species.

In the testis of both frog (*Pelophylax esculentus*) and lizard (*Podarcis sicula*), P450 aro gene and/or protein expressions change in relation to the reproductive status showing the highest levels in the post-reproductive period. A positive correlation was observed between P450 aro expressions and serum E2 levels through the seasons. Further, P450 aro was prevalently immunolocalized in Sertoli and Leydig cells as well as in spermatids and spermatozoa. Our studies have also demonstrated seasonal fluctuations in expression levels of P450 aro in both frog and lizard brains with higher levels in post-reproductive period. P450 aro was principally detected in a variety of telencephalic and mesencephalic areas including the medial preoptic area, the ventro-medial nucleus of the hypothalamus and the amygdala, i.e. areas involved in sexual behavior and reproduction. Interestingly, it is well known that in song birds brain P450 aro activity plays an important role in activating the vocalization during reproductive season. In non-breeding season P450 aro activity is lower as well as E2 levels. During this period brain-produced estrogens seem to have a direct role in aggression behavior. In conclusion, the seasonal cyclic expression of P450 aro strongly supports the role of this enzyme in modulating the reproductive mechanisms in the seasonally-breeding species, confirming the role of estrogens in the endocrine regulation of the reproductive axis.

## GRAPHENE OXIDE EFFECT ON THE INTERACTION BETWEEN SPERMATOZOA AND FALLOPIAN TUBAL CELLS

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The sperm-uterine tube interaction has been a subject of interest for many years. It is well known that the isthmus of the fallopian tubes acts *in vivo* as a sperm reservoir. Furthermore, this interaction increases sperm capacitation, motility and fertilising ability.<sup>1</sup> Using the unique characteristics of the cells from the tubal epithelium an enhanced fertilization system can be design to improve *in vitro* fertilization success rate. To this purpose, cells have been isolated from both the isthmus and ampullary region of a prepubescent gilt and grown in a culture media in a 5% CO<sub>2</sub> humidified atmosphere at 38.5°C.

Previous work has shown that graphene oxide(GO) can aid spermatozoa capacitation and increase their fertilising ability.<sup>2</sup> Therefore, to establish if the interaction between spermatozoa and graphene oxide interferes with the sperm-tube interaction spermatozoa treated with GO or in control conditions were incubated with the cells isolated from the tube. After incubation the cell/sperm were stained with DilC12 for the membrane, CFDA has viability marker and Hoescht for the nucleus. Comparison between GO and control show no difference in the attachment of spermatozoa to the epithelial cells of gilt tube. These results indicate that GO is not hindering the spermatozoa normal function.

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## THE OVARIAN ADAPTIVE RESPONSE AGAINST DIETARY-INDUCED DICARBONYL STRESS: INSIGHTS FROM THE MOUSE MODEL

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Dicarbonyl stress is a condition characterized by increased levels of highly reactive dicarbonyl compounds, such as methylglyoxal (MG), which interact with biomolecules leading to formation of advanced glycation end products (AGEs). MG and AGEs are known to increase during aging and under conditions of impaired glucose metabolism and/or oxidative stress (OS). In addition, exogenous factors, such as tobacco smoking and diet, may also contribute to increase MG and AGE levels. In the ovary, dicarbonyl stress occurs during reproductive aging and polycystic ovarian syndrome (PCOS). Based on previous studies on in vitro systems,<sup>1</sup> in this work we investigated the ovarian toxicity of dietary MG. Four-week-aged female CD1 mice received water (n=9) or 100 mg/kg MG (n=9) by gastric administration for 28 days.<sup>2</sup> Analysis of follicle population revealed no differences in the number of ovarian follicles, although a reduction of PCNA levels suggested negative effects of MG on ovarian stroma. Moreover, MG increased SIRT1 ovarian levels along with over-expression of catalase, superoxide dismutase 2, SIRT3, and PGC1 $\alpha$ . Finally, similar levels of ovarian MG-AGEs were observed in the two groups, along with enhanced protein expression of glyoxalase 1, the main MG detoxifying enzyme, in MG mice. Although control and MG mice showed similar ovulation rate, immunofluorescence analysis revealed that oocytes ovulated by MG mice exhibited abnormal meiotic spindles, a condition predisposing to embryo aneuploidy. Taken together our results suggest that MG intake triggers an ovarian adaptive response involving a SIRT1 signalling and anti-glycation defence which prevents MG-AGE accumulation, but negatively affects the development of competent oocytes.

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## CUMULUS CELL DETERMINANTS OF OOCYTES DEVELOPMENTAL COMPETENCE

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A third of women undergoing oncological treatment is at risk of premature ovarian failure. *In vitro* maturation of denuded fully-grown antral oocytes (DOs) to metaphase II in the presence of cumulus cells (CCs) and their further cryopreservation is a strategy for preserving women fertility.<sup>1,2</sup> Attempts have been made to culture DOs upon a feeder layer of CCs (FL-CCs) with meager results.<sup>3-5</sup> The purpose of this study was to test whether a selection of CCs prior to the preparation of the FL improves the quality of oocytes maturation. We classified CCs based on their association with developmentally competent (SN) or incompetent (NSN) mouse fully-grown antral oocytes and prepared a FL-SN-CCs or FL-NSN-CCs, respectively. We show that maturation of DOs upon FL-SN-CCs significantly better contributes to the acquisition of oocytes meiotic and developmental competence, with a developmental rate to blastocyst equal to that obtained after the maturation of intact cumulus oocyte complexes.

Instead, DOs matured in the absence of CCs (NO-FL) or upon FL-NSN-CCs undergo meiotic and developmental failure, with embryos arresting either at the 4-cell or morula stage. Our experiments suggest the presence of a crucial developmental CC-factor/s released in the presence of FL-SN-CCs during oocyte maturation. These results set the bases to unravel the molecules involved in the cross-talk between the gamete and its companion cumulus cells during the GV-to-MII transition and to further improve the protocols of antral oocytes maturation in animal studies and human clinical applications.

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## ULTRASTRUCTURAL ANALYSIS OF HUMAN GERMINAL VESICLE-STAGE OOCYTES RETRIEVED AFTER CONVENTIONAL AND MILD OVARIAN STIMULATION

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Immature oocytes can be an alternative source of oocytes for Assisted Reproductive Technologies (ARTs).<sup>1</sup> They can be retrieved by patients undergoing conventional (COS) or mild ovarian stimulation (MOS)<sup>2</sup> protocols. These "leftover" oocytes can increase the yield of total available oocytes in low responder patients or those having an unsynchronized cohort of follicles. This study aimed to describe the ultrastructure of fresh germinal vesicle (GV)-stage oocytes obtained after COS and MOS, to consider the ultrastructure of wellpreserved organelles as indicator of GV quality preservation. GVs were retrieved from consenting donor women undergoing ARTs due to tubal or male infertility factors. Ovarian stimulation (OS) was achieved by a standard long protocol (COS), with GnRH agonists or a mild protocol (MOS), with GnRH antagonists. After retrieval, oocyte were processed for transmission electron microscopy (TEM).<sup>3</sup> Most of COS and MOS oocytes were well preserved and showed a roundish, centrally located nucleus. Mitochondria (Mt) were usually located in a perinuclear position, sometimes associated to small vesicles. Mt appeared more numerous in MOS. Some vacuoles (V) were also located close to the nucleus in both groups, but they appeared more abundant in COS. Migrating cortical granules were dispersed throughout the whole ooplasm. An intact and continuous zona pellucida and uniformly distributed, short microvilli were found in both groups. In conclusion, data obtained showed a variety of ultrastructural alterations, possibly related, at least in part, to the applied OS protocol. In particular, the presence of a reduced number of Mt and of an increased amount of V in COS oocytes suggest that high doses of hormones during COH may affect some microdomains of GV's cytoplasm.

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## **OXYGEN CONCENTRATION ALTERS MITOCHONDRIAL ULTRASTRUCTURE IN PREIMPLANTATION MOUSE EMBRYOS *IN VITRO***

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Different culture media and oxygen (O<sub>2</sub>) concentration are used to culture embryos in Assisted Reproductive Technologies. Embryos cultured under a physiological O<sub>2</sub> tension (5%) give better developmental performance and higher embryo production rates than those cultured under atmospheric O<sub>2</sub> conditions (20%).<sup>1</sup> The mechanisms responsible for these effects linked to reduced O<sub>2</sub> tension in pre-implantation embryogenesis remain unclear but mitochondria (Mt) are believed to play an essential role. The aim of this study was to evaluate the effect of physiologic or atmospheric O<sub>2</sub> tension on the ultrastructure of Mt in mouse embryos. Embryos ovulated *in vivo* were used as control. Zygotes, 2-cells, 4-cells, morulae and blastocysts were flushed out of the uterus after natural fertilization and used as control. *In vitro* fertilization (IVF) was performed using KSOM medium and embryos cultured under different O<sub>2</sub> tension (5% and 20%) until the blastocyst stage.<sup>2</sup> After collection, embryos were washed in PBS, fixed in 2.5% glutaraldehyde/PBS and subjected to standard preparative for transmission electron microscopy observations.<sup>3</sup> The results showed a well-preserved embryo ultrastructure. Mt analysis revealed that embryos cultured in 20% O<sub>2</sub> have a decrease in mitochondrial numerical density, an increase in abnormally shaped Mt and an increase of vacuolization. These morphological alterations in IVF embryos could be associated with a lower mitochondrial membrane potential, lower ATP levels and more ROS levels and could be a major cause of delayed/missed development of pre-implantation mouse embryos during IVF. In addition, this study suggests that changes in the Mt ultrastructure may be part of the mechanism by which lower O<sub>2</sub> concentration leads to better pre-implantation embryo development.

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## **THE IMPACT OF FOOD INTAKE ON SEMEN AND OOCYTE QUALITY**

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A nutritionally unbalanced diet characterized by low intake of proteins, minerals, vitamins and antioxidants has been associated with infertility.<sup>1</sup> Obesity and low body weight may also impair fertility.<sup>2</sup> One of the most involved factors is hyperhomocysteinemia.<sup>1</sup>

The men, who have a low intake of antioxidant nutrients, have less sperm quality.<sup>3</sup> The poor semen quality is associated with a higher intake of products that may incorporate xenobiotics, mainly xenoestrogens or certain anabolic steroids.<sup>4</sup> Several studies reported that Oxidative stress in antral follicle has a deleterious effect on the developmental competence of oocytes. This nutritional effect on oocyte quality can originate

when ovarian follicles emerge from the primordial pool and become committed to growth.<sup>5</sup> Undernutrition at this time reduces the number of follicles that emerge and therefore the number available to ovulate.<sup>6</sup> Nutrition affects not only the number of oocytes that ovulate but also their quality.<sup>5</sup> Good nutrition is an essential component of attaining a healthy pregnancy and birth outcome. The couple should have a well-balanced diet including fruits and vegetables, calcium-rich foods, and protein-containing foods daily and increase their consumption of iron-rich foods, vitamin C-rich foods.<sup>7</sup> A high adherence to the "Mediterranean" diet by couple may improve the chance of pregnancy.<sup>1</sup>

In my clinical experience, couples that changed their diet, reducing refined sugars, increasing the consumption of vegetables and proteins, had a greater number of successful pregnancies. Women, who changed their diet before hormonal stimulation during *in vitro* fertilization (IVF), produced higher quality oocytes with reduced risk of hyperstimol. Most of my patients who have followed low carbohydrate diets experienced increasing chances of embryo implantation.

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## **SIRT1 AND THE ADAPTIVE RESPONSE TO OXIDATIVE STRESS OF HUMAN GRANULOSA CELLS**

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SIRT1 is a NAD-dependent deacetylase that plays a key role in fundamental cellular processes through its activity on histones, transcriptional factors and cell cycle regulators. SIRT1 is a nucleocytoplasmic shuttling protein: is predominantly expressed in the cytoplasm but its nuclear localization is necessary for it to regulate gene expression in the stress response. Under stressing conditions, SIRT1 activates ROS detoxification through up-regulation of scavenging enzymes such as superoxide dismutase (SOD2) through FOXO3a. Since ovarian function can be compromised by stress conditions induced by physiological events such as reproductive aging, the present study was undertaken to determine whether SIRT1 and its signalling pathway are involved in OS response in human granulosa cells (GCs). In the first part, we investigated SIRT1 protein expression and cellular localization in human GCs isolated from young (28-31 years) and aged (39-42 years) women. Although both groups presented similar amounts of SIRT1 protein, it had a different cellular localization. When young GCs were exposed to H<sub>2</sub>O<sub>2</sub>, SIRT1 promptly relocated in the nucleus (nuclear SIRT1: young CTRL 38% vs. young H<sub>2</sub>O<sub>2</sub> 100%). By contrast aged GCs were unable to cope with OS and SIRT1 localization remained unchanged (nuclear SIRT1: aged CTRL 11% vs. aged H<sub>2</sub>O<sub>2</sub> 15%). To better elucidate the role of Sirt1 in OS response, we employed COV434, an immortalized cell line commonly used as experimental model of human granulosa cells. To verify the impact of SIRT1 activity on cell survival, rate of apoptosis, content of oxidized DNA and protein expression, COV434 were exposed to hydrogen peroxide



(H<sub>2</sub>O<sub>2</sub>) in condition of SIRT1 inhibition by EX527. Co-culture with EX527 and H<sub>2</sub>O<sub>2</sub> reduced cell proliferation without affecting the apoptosis rate or increasing FOXO3a and SOD2 protein expression.

Our results reveal a role for SIRT1 in sensing oxidative damage and promoting apoptosis. This can be considered an important starting point for defining human GC response to stress conditions associated with ovarian ageing and dysfunctions.

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## BIOMATERIALS, NANOPARTICLES, NANOSTRUCTURES

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### **NANOACTUATION OF THERMOPHILIC ENZYMES BY ALTERNATE MAGNETIC FIELD: POSSIBLE APPLICATIONS IN INDUSTRIAL BIONANOTECHNOLOGY AND IN NANOMEDICINE**

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We are combining the capacity of magnetic nanoparticles (NPs) to generate thermal energy under the influence of an alternate magnetic field (AMF) with the characteristics of thermophilic enzymes to obtain NP-enzyme systems<sup>1</sup> possessing optimal activity at high temperatures. We have demonstrated that the catalytic activity of these systems can be modulated in a wireless fashion by applying an AMF. In particular, we have synthesized 10 nm iron oxide NPs that we have functionalized with the thermophilic enzymes  $\alpha$ -amylase (AMY) or L-aspartate oxidase (LASPO). Exposing these two NP-enzyme systems (i.e., NP-AMY and NP-LASPO) to an alternate AMF, we have obtained enzymatic activities that are normally reached at 80-90°C without recording a significant raise of the global temperature of the solution in which NP-enzyme systems were suspended.

We are convinced that nanoactuation of thermophilic enzymes by AFM may lead to interesting applications both in industry and in nanomedicine. In industrial applications, enzymatic or even multi-enzymatic processes for *in vitro* synthetic biology could benefit from AFM nanoactuation especially when products, substrates or intermediates are heat-labile. In nanomedicine, NP-enzyme systems might be delivered in the cell cytoplasm or in other cellular compartments and switched on or off with an AMF of appropriate frequency and intensity. This could be exploited to replace missing or non-functional enzymes and regulate them in a remote control fashion. Alternatively, we could avail of these NP-enzyme systems to kill cancer cells<sup>2</sup> or to dissolve atherosclerotic plaques.

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### **EXPOSURE TO COPPER OXIDE NANOPARTICLES (CuO NPs) MODULATES THE OXIDATIVE STRESS RELATED GENES IN *ARBACIA LIXULA* EMBRYOS**

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The development of nanotechnology and the increasing applications of metal oxide nanoparticles (NPs) in a wide range of fields have been rising increasing concerns about their environmental fate and potential toxicity. Although several studies focused on the potential hazards of CuO NPs to aquatic life, their environmental impacts and toxicity mechanisms still have been poorly elucidated.<sup>1,2</sup> Developmental abnormalities such as alterations in larval skeletogenesis and neurotransmission pathways have been

reported in sea urchins exposed to copper oxide nanoparticles<sup>3,4,5</sup> but their effects on antioxidant defences have not been investigated.

In the current study, the potential role of oxidative stress in CuO NPs toxicity was evaluated in sea urchin *Arbacia lixula* embryos exposed to three CuO NPs concentrations (0.7, 10, 20 ppb) until the pluteus larval stage (72 hours post-fertilization, hpf).

Quantitative real time PCR revealed a time- and concentration-dependent modulation of oxidative stress-related genes, i.e. *Cu/Zn-superoxide dismutase (Cu/ZnSod)* and *catalase (cat)* together with *metallothionein (mt)*, here cloned and molecularly characterized for the first time. These transcriptional responses strongly support the hypothesis that the toxicity of CuO NPs is related to reactive oxygen species (ROS)-mediated pathway and provide insight into the possible molecular mechanisms underlying copper nanoparticles toxicity in *A. lixula* sea urchins. The obtained results provide new biomarkers for monitoring of aquatic environments while corroborating the suitability of *A. lixula* embryotoxicity assay<sup>6</sup> for future ecotoxicological investigations of impacted marine areas.

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## BIOLOGICAL INTERACTIONS AND EFFECTS OF METAL OXIDE NANOCOLLOIDS IN *IN VITRO* AND *IN VIVO* SYSTEMS

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In recent decades, metal oxide nanoparticles (MONPs) have found widespread applications in the biomedical and agricultural fields due to their strong biocidal activity, while their biocompatibility and adverse outcome pathways are still debated. Their effectiveness, based on the high volume surface ratio can be reduced or modified by agglomeration phenomena, which compromise the stability of NPs suspensions. In this matter, the synthesis of MONPs coated with capping agents can help to improve NP stability and avoid agglomeration. Moreover, the application of MONPs in colloidal form is inevitable for better assimilation and functioning of these agents in the bio-systems.

With the aim to investigate the comparative toxicity of colloidal suspensions of CuO and ZnO NPs, coated with different polymers (PEI, PEG or PVA), and to contribute in nanotechnology safety aspects, in this study we used human lung A549 cells and *Xenopus laevis* embryos as *in vitro* model for inhalation toxicity and *in vivo* model for aquatic toxicity respectively.

A549 viability results showed that all coated ZnO NPs and PEI-CuO NPs (>10 µg/mL) were strongly cytotoxic, while PEG-CuO NPs were less effective even at the highest doses. Unexpectedly, the proinflammatory response (IL-8 levels) increased in a dose-dependent manner after treatment with both CuO NPs, regardless of the coating.

The standard Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) evidenced that all coated-ZnO NPs were not embryolethal but able of inducing malformations (mainly abnormal gut coiling and abdominal edema). While the PEG-CuO NPs were the safest, PEI-CuO NPs showed the highest developmental hazard with an LC50 of 7.5 mg/L and a TI of 1.53. Also, the ICP analy-

sis showed that PEG coated ZnO and CuO NPs were less concentrated in stage 46 embryos.

Taken together the results suggest that the effects and modality of bio-interactions of colloidal CuO and ZnO NPs is dependent on the type of coating polymer and that PEGylation of CuO NPs in particular is promising in a safe-by-design approach.

## PACAP-LOADED LIPOSOME DELIVERY ACROSS THE BBB: A LIGHT-SHEET MICROSCOPY STUDY

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The blood-brain barrier (BBB) impermeability and selectivity prevent the transport of many therapeutic molecules into the brain, making ineffective their use for treatment of neurological diseases.<sup>1</sup> Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuroprotective peptide proposed for treatment of central nervous system (CNS) diseases.<sup>2</sup> However, its clinical use is limited by the efflux component of peptide transport system-6 (PTS-6), which reduces its brain uptake<sup>3</sup>, and also for its low stability in human plasma, rapid degradation and peripheral actions.<sup>4</sup> Nanocarrier-mediated method is a non-invasive strategy to explore for brain drug delivery; among them, liposomes are attractive tools that can be easily modified to improve their delivery.<sup>5</sup> We developed liposomes loaded with PACAP and functionalized on the surface with gH625 peptide, a membrane-perturbing domain in glycoprotein H of *Herpes simplex virus 1*. gH625 can traverse the membrane bilayer and deliver several cargoes across cell membranes *in vitro*<sup>6</sup> and crosses the BBB *in vivo*.<sup>7</sup> We evaluated the efficiency of gH625-liposomes to deliver PACAP to the brain in Swiss CD1 mice after intravenous administration using light sheet fluorescence microscopy. Our results show that gH625-liposomes ameliorate both PACAP reaching and crossing the BBB, increasing the number of neuronal cells labeled with PACAP. These data suggest that gH625-liposomes represent a promising strategy to deliver therapeutic agents to CNS for the treatment of neurological diseases but also to provide an effective imaging and/or diagnostic tool for the brain.

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## CAN ENGINEERED NANOPARTICLES CROSS THE SKIN BARRIER IN EMBRYOS OF THE WATER FROG *Pelophylax kl. esculentus*?

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Engineered metallic nanoparticles (NP) can enter the natural environment via several routes<sup>1</sup> and accumulate in living organisms.<sup>2,3</sup> In the water frog *Pelophylax kl. esculentus* treatments

with iron, cobalt and nickel NP resulted in a marked retard in growth as well as an increase of malformations, while the epidermal cells showed a stressed condition as indicated by hypertrophy and mitochondrial swelling.<sup>4</sup> It is not clear how the cited effects were caused and in particular whether the NP entered the cells and altered the metabolic pathways. In the present contribution we report our attempts to observe the passage of NP across the epithelial cell membranes by TEM observations. Embryos at the developmental stage 10 (earliest involution of blastopore dorsal lip) of *Pelophylax kl. esculentus* from an artificial tank in the University Botanic Garden in Bari were treated with iron, nickel or cobalt NP. A control group and three treatments per NP at concentration of 0.1 mg/mL, were considered, for a total of four groups. Each group included about 10 individuals. Groups were monitored for the following ten days. Animals showing malformations were selected for the TEM analysis. Samples were fixed in 4% glutaraldehyde and then embedded in Epoxy Resin-Araldite. Ultra-thin sections were stained with uranyl acetate.<sup>5</sup> The epidermis of the embryos presented four cell types, i.e. ciliated cells, muciparous cells and two types of ionocytes, indicated as I and II, respectively. In the ionocytes, aggregates of NP were observed out of the cell membranes and in endocytotic vesicles in the cytoplasm. Thus, there would seem that NP actively enter the ionocytes via an endocytotic pathway.

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## BIOMATERIALS AND TISSUE REGENERATION

### BIOCOMPATIBLE SCAFFOLDS FOR TISSUE REGENERATION

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The design of scaffolds based on biocompatible materials is a great challenge in tissue regeneration aiming to obtain biomaterials with structural and functional characteristics adapted to the desired tissue. The building block of this project was a system based on the use of a protein, 2-cys peroxiredoxin (Prx) and graphene oxide in combination with various biocompatible supports. A Prx mutant (from the parasite *S. mansoni*) can grow as an array of protein nanotubes<sup>1</sup> triggering the differentiation of SH-SY5Y cells and sustain the growth and development of cortical neurons as well as the spread-out of glioma cancer stem cells.<sup>2</sup> In this work the protein was combined by a self-assembly process with graphene oxide. The porous scaffold obtained is still able to trigger morphological changes in neuronal precursors, such as SH-SY5Y, towards a neuronal-like phenotype. Similar results were obtained by temperature-triggered reduction of GO, suggesting a redox process as one of the basis of the biological stimulation. The expression of typical differentiation markers and factors that decide cell fate in relation to adaptation to the external environment, such as YAP/TAZ, confirmed the activation of the differentiation pathway<sup>3</sup> in cells grown on GO/ Prx composite materials. These composites were effective also once deposited onto decellularized bovine pericardium, chitosan or alginate.<sup>4</sup> In addition, graphene foams<sup>5</sup> have been linked with smPrxI to obtain a 3D implantable scaffold. All of these approaches have proved suitable for the growth and differentiation of neuronal precursors thus encouraging further ongoing experiments.

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### TISSUE ENGINEERING APPROACHES FOR BRAIN INJURY APPLICATIONS

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Disruptions of central nervous system (CNS) architecture are devastating, due to the irreversible process of neuronal death, the limited regenerative capabilities of brain and the current lack of effective treatments. Nowadays, regenerative medicine and cell replacement therapies are very promising approaches to treat

the damaged brain.<sup>1</sup> In this context, stem cells (SC) represent an important cell source for transplantation therapies, and biomaterials can help in recapitulating the three-dimensional environment of the brain that usually gets compromised in the injured site.

Among the different types of scaffold, hydrogels are very interesting for brain tissue engineering. We are currently working on alginate hydrogels to support human neural- and mesenchymal-SCs injection into the brain. Indeed, regarding the cells source, the transplantation of both NSCs and MSCs has been reported to elicit beneficial effects by regenerating neurons or secreting molecules and factors that help in the healing process.<sup>2,3</sup> At the moment we are focusing on the identification of the best conditions for NSCs and MSCs coculture in alginate hydrogels. We have thus started to investigate whether we can create "compartmentalized" co-cultures that would at least partially retain serum in one compartment only. We have also shown that MSCs can survive, proliferate and maintain their stemness even in absence of serum, supporting the hypothesis that the use of "compartmentalized" co-cultures with low serum content would likely be effective.

In the future, the coculture of MSCs and NSCs would allow *in vivo* applications such as transplantation in injured animal models for CNS tissue regeneration.

#### **A MODEL OF CARDIAC REGENERATION AND LONG TERM CULTURE OF CARDIAC-DERIVED CELLS IN ZEBRAFISH**

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Zebrafish hearts can regenerate through activation of growth factors and trans-differentiation of fibroblasts, epicardial, myocardial and endocardial cells,<sup>1,2,3</sup> all positive for GATA4 during the process.<sup>4,5</sup> A innovative model of regeneration of the whole heart and the regenerating cells in *ex vivo* culture is developed by a stimulation of cocktail of growth factors. In *ex vivo* growth-factors-supplemented culture the heart regeneration was quite complete without signs of fibrosis. Epicardial- and endocardial-derived cells have shown capability to moving out of the hearts in culture and were analysed and then expanded to obtain long term culture. Electron microscopy evidencing two main types of : 1) larger/prismatic and 2) small/rounded. Type(1) showed on the surface protein-sculptures, while type(2) was smooth with sparse globular proteins. To confirm their nature we have contemporarily analysed their proliferative capability and markers-positivity. The cells treated by growth factors have at least two-fold more proliferation with GATA4-positivity. The type (1) cell evidenced WT1+ (marker of embryonic epicardium); the type (2) showed NFTA2+(marker of embryonic endocardium); whereas cTNT-cardiotroponin (marker of cardiomyocytes) was negative. Under growth factors stimulation, GATA4+/WT1+ and GATA4+/NFTA2+ could be suitable candidates to be the cells with capability to move in/out of the tissue, probably by using their integrins. The model of regeneration in *ex vivo* open the possibility to have long term selected organ/culture to future translationa studies.

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## **DEVELOPMENTAL TOXICITY**

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#### **COCAINE EFFECTS ON THE GILLS OF THE EUROPEAN EEL, *ANGUILLA ANGUILLA***

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Many illicit drugs and their breakdown products are detected in the aquatic environment due to the worldwide use of these substances and the variable efficiency with which they are removed from sewage effluent in sewage treatment plants.<sup>1</sup> These substances have strong pharmacological activity; indeed, despite their low concentrations in surface waters, the first studies showed toxic effects for aquatic organisms.<sup>2,3,4,5</sup> The aim of this study was to evaluate the influence of environmental concentrations of cocaine, an illicit drug widespread in surface waters, on the gills of the European eel (*Anguilla anguilla*). A stock solution of 0.006 mg mL<sup>-1</sup> cocaine free-base in ethanol was prepared. Male silver eels were exposed for fifty days to 20 ng L<sup>-1</sup> of cocaine, corresponding to the mean cocaine concentration detected in surface waters.<sup>1</sup> The gills of cocaine exposed eels were compared to untreated control and vehicle control groups. The morphology of the gills and plasma levels of prolactin and cortisol, involved in gill physiology regulation, were evaluated. The gills of cocaine-exposed eels showed an hyperplasic epithelium, in which many mucous cells were observed, and lamellar fusion. Moreover, cocaine increased plasma prolactin and cortisol levels. The changes observed in the gills epithelium agree with those observed in the intestine<sup>4</sup> of cocaine-exposed eels. Since in fish prolactin and cortisol stimulate epithelial cell proliferation and the differentiation and proliferation of mucous cells, these hormones might be involved in the changes induced by cocaine. Fish gills are involved in gas exchange, in the exchange of salt and water and excretion of nitrogenous waste products; therefore, even slight structural changes can render a fish vulnerable to osmoregulatory and respiratory difficulties. This study shows that even low cocaine concentrations affect the gills, suggesting potential impact on the survival of this species.

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#### **EFFECTS OF CADMIUM AND ALUMINIUM ON GROWTH AND MOTILITY IN *Danio rerio* EMBRYOS**

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Cadmium (Cd) is known as a potent toxic metal also at low concentrations. It is biologically non-essential and subject to the bioaccumulation phenomenon. Aluminium (Al) is the third most abundant element of the earth. Some investigations on environmental toxicology revealed that high concentrations of Al may present a major threat for humans, animals and plants causing



many diseases.<sup>1</sup> Our previous studies showed that both Cd and Al affect development of *Danio rerio* (zebrafish) and damage the nervous system although at significantly higher concentrations for Al.<sup>2,3</sup> *D. rerio* is becoming a focus of neurobehavioral studies.<sup>4</sup> In the present study we have analysed the effects of Cd and Al at sublethal concentrations on the development of *D. rerio* by the observation of embryos dechorionation and motility at 78 hpf (hours post fertilization). The treatments were performed on embryos at 6 hpf for 72 hours. Four groups of embryos were exposed to Cd at the concentrations of 9, 18, 36 and 72 µM. Other three groups were instead exposed to Al at concentrations of 50, 100 and 200 µM. Another group of embryos was kept as control. Cadmium harmed the embryos at early stages of development by a delay of the exit from the chorion and this effect increased significantly at higher concentrations. Also Al caused a similar delay but this inhibition appeared particularly evident at lower concentrations. Moreover we tested different parameters for the study of embryos motility at 78 hpf using the DanioVision. Both metals, albeit in different way, affected significantly the behavioral parameters such as Distance moved, Velocity mean, Cumulative movement, Meander and Heading. These results are indicative of the toxic effect of Cd and Al at sublethal concentrations on embryos development of *D. rerio* suggesting the need for further experiments to elucidate the different mechanisms underlying such alterations.

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## DEHP INVOLVEMENT ON RAT SPERMATOGENESIS IMPAIRMENT

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In recent decades, high incidences of male reproductive dysfunctions have been associated with exposure to Endocrine Disruptors Chemicals (EDCs) during perinatal and neonatal life. Among EDCs, phthalates are an important group of multifunctional and environmental chemicals widely used as plasticizers and solvents in many different applications.

Di-(2-ethylhexyl)phthalate (DEHP) is one of the most found phthalate in the environment; it is well known for being toxicant for the male reproductive system. It exerts antiandrogenic effects by suppressing fetal testosterone biosynthesis which in turn led to male reproductive tract anomalies. DEHP effects depend on developmental stage of organism at the time of exposure, hence in this study, after gestation and lactation exposure, we evaluated the effects of different doses of DEHP on testicular histopathology during different periods of development. First, testes were stained with hematoxylin and eosin for histopathological evaluation. To better investigate DEHP effects on germ cells and Leydig cells proliferation, we performed immunohistochemistry for ki-67. Moreover, to understand the potential mechanism for phthalate impairing testis development, we also performed AR immunostaining. DEHP treatment did not cause AR mislocalization indicating that DEHP is not AR antagonist.

Moreover, we also analyzed whether DEHP was able to induce alteration in the expression of Sertoli cells junctions such as gap (GJ) and tight junctions (TJ) which are important for maintain-

ing spermatogenesis and establish the blood-testis barrier (BTB).

We showed that DEHP effects were age and dose related and may induce perturbation on junctions that can be one of the contributing factors that lead to impairments in spermatogenesis of treated rats.

## DOSE-RESPONSE EFFECTS OF THE MIXTURE OF TWO ANTIFUNGAL AZOLES (CYPROCONAZOLE AND TRIADIMEFON) RECORDED AFTER *IN VITRO* OR *IN UTERO* EXPOSURE

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In modern developmental toxicology, the evaluation alternative tests to assess the hazard derived from mixture exposure to molecules of the same chemical class or showing a common mode of action is a nodal issue. Cyproconazole (CYPRO) and Triadimefon (FON) are antifungal azoles used alone and in mixture in agriculture and in post manufacture treating. Both molecules have been related to teratogenic effects (cranio-facial defects) in experimental models. The aim of the present work is to compare the effects of the CYPRO and FON after single exposure and in mixture using two different animal models: the traditional mouse *in utero* exposure and the postimplantation rat whole embryo culture (WEC) methods. Pregnant mice were orally treated (gavage) at day 8 of gestation (E8) with a single dose of vehicle (DMSO: corn oil 1:9) or with crescent doses of CYPRO, FON or their mixture. At E18 maternal and foetal parameters were recorded. Gross malformations (including cleft palate) were recorded. Postimplantation rat embryos were explanted at day 9 (E9, corresponding to the developmental E8 stage in mice) and cultured in presence of increasing concentrations of CYPRO, FON or their mixture. After 48 hours of culture, any embryo abnormality (including defects at the level of cranio-facial primordia, the branchial structures) was evaluated. Data obtained by the two different models were modelled by PROAST 65.2 software in order to characterize the dose-response curves and to obtain the relative potency factor (RPF) and evaluate if dose-addiction occurs.

Specific concentration-related defects at the level of cranio-facial structures were described both after *in utero* and *in vitro* exposure in a dose-related manner. Additive effects were recorded after mixture exposure. Our results show that both the *in utero* and the *in vitro* exposure to the test molecules or to their mixture are effective to induce specific cranio-facial defects. Our results suggest the use of WEC as a valid alternative method for the study of mixture effects of pesticides and also suggest the inclusion of the two tested azoles in a cumulative assessment group for risk assessment.

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## **BIOCIDE TRICLOSAN DAMAGES BYSSAL APPARATUS IN MYTILUS GALLOPROVINCIALIS**

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In this study we describe the effects of a common environmental contaminant, the Triclosan<sup>1</sup>, on the byssal apparatus of the marine mussel *Mytilus galloprovincialis*. The aim in particular was to understand if this biocide, largely used in personal care products, impairs byssal threads formation, structure and/or anchoring efficiency. To this end, the mussels were exposed for 7 days to 10 µg/L triclosan<sup>2,3</sup> and the effects were monitored by a multidisciplinary approach. The effects on byssal glands cytoanatomy were studied by light and electron microscopy while the biochemical effects were investigated in situ by PAS staining and in protein extracts. Functional impairment of byssal glands was assessed by determining changes in thread growth rate and resistance to traction after cut off;<sup>3</sup> the organization of regrown byssal threads was also verified in histological sections.

Experimental evidences indicate that marked alterations are induced by the biocide, primarily in collagen polymerization. This interference causes a significant loss in threads resistance and also a delay in regrowth. Such alterations would cause a consistent loss in the ecological fitness of mussels in nature since they typically live in areas exposed to tidal and waves action, protecting the coastline from erosion. Triclosan release in coastal environments therefore should be more carefully monitored so to prevent unwanted drastic consequences.

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## **IN VIVO EVALUATION OF ELLAGIC ACID EFFECT IN DANIO RERIO EMBRYOS**

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Reactive oxygen species (ROS) play a fundamental role in many cellular processes, including proliferation and differentiation. However, when the ROS are in excess, the endogenous antioxidants are unable to restore the basal level, leading to oxidative stress (OS). The OS can cause structural and functional alterations of DNA during delicate life phases of organisms as embryonic development. In this regard, the aim of this work was to evaluate the protective effect of an antioxidant molecule, ie ellagic acid ((EA)(2,5 – 5 – 10 mM)), in *Danio rerio* embryos against to a genotoxic agent (H<sub>2</sub>O<sub>2</sub> 15 µM). The evaluation of cytotoxicity and genotoxicity of H<sub>2</sub>O<sub>2</sub> and the protective role of EA, were performed by observing embryos morphology and larvae behavior, and estimating the oxidative and DNA damage by RAPD-PCR and DCF Assay. Embryos exposed to H<sub>2</sub>O<sub>2</sub> showed morphological alterations, such as body hypopigmentation, calf sac edema and altered natal movements after larval hatching (72hpf). The genotoxicity tests confirmed the negative activity of H<sub>2</sub>O<sub>2</sub> by reducing GTS% and inducing high production of intracytoplasmic ROS for two exposure times (8h and 20h). The embryos exposure to EA did not induce damage to any biological

level, except for 10 mM EA treatment that denotes a statistically significant mortality at 24hpf. The combined treatment with EA (2.5 and 5 mM) + H<sub>2</sub>O<sub>2</sub> demonstrated the capacity of EA to protect the embryo development and DNA from the H<sub>2</sub>O<sub>2</sub> negative action. In particular, the co-exposure to EA 5 mM + H<sub>2</sub>O<sub>2</sub> determined an increase in survival for all exposure times. No morphological alteration, pigmentation changes or larvae abnormal movements were observed. An increase of 56% in GTS% and a drastic reduction of intracellular ROS% compared to the positive control has been highlighted for the two coexposure times. In conclusion, EA is a powerful antigenotoxic agent and it is able to protect the earliest stages of embryonic development from damage induced by mutagenic substances. We believe that these results can stimulate a great scientific interest because EA is a natural substance which could be used in the prevention of many oxidative stress diseases during ontogenesis.

## **GENOTOXICITY AND TOXICOLOGICAL EVALUATION OF ENDOCRINE DISRUPTORS ON ZEBRAFISH (*DANIO RERIO*) EMBRYOS**

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The aim of the present study was to evaluate *in vitro* genotoxicity via SOS-chromotest<sup>1</sup> model for three endocrine disruptors [Bisphenol-A (BPA)<sup>2</sup>, pentabromodiphenyl ether (BDE-99) and di-*N*-hexyl phthalate (DnHP)] and toxic effects (lethal and sub-lethal effects) of BPA (0.5, 2, 4, 8, 16 and 32 mg/L) and BDE-99 (0.6, 5.7, 14.20, 28.30, 42.40, and 56.50 µg/L) exposure along with positive and negative control groups on zebrafish (*Danio rerio*) embryos via Fish Embryo Toxicity (FET)<sup>3</sup> test according to OECD n.236. Results of SOS-chromotest have been shown that DnHP activating with rat enzyme has significantly higher genotoxicity [2.63463 induction factors (IF)] as compared to control, however, as we are interested in zebrafish model, so BPA activated with trout enzyme showed high genotoxicity (0.48614 IF) than BDE-99 and DnHP. In the second part of the research, FET test have shown that BPA at 16 and 32 mg/kg reported 100% mortality and EC50 is 7.350 mg/L at 95% confidence limits (0-96 hpf) along with delay in development, multiple deformations (blood stasis, tail deformation, deformed head, yolk edema, pericardial edema and no blood circulation) and low heartbeat in lower doses. For BDE-99, however all the tested groups shown sub-lethal effects and didn't reach to EC50 while the mortality decreased from 25 to 5% while increasing the dose. In conclusion, BPA revealed a great genotoxicity and reported also a high toxicity versus zebrafish embryos, while acute lethal and sub-lethal effects on zebrafish embryos development by high doses of BPA and lowest dose of BDE-99 highlight the risk posed to fish development upon exposure to environmentally relevant concentrations of pollutants.

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## EFFECTS OF OCHRATOXIN A ON DEVELOPMENTAL POTENTIAL OF LAMB OOCYTES

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The aim of the study was to evaluate the effects of OTA, a mycotoxin produced by *Aspergillus* and *Penicillium* fungi and reported as an ubiquitous contaminant of food and feed<sup>1,2</sup> on: 1) nuclear and cytoplasmic maturation and 2) developmental potential of lamb oocytes. Cumulus-oocyte complexes (COCs) were recovered at local slaughterhouses from lamb ovaries. During in vitro maturation (IVM)<sup>3</sup>, COCs were exposed to 10 µM OTA, as in a previous study in mice.<sup>4</sup> Vehicle controls (IVM medium with 1% methanol) and standard controls (IVM medium without vehicle) were used. In Experiment 1 (Exp.1), after IVM, COCs underwent cumulus removal and staining of nuclear chromatin and mitochondria (mt)<sup>3</sup>. Oocytes in metaphase II underwent confocal microscopy to analyze their mt distribution pattern.<sup>3</sup> In Exp.2, oocytes underwent IVM, in vitro fertilization (IVF) and embryo culture up to day 7.<sup>3</sup> Cleavage and blastocyst formation rates were recorded. Data were analysed by Chi-square test (differences significant when P<0.05). In Exp.1, 218 oocytes were analyzed. Lack of vehicle-related effects was noticed (23/37, 62% vs 62/96, 65%, for oocytes cultured with or without vehicle; NS). OTA tended to reduce the maturation rate (39/85, 46% vs 23/37, 62%, for exposed and controls, respectively) even without statistical significance. Instead, it reduced the rate of oocytes with healthy perinuclear/pericortical mt pattern (4/39, 10% vs 9/23, 39%, P<0.05). In Exp.2, (n=275 oocytes analyzed) no effects were noticed on cleavage (22/90, 24%; 24/92, 26%; 31/93, 33%) and blastocyst rates (4/90, 4%; 1/92, 1%; 2/93, 2%) between OTA and controls with or without vehicle (P>0.05). In conclusion, OTA hindered nuclear and cytoplasmic maturation of lamb oocytes but do not affect their developmental potential.

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## THE USE OF THE XENOPUS LAEVIS AS A SENSITIVE ALTERNATIVE TEST FOR THE STUDY OF THE EMBRYOTOXICITY OF THE ANTI-EPILEPTIC DRUG VALPROIC ACID

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Even if the teratogenic effects of the anti-epileptic drug sodium valproate (VPA) are known also in humans, where the Fetal Valproate Syndrome (FVS) has been identified, a sensitive alternative animal model was not described till now. FVS characteristics include facial features and defects (including cleft lip/palate), neural tube defects (NTDs), heart defects, genital

and skeletal defects and developmental delays. VPA exposure is related to teratogenesis (mainly skeletal anomalies) in different mammals while NTDs have been reported only in sensitive mouse strains. A complete FVS picture, including both cranio-facial and neural tube defects, has never been described in alternative animal models. We propose a modified Frog Embryo Teratogenicity Assay: Xenopus (FETAX) as an animal free model for FVS evaluation.

Early post fertilization embryos, obtained by natural mating, were exposed to VPA (sodium salt) diluted in FETAX (VPA 0-500-750-1000-1500 mM). Continuous exposures from Nieuwkoop and Faber stage (NF) 13 to 46 or pulse exposure (NF 13-26 or NF 26-46) were performed. NF 13 corresponds to late gastrulation/initial neurulation, NF 26 to the phylotypic stage, NF 46 to the tadpole stage considered the end of the classical FETAX test. Mortality and external morphology were evaluated during the entire test period. At the end of the test tadpoles processed for the staining of cartilage.

High rate mortality was observed after the continuous exposure at any tested concentration and at the highest tested concentration after NF 26-46 exposure regimen. Mortality was not significant after NF 13-26 exposure. Dose dependent abnormalities at the head were observed in tadpoles exposed in NF 13-46 window. The head defects were dose-dependent and related to FVS picture (shortened and abnormal anterior region related to abnormal cartilaginous elements and swollen, bent and shortened encephalon). The proposed alternative model resulted adequate for the study of FVS at concentrations similar to those reported in patients (280-700 mM). We suggest the use of this alternative animal model in order to evaluate VPA analogues and VPA-related teratogenic pathway.

## THE DARK SIDE OF FOOD COLORS

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Food colors, or color additives, are substances imparting color to commercial food and drinks and to a variety of non-food applications such as cosmetics, pharmaceuticals, home craft projects and medical devices. Of natural or artificial origin, their safety has been long discussed and concerns about consumer health led to testing for safety and to certification. From 1973 interferences with activity in children is generally accepted and warnings are now present on products labels. Much less attention however has been dedicated to the possible effects of these additives on natural flora and fauna and, in particular, on the aquatic ones. In our work we have tested the toxicity of 4 different commercially available food colors: one natural, cochineal red E120 and three of synthesis: Ponceau red E124, tartrazine yellow E102 and patent blue E131. Concentrations were the same suggested in labels for preparing food (650 mg/500 ml milk or cream). Toxicity was tested on *Cucumis sativus* (Cucurbitales), *Artemia salina* (Crustacea Anostraca) and *Danio rerio* (Actinopterygii Cyprinidae) development. Results have demonstrated that the four food colors significantly interfere with *Cucumis* germination and rootlets formation and that they moderately alter toracopods development in *Artemia*. In *Danio rerio*, embryos show significant alterations with pericardial oedema, hypopigmentation and anomalous development of tail and body axis. Results, though preliminary, suggest that food colors are potentially toxic to the flora and aquatic fauna and that attention should be devoted to this so far ignored consequence of our habit of artificially coloring the world around us.

## GLYPHOSATE: TOXIC OR NOT TOXIC, THIS IS THE QUESTION

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The potential toxicity of Glyphosate-based herbicides (GBHs), a broad spectrum herbicides widely used in agricultural, industrial and urban areas, is a great matter of debate. Although considered *non-toxic and not an irritant* (EPA) and *no carcinogenic to humans* (EFSA), converging evidence suggests that GBHs pose serious health risk on non-target wildlife. Many studies demonstrate that GBHs threaten the reproduction interfering with the activity of aromatase, an important enzyme involved in the production of estrogen.<sup>1</sup> Moreover, in fish and mammals GBHs cause the raise of oxidative stress markers and tissues alterations.<sup>2,3</sup> Hence, the question about the glyphosate (Gly) toxicity is still open. To concur to answer to this question, we investigated the effects of Gly exposure on the wall lizard *Podarcis sicula*, a suitable bioindicator of soil pollution. Adult *P. sicula* specimens were divided in 3 groups (n=6): 2 groups exposed to pure Gly 0.1 and 1 µg/L, respectively, via gavage every other day for 3 weeks; group 3 received by gavage the same dose of tap water (100 µL). The results demonstrate that both Gly doses affect the male gonad. Spermatogenesis is slightly slower, spermatocytes II fuse to form rosette-shaped arrangement, spermatids appear damaged and cells in degeneration are evident in the tubules lumen. Changes in the expression of estrogen and androgen receptors and aromatase have been also detected. In females, the ovary is not affected by Gly exposure, no matter the dose.

Livers show signs of suffering, regardless of the animals sex. The increase in melanocytes degranulation and nodular/cystic formations, mainly consisting of collagen fibers, have been observed. The liver of Gly-treated males also displays the biosynthetic alterations typical of an estrogenic contamination: hepatocytes, in fact, contain transcripts for both vitellogenin and estrogen receptors. Data suggest that Gly exposure in a vertebrate commonly inhabiting the fields potentially exposed to GBHs causes reproductive and tissue toxicity, with possible health implications for wild and breeding animals, as well human populations.

## STEM CELLS AND CANCER STEM CELLS

### THE POTENTIAL OF MASS CYTOMETRY TO LOOK INTO THE RHABDOMYOSARCOMA HETEROGENEITY AND CELL ORIGIN

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Over recent years it has become even more evident that cell populations, considered homogenous, are instead characterized by intrinsic heterogeneity. This observation demanded the development of technologies able to analyze cell populations at single cell level. Mass cytometry is a new technique that offers the possibility to do a multi-parametric analysis of complex cell populations with single-cell resolution. The Cytof 2 instrument permits to analyze up to 40 cell surface or intracellular markers by tagging antigen specific antibodies with stable heavy metal isotopes.<sup>1,2</sup>

We applied this technique, to evaluate the changes in the mononuclear cell populations composition in the muscle during tumor development. We focused on the rhabdomyosarcoma (RMS), a soft tissue sarcoma which has an incidence of 4.5 cases per million of adolescents. It generates in various body regions, most commonly in the head and neck, in the extremities and in the genitourinary tract.<sup>3</sup> RMS, which can be classified in two major subtypes, embryonal (eRMS) and alveolar (aRMS), on the base of histological and pathological characteristics, may share the same originating cell(s). This conclusion, however, is still debated. Therefore, we decided to investigate the variations in the mononuclear population profiles during cancer development in order to identify the cell type involved. To this end, we have compared the cell populations of healthy muscles with that of rhabdomyosarcoma. To achieve our objective, we adopted the KRAS<sup>LSLG12D::Tp53<sup>fl/fl</sup></sup> conditional mouse model in which the undifferentiated myosarcomas are induced in a spatio-temporal controlled manner by using an adenovirus expressing the CRE recombinase.<sup>4</sup> In this mouse model, by inducing chromosomal rearrangements it is possible to achieve constitutive activation of KRAS along with the inactivation of the Tp53. Hence exploiting this inducible cancer model, we were able to analyze tumor progression and composition, unravelling differences in terms of initiating cell population.

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### HUMAN NEUROEPITHELIAL STEM CELLS IN NEURODEVELOPMENTAL AND NEURODEGENERATIVE DISORDERS

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Human Neuroepithelial Stem (NES) cells are long-term, self-renewing neuroepithelial stem cells forming neural rosettes reminiscent of the radial arrangement and apico-basal polarization established by neuroepithelial cells in the native neural tube. Single-cell RNA-sequencing on expanded NES cells and cells from donor-matched brains demonstrated that NES cells exhibit a close transcriptional signature of early neural stem cells as their donor-matched isogenic brain cells<sup>1</sup>. NES cells represent a unique model for early human neurodevelopment and pathobiology, which we have used to unravel Zika virus (ZIKV)-infection and related neuropathogenesis, including microcephaly. We showed that ZIKV infects neocortical NES cells, causing disrupted mitoses, supernumerary centrosomes, structural disorganization, and cell death. ZIKV infection caused centrosomal depletion and mitochondrial sequestration of phospho-TBK1 (pTBK1) during mitosis. We also found that nucleoside analogs inhibit ZIKV replication in NES cells, protecting them from ZIKV-induced pTBK1 relocalization and cell death<sup>1</sup>.

NES cells were also derived from human developing spinal cord, to investigate traumatic spinal cord injury, a condition resulting in persistent disability due to disconnection of surviving neural elements. We describe robust engraftment of human NES cells into rodent spinal cord injury lesions. Extensive elongation of both graft and host axons occurs. Thus, human NES cells provide a platform to interrogate neurodevelopmental and degenerative human conditions.

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### PLURIPOTENCY GENE EXPRESSION DURING REVERSE DEVELOPMENT OF THE “IMMORTAL” HYDROMEDUSA, *Turritopsis dohrnii* (Cnidaria)

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Hydrozoan life cycles typically encompass a swimming larva, a colonial asexual stage, the sessile benthic polyp, and a pelagic, sexually competent stage, the planktonic medusa. Medusae are typically semelparous free-spawners, and rapidly die after reproduction. However, medusae of *Turritopsis dohrnii* are able to fully rejuvenate by asexually reverting the medusa morph into the polyp colony, through an intermediate, cyst-like stage. Also known as reverse development (RD), this process is induced by different kinds of stress, including lack of food, reduction of seawater salinity, physical damage, as well as sexual reproduction and aging. The RD involves cell transdifferentiation, i.e. a change in commitment and gene expression of well differentiated, somatic cell types.

By TEM and confocal microscopy analyses, we studied the dynamics of cell and tissue reorganization throughout the RD course. To shed light on the molecular mechanism driving the RD, we analysed the spatial and temporal expression patterns of some pluripotency genes identified in the transcriptomes of *T. dohrnii* across different RD stages. The putative orthologs of *Sox2*, *Oct3/4*, *cMyc* and *Nanog*, four transcription factors governing the *in vitro* induction of pluripotency in mammals, showed highly dynamic expression patterns and, overall, the observed changes suggest their involvement in the reprogramming activity.

### CHOLINERGIC RECEPTORS CONTRIBUTE TO MAINTAIN THE QUIESCENT STATUS OF ADIPOSE MESENCHYMAL STEM CELLS

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Mesenchymal stem cells (MSCs), also known as stromal mesenchymal stem cells, are multipotent cells, which can be found in many tissues and organs as bone marrow and adipose tissue. In particular MSCs derived from Adipose tissue (ASCs) are an attractive cell source for regenerative medicine. Acetylcholine (ACh), the most important neurotransmitter in Central (CNS) and peripheral nervous system (PNS), plays key roles also in non-neural tissue. Although MSCs express cholinergic receptors, their role has been poorly investigated. Analysis by RT-PCR have demonstrated that ASCs express several muscarinic and nicotinic receptor subtypes. In particular M2 mAChR and alpha7 nAChR expression was also confirmed by western blot analysis. In present work cholinergic effects were studied on rat ASCs. By MTT and FACS analysis we have demonstrated that M2 receptor activation caused a reversible reduction of cell proliferation. Moreover, by wound healing and transwell assays, we have also demonstrated that M2 receptors caused an inhibition of cell migration, indicating the ACh as possible modulator of ASCs proliferation and migration. Similarly to that observed for M2 receptor, preliminary data on alpha-7 nAChR demonstrate that this receptor is able to modulate cell proliferation and migration. Interestingly the activation of alpha-7 nAChR appears also up-regulate the expression of M2 receptor, suggesting a feedback positive loop between the muscarinic and nicotinic receptors. Our results indicate that ACh via M2 mAChR and alpha-7 nAChR, may contribute to the maintaining of the ASCs quiescent status. These data are the first evidence that ACh, might contribute to control ASCs physiology.

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### ALLOCATING SELF-RENEWAL AND DIFFERENTIATION DURING ASYMMETRIC CELL DIVISION AND RETINOGENESIS

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One focus of regenerative medicine is to efficiently and safely replace retinal ganglion cells (RGCs), output neurons of the retina that are lost upon optic neuropathies leading to irreversible blindness. Major efforts are underway to understand how a source cell (stem, progenitor or even differentiated resident cells) can be efficiently expanded and reprogrammed into RGCs to restore functional retinal circuits and vision. Much of this knowledge finds its foundation in developmental biology. During the normal genesis of RGCs in the embryo, a complex crosstalk

of cell fate determinants and self-renewal factors is at work in dividing retinal progenitor cells (the stem cells of the embryonic retina). This concurrently ensures timely regulated expansion of retinal progenitor pools and their correct specification into nerve cell subtypes composing the mature retinal circuits. To disentangle these still elusive molecular and cellular interplays *in vivo* we exploit the optical transparency of the zebrafish embryo and apply genetic manipulations to interrogate regulatory networks controlling self-renewal, specification and differentiation of RGC progenitors. Performing 3D time-lapse cell profiling<sup>1</sup> we investigate when and how these factors become active in the physiological cellular context of the *in vivo* developing retina. We uncovered reciprocal feedbacks between RGC-fate determining factors and factors influencing multipotency, self-renewal and daughter cell inheritance<sup>2,3</sup>. We aim to understand how these factors intersect during asymmetric cell division to restrict the RGC fate choice of retinal progenitor/stem cells.

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### ULTRASTRUCTURAL ANALYSIS OF MOTOR NEURONS DERIVED FROM INDUCED PLURIPOTENT STEM CELLS OF PATIENTS WITH BROWN-VIALETTA-VAN LAERE SYNDROME

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Brown-Vialetto-Van Laere syndrome (BVVL) is a rare neurological disorder characterized by hearing loss, sensory ataxia and spinal motor neuron (MN) neurodegeneration,<sup>1</sup> caused by mutations in two riboflavin transporters (RFT2 and RFT3). Riboflavin is the precursor for FMN and FAD, i.e., cofactors of flavoproteins, involved in several energy metabolism pathways. Impaired mitochondrial functionality is likely to contribute to the disease, however the role of oxidative stress and, more generally, the pathogenic mechanisms underlying BVVL syndrome are still unclear. Even data on mitochondrial involvement in BVVL are lacking, encouraging research addressing this issue. On the other hand, the cellular effects of riboflavin, empirically used in systemic therapeutic treatment of patients, are so far unexplored.<sup>2</sup>

In the present work, we took advantage of induced pluripotent stem cells (iPSCs) technology to perform ultrastructural analyses of BVVL cells, before and after treatment with riboflavin and the antioxidant N-acetyl-cysteine (NAC). Patient-specific iPSCs and iPSCs-derived MNs were analyzed by Focused Ion Beam/Scanning Electron Microscopy (FIB/SEM)<sup>3</sup> and compared with their healthy counterparts. Diseased iPSCs and derived MNs displayed aberrant mitochondrial features, which

were reverted by combined riboflavin/NAC treatment.

Consistent with ultrastructural results, molecular data obtained by employing MitoSOX™Red probe, demonstrate significantly higher levels of superoxide anion in BVVL cells vs. controls. Also in agreement with FIB/SEM observations, riboflavin and/or NAC supplementation restored normal O<sub>2</sub><sup>-</sup> levels.

Our data support the use of iPSCs for *in vitro* modeling of BVVL syndrome highlighting the pathogenic role of oxidative stress generated by mitochondrial dysfunction. Restoring redox balance by riboflavin/NAC treatment encourages antioxidants-based therapeutic strategies aimed at ameliorating symptoms of BVVL syndrome.

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### CELLULAR AND MOLECULAR BASIS OF NEUROMUSCULAR JUNCTION FORMATION *IN VITRO*

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The formation of the neuromuscular junction (NMJ) at the interface between motoneurons and skeletal muscle, is a complex multistep process involving a variety of signaling molecules and pathways. A derangement in NMJ integrity and signaling can be caused by both neurodegenerative diseases and muscular pathologies. *In vitro* modeling of this complex structure could represent a powerful tool to help unravel the mechanisms leading to its degeneration and repair.<sup>1</sup> Nonetheless, to date, no reliable and predictive *in vitro* human models of NMJ in physiological and pathological conditions exist. It is possible to obtain human motoneurons out of induced Pluripotent Stem Cells<sup>2</sup> and human skeletal muscle from perivascular muscle progenitors, namely Pericytes, can be isolated from muscle biopsies.<sup>3</sup> Additionally, the microfluidic technology, unlike mass co-cultures, allows spatial and temporal control over microenvironments by manipulating either one or the other cell population independently.<sup>4</sup> Our preliminary results demonstrate that it is possible to successfully co-culture human skeletal muscle differentiated from Pericytes with iPSCs-derived motoneurons. Hence, exploiting an organ-on-a-chip approach, we propose a set up for a novel human NMJ model system to investigate the occurrence of NMJ mismatches in disease. Our system is composed of two separated chambers linked through microchannels to enable the axonal outgrowth towards muscle chamber. While being designed as a reliable platform to investigate the molecular actors of NMJ processes, the setup is versatile enough to host patient-specific cells and perform functional and molecular analysis.

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## NITRIC OXIDE SYNTHASE 2 INVOLVEMENT ON NEUROSPHERE GENERATION IN GLIOMA CELL CULTURES

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Gliomas (GM) are characterized by a markedly inflammatory environment and the inflammation seems to be involved in all steps of tumorigenesis.<sup>1</sup> We previously proposed inducible nitric oxide synthase (NOS2) as a component of molecular profile of GM. NOS2 up-modulation in human GM primary cultures able to arise neurospheres significantly correlates with SOX-2 expression, suggesting the interplay between inflammation and stemness potential.<sup>2</sup> Aim of this study is investigate the NOS2 involvement in neurosphere generation, using an highly selective NOS2 inhibitor, 1400W. T98G and U87-MG cell lines were cultured in standard medium (St-M) for 24 and 48h or in glioma stem cell medium (GSC-M) for 10-20-30 days in the presence or the absence of 1400W. NOS2 inhibitor did not cause morphological changes in both St-M cell lines. Of note, NOS2 protein levels significantly decreased in 1400W-treated cells at 24 and 48h; this effect was associated with a decrease in COX-2 protein expression at 48h. On the contrary, NOS2 inhibition did not affect SOX-2 expression. Exposure to 1400W at different time points strongly influenced the neurosphere size in both GSC-M cell lines. As expected, NOS2 activity, measured as nitrite concentration by Griess assay, was clearly reduced after 1400W exposure. The NOS2 role on cell proliferation was determined by a colony-formation assay. Colony size was smaller and less numerous in the 1400W-group than the untreated-group confirming the NO important role in glioma proliferation. Preliminary results on neurospheres from a primary culture confirmed that NOS2 pharmacological inhibition significantly affects the sphere size already by 10 days treatment strengthening the idea of NOS2 involvement in neurosphere generation.

## INHIBITORY EFFECTS ON DRUG RESISTANCE MEDIATED BY M2 MUSCARINIC RECEPTORS: STUDIES IN HUMAN GLIOBLASTOMA CANCER STEM CELLS AND IN NEUROBLASTOMA

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Muscarinic receptors have been involved in cancer. While M3 mAChR results to promote tumor growth and progression, M2 subtype negatively modulates cell growth and survival in different tumor types. In particular our previous studies demonstrated that M2 selective agonist Arecaidine Propargyl Ester (APE), arrested cell proliferation and induced apoptosis in glioblastoma (GB) cell lines and in glioblastoma cancer stem cells (GSC), an undifferentiated GB subpopulation characterized by high chemoresistance. Studies on GB cell lines have demonstrated that low doses of M2 agonist APE were able to counteract the drug resistance for conventional drugs such as doxorubicin (Doxo) and Temozolomide, negatively modulating the drug efflux pump AGCG2. Similarly, in GSC, treatment with the M2 agonists APE and N8-Iper decreased cell proliferation in a time and dose dependent manner. Muscarinic agonists decreased, the expression of ABC drug efflux pumps (C1 and G2) both as transcript and as protein. Moreover the co-treatment of M2 agonists with low doses of Doxo (6.2  $\mu$ M) significantly affected cell growth compared with the treatment with Doxo alone.

Comparable results have been obtained also in neuroblastoma cells, where M2 agonist APE down-regulated the ABCB1 and ABCC1 pumps expression and inhibited cell growth when the cells were co-treated with low doses of Doxo (0.01  $\mu$ M) or Cisplatin (3  $\mu$ M).

Our data suggest that M2 receptor agonists, decreasing the drug efflux pumps expression, may impair the GSC chemoresistance, making the tumor cells more responsive to low doses of conventional drugs, reducing the side effects induced by chemotherapy.

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## MISCELLANEOUS

### UNRAVELLING THE FUNCTIONS OF FAM46C, A NOVEL TUMOUR SUPPRESSOR WHICH CONTROLS MM CELL SURVIVAL BY REGULATING PROTEOSTASIS AND AUTOPHAGY

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FAM46C is a gene whose functions still remain elusive and which is found mutated in more than 10% of Multiple Myeloma (MM) patients. Intriguingly, FAM46C mutations are not present in other tumours. Given that MM cells are uniquely sensitive to proteasome inhibition due to their unconventionally high quantities of unfolded proteins requiring degradation, we hypothesized a role for FAM46C in proteostasis. We re-expressed FAM46C in MM cell lines lacking its expression and found that FAM46C induced an Unfolded Protein Response (UPR) followed by apoptosis. In order to address the molecular function of FAM46C we studied its localization and its molecular partners. We found that FAM46C is part of a high molecular weight complex and that it localizes in the cytoplasmic side of the ER through interaction with a novel ER-resident protein. This newly-identified complex regulates apoptosis of MM cells since: 1) components of the complex are also frequently found mutated in MM patients and 2) reconstitution of a functional complex in MM cells induces apoptosis.

By directly analysing the effects of the FAM46C complex on proteostasis we found that it ultimately regulates autophagy, which in this particular scenario drives the clearing of toxic protein aggregates.

Future work will focus on the impact of proteostasis on secretory pathways and on the interaction between FAM46C and components of the autophagic machinery.

### MITOCHONDRIAL TOXICITY INDUCED BY DICHLORODIPHENYLETHYLENE IN RAT LIVER AND IN HUMAN HEPATOCYTES

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Nutrition represents a crucial point to maintain a good health status. The intake of harmful substances through contaminated foods can generate toxicity in many organs, especially in the liver. Our study was focused on the effects of Dichlorodiphenylethylene (DDE), the main metabolite of Dichlorodiphenylethane (DDT), *in vivo*, on rat liver, and *in vitro*, on the hepatocarcinoma cell line *Huh7*, to compare the cellular responses activated by the two different experimental models.

*In vivo* study was performed following a treatment of 4 weeks using 10 mg/kg b.w. DDE daily via gavage. The results showed that DDE induces mitochondrial ROS production, particularly H<sub>2</sub>O<sub>2</sub>, followed by oxidative damage. In addition, DDE showed a

pro-inflammatory activity with the recruitment and activation of monocytes/macrophages in the liver and stimulated mitochondrial pathways of apoptosis.

*In vitro* studies were carried out after a dose-response pilot experiment (increasing concentration of DDE, with a constant time of 24h exposure). The DDE dose chosen (30µM) for the subsequent experiments was the lowest able to generate the increase of mitochondrial ROS, as occurred *in vivo*. The results showed H<sub>2</sub>O<sub>2</sub> and cellular ROS accumulation in presence of DDE. In addition, reduction of mitochondrial respiratory capacity and ATP production were observed, together with the activation of the mitochondrial apoptotic pathway. These data indicate that DDE acts predominantly on mitochondria, producing responses linked to the oxidative damage. Further data showed variations in mitochondrial dynamics and biogenesis, and activation of UPR signalling, to partially contain the oxidative damage.

Collecting *in vivo* and *in vitro* data allowed us to conclude that DDE, at low doses, give rise to prooxidant, pro-inflammatory and pro-apoptotic effects. Cells try to limit the damages activating a cascade of responses able to regulate cellular metabolism towards survival or death. Finally, our data also demonstrated that the *in vitro* hepatocarcinoma cell line *Huh7* is a good model for preliminary studies on hepatic toxicology.

### NON-INTERNALIZING SITE SPECIFIC ANTIBODY-DRUG CONJUGATES BASED ON MAYTANSINOIDS DISPLAY CURATIVE PROPERTIES

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Antibody-drug conjugates (ADC) represent an attractive class of biopharmaceuticals, which has gained considerable attention for the development of anticancer products. The ADCs use the targeting ability of monoclonal antibodies to selectively bind to tumor cells where the conjugated cancer drug is released. It is generally believed that the anticancer efficacy of ADCs relies on their selective targeting and internalization by cancer cells.

However, it has recently been shown that non-internalizing, linkerless ADCs targeting the tumor extracellular environment can display a potent therapeutic activity. LGALS3BP (aka Mac-2 BP or 90K) represents an interesting target for non-internalizing ADC development, as the protein is largely secreted by the majority of human tumors, while being virtually undetectable in normal adult tissues except for the prostate and the colorectum.

In the present work we show that an engineered variant of the humanized 1959 antibody to human LGALS3BP selectively localizes around the tumor cells and once site-specifically coupled to the maytansinoid drugs (DM3 or DM4) by means of disulfide linker can display a potent therapeutic activity. Both the 1959sss-DM3 and -DM4 conjugates mediated potent antitumor activity in mice bearing human tumor xenografts. Quantitative biodistribution studies confirmed a preferential accumulation of 1959sss-DM3 and -DM4 conjugates around the tumor cells. Notably, we found that both conjugates were well tolerated and cured 100% of tumor-bearing mice. Our results offer a preclinical proof-of-concept for curative non-internalizing ADC endowed with an innovative mechanism of action.



## MICROPARTICLES FROM WASTE PLASTICS: DO PHYSICO-CHEMICAL PROPERTIES AFFECT BIOLOGICAL RESPONSES IN *IN VITRO* AND *IN VIVO* SYSTEMS?

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Due to intentional or unintentional release into the environment, and especially in water systems, where they tend to accumulate, microplastics (MPs) represent an emerging environmental health concern worldwide. MPs gather together all the organic polymers with dimensions from 1 µm to 5 mm, and can be distinguished in engineered, or primary, MPs (such as microbeads in toothpaste and cosmetics) and secondary MPs, materials obtained by fragmentation from any plastic good.

Most of the biological evidences on MP toxicity on environmental organisms are achieved using reference commercial materials, represented by uniform well-characterized MPs.

By taking advantage from an ongoing project devoted to the recycling of miscellaneous waste plastics, actually destined to waste-to-energy conversion, we propose to investigate the potentially different biological responses to differently sized and shaped MPs contained in waste plastic granulates produced in recycling plants.

A preliminary chemical characterization revealed that the plastic granulates are composed by polyolefin (> 85%), with almost no contamination of trace metals. The sieving of granulate has allowed to obtain finer fractions whose size and shape are under characterization by light and scanning electron microscopy. FTIR spectroscopy will also be used to determine the polymeric composition.

*In vitro* cell cultures representative of human exposure through inhalation and ingestion (A549, Caco2 in mono or 3D culture conditions), in parallel with *in vivo* alternative models, represented by *X. laevis* and zebrafish embryos, are here proposed as valuable sensitive MPs target biological systems.

To the best of our knowledge it is the first attempt to investigate the comparative toxicity and MoA of "real" MPs in *in vitro* and *in vivo* systems. The results will be of high relevance for both the basic biological mechanisms governing the cell-MP interactions and the managing of the MP-related environmental concerns.

## BIOLOGICAL EFFECTS IN IRRADIATED CARDIAC TISSUE USING CARBON IONS FOR CARDIAC ABLATION TREATMENT IN A PORCINE MODEL

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Cardiac arrhythmias are causes of mortality in the Western world. Catheter ablation in which energy is locally applied in the heart to destroy the origin of the arrhythmia represents a therapeutic alternative to drug treatment, however carries risks for a number of complications like thrombosis and pulmonary vein stenosis. This motivates investigation of alternative, non-invasive treatments using beams of ionizing radiation as carbon ions, which are successfully used for radiotherapy of deep seated and radio resistant tumors, allow for irradiation with high volume conformity, sparing critical structures and enabling irradiation of

small volumes. Side effects occur commonly in the tissue that is located in the path of the treatment beam (entrance channel, EC). The aim of our study is to investigate the biological mechanisms underlying interruption of electrical impulse propagation in cardiac tissue following high-doses exposure (25-55 Gy) using a pig model. Three main cardiac ablation targets have been chosen, atrioventricular node, pulmonary vein and left ventricular free wall. Cardiac tissue was taken 3 and 6 months after irradiation, from the target area, and from areas of the EC of the beam, and out of the irradiation field. Macroscopic examination was performed on the hearts and on the organs close to the EC: no irradiation-induced side effects in organs at risk such as skin, oesophagus aorta or trachea were observed. Scar formation in the target areas was detected concomitantly to electrophysiological changes in conductivity. Histological analysis showed in the tissue of sham irradiated animals the expected well-organized structure of cardiomyocytes, whereas the analysis of tissue taken from the target area in irradiated animals revealed vascular, inflammatory and fibrotic changes.

These investigations will complement the medical and technical part of the study, dedicated to investigate if carbon ions might be a suitable non-invasive means for cardiac ablation treatment.

## FECAL MICROBIOTA AND ITS METABOLITES IN OBESE PET OWNERS AND THEIR OBESE PETS

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One of the most significant healthcare issues facing the human population, and shared by small companion animals, is the growing problem of obesity.<sup>1</sup> Defined as an accumulation of excessive amounts of adipose tissue in the body, obesity is related to the development of other metabolic disorders such as type 2 diabetes.<sup>2</sup>

Despite decades of research into causes and risk factors, obesity remains a serious concern for people and companion animals. The quality and composition of diet and an individual's genetic endowments may impact on the types of microbes that exist in the gastrointestinal tract, which are directly implicated in food absorption, and can alter energy utilization<sup>3</sup>.

Studies in animal models and humans revealed a different gut microbiota pattern between obese and lean groups,<sup>4-6</sup> showing that obesity may affect the diversity of the gut microbiota. However, other studies, both in humans and dogs, did not support these findings, not clarifying the relationship between this pathology and the microbiota.<sup>7,8</sup> These discordant results stand out the necessity of studying better the microbial composition, as well as the role of its metabolites in obese humans and companion animals. As prevalence of obesity in people and pets is rising in the last years, one approach to study this problem is by taking a "One Health" perspective. On that basis, our study is focus in evaluating the microbiota and the metabolites presents in fecal samples of obese/lean pets and humans living in the same house, to know if them share a similar gut microbiota either in health or disease conditions, and analyse if factors such as diet, lifestyle and environment could influence microbiota composition even though among different species.

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## COPPER CONTRIBUTES TO THE AMPHIBIAN DECLINE

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Although copper is an essential micronutrient normally subject to effective homeostatic control, excess dietary intakes can in some circumstances be toxic. Many of the toxic effects of copper, such as increased lipid peroxidation in cell membranes and DNA damage, are related to its role in the generation of oxygen free radicals. Both Cu deficiency and toxicity can occur in natural conditions and may lead to diminished animal reproduction,<sup>1</sup> various organs dysfunctions,<sup>2</sup> development of pathological lesions and, ultimately, to death.<sup>3</sup> Copper can be released into the environment by both natural sources and human activities and it is very widespread in the environment. Aquatic environment is a concentrator for this metal. Soluble copper compounds is one of the most toxic metals to aquatic organisms and ecosystems.<sup>4</sup> Our aim is to study the effects of copper accumulation in aquatic organisms. We have utilized adult *Xenopus laevis* and a concentration of 1mg/L of CuCl for 3 weeks which is the lower concentration required in the daily human diet. In aquatic environment the concentration of copper reaches higher values. Our preliminary data showed that, in treated *Xenopus*, the erythrocytes were smaller in size but more numerous. These data support the hypothesis of a possible microcytic anemia, whose symptoms are not just smaller size of the erythrocytes, but also their greater number to compensate the transport of oxygen. Moreover the dorsal skin showed morphological changes such as: erosion of the stratum corneum, disorder of the various layers that form the epidermis, increase in melanocytes and emptying of the mucous glands cells. Our data show that the Cu causes suffering in treated *Xenopus* suggesting that it is one of the pollutants involved in the decline of the amphibians.

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## TRAQ: A VERSATILE AND AUTOMATED TRACKING SOFTWARE FOR THE CHARACTERIZATION OF BEHAVIOURAL RODENT MODELS

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Quantitative metrics of laboratory animals' locomotion from video footage are crucial data in behavioural and neuroscience studies. Commercial tracking software are stable and versatile but quite expensive, requiring various degrees of user's interaction. Open source tracking software are often task specific.<sup>1</sup> TrAQ

(Tracking rodents in L'Aquila) is a MATLAB-based tracking software for off-line video analysis, developed to minimize the user intervention, allowing single-click multiple-videos processing. TrAQ permits automatic recognition of the animal and arena, providing positioning of the animal's centroid, head and tail, as well as other two-dimensional behavioural parameters (among which the number of body-centred turns). The MATLAB environment allows any kind of data analysis and we plan to distribute TrAQ as an open-source software. We tested TrAQ with smartphone-recorded videos of 6-hydroxydopamine unilaterally injected rats in the *SNpc*. In this model the number of body-centred turns performed after the administration of dopaminergic agonists correlates with neurodegeneration degree.<sup>2</sup> The compulsory pivoting and high deformation of the animal shape represent a worst-case condition for tracking software. TrAQ was validated both with drug-free and apomorphine administered animals, against the commercial Ethovision XT 13.0.1220.<sup>3</sup> The check included tracking coordinates, total distance travelled and body turning counting, the latter compared with manual counting as well. Results are consistent with manual assessment and commercial software demonstrating the high reliability and versatility of TrAQ.

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## FERMENTED FOODS, FROM MICROBE TO FUNCTIONALITY

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Since the introduction of the concept of "functional food", food industry has focused its efforts towards the production of health-promoting foods that not only provide nutrients but also give healthy benefits preventing diseases and promoting human health. In this way, the interest on probiotics has also gained special attention. Given the wide variety of autochthonous microbes found in fermented-foods, they have been considered as valid heterogeneous source of "safe" and active microorganisms. Moreover, it has recently been clarified that food-associated microbes share genetic and physiological traits with probiotic strains.

The present study aims to evaluate a collection of 22 *Lactobacillus plantarum* strains, already characterized for some functional properties<sup>1</sup>, for the following probiotics traits: <sup>1</sup>ability to express *bsh* genes, <sup>2</sup>to adhere to intestinal epithelium and <sup>3</sup>to modulate the host immunity.

Data from expression of *bsh* genes showed that *bsh1* gene displayed the highest fold changes, for the two food-associated strains O5 and LT52, suggesting a better ability to hydrolyse bile acids in comparison with the other strains evaluated. Regarding adhesion efficiency, assays confirmed that food-borne *Lb. plantarum* strains displayed a strong ability to adhere intestinal cell lines and mucus, showing a strain-dependent behaviour with values between 77-98 adhesion percentage in both cases. Interaction of *Lb. plantarum* with intestinal cells showed a modulation of different pro- and anti-inflammatory cytokines that could lead to a potential reduction of the induced-inflammation status.

All above highlight the strain-dependent feature of all properties evaluated, emphasizing the necessity to test every feature at strain-level for a functional characterization of a microbe candi-

date as probiotic. Moreover, the majority of *Lb. plantarum* strains isolated from foods displayed a similar behavior to those of human origin, revealing a promising interaction with host cells. These findings support a potential cross-talk with the host immune system, and hence, better prospects of exerting health benefits.

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## ENCAPSULATION TECHNOLOGIES FOR STABILIZATION AND FUNCTIONALITY OF OLIVE LEAVES BIOACTIVE COMPOUNDS

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Olive phenolic compounds health properties have been linked to their antioxidant capacity, acting as defence against free radicals in cellular and extracellular compartments, that may prevent oxidative physiological changes taking place in oxidative-stress associated diseases like coronary heart diseases, cancer or neurodegenerative pathologies. Recent studies have investigated the effects of on gene expression modulation via epigenetic mechanisms both in vitro and in vivo, supporting the potential role of extra virgin olive oil and its major phenolic compounds (oleuropein and hydroxytyrosol) as preventive antitumor agents.<sup>1</sup>

The high reactivity and sensitivity of these compounds have been overcome by applying micro-/nanoencapsulation technologies by which bioactives are coated in a capsule or matrix of a carrier to protect from degradation by reducing reactivity with environment, modification of physicochemical characteristics and controlled release of microcapsule contents.<sup>2</sup>

Encapsulates obtained by moisture removal as in spray-/freeze-drying are most commonly applied in food industry for plant polyphenols.

In this work, encapsulated olive leaf phenolic-rich extracts (OLE) were produced by freeze-drying using maltodextrin/trehalose as carriers and their structural (microscopy), physicochemical and thermal stability were investigated.

A response surface methodology approach was used to study effect of total solids, matrix component and ratio core:wall in freeze-drying encapsulation. Experimental values fitted well the predicted model, showing that presence of trehalose decreased encapsulation efficiency and also depressed the glass transition temperature, while lower core:wall ratio further retained phenolic compounds. The results of this research will contribute to better understand the effect of matrix component on core retention and physicochemical stability of amorphous, low moisture encapsulated matrices.

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## THE ERYTHROCYTE MEMBRANE LIPIDOME PROFILE IN HEALTHY DOGS AND CHANGES IN DOGS WITH DIABETES MELLITUS AND CHRONIC ENTEROPATHY

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Analysis of red blood cells (RBC) membrane lipidome is a powerful diagnostic tool for the follow-up of the membrane remodeling under physiological and pathological conditions in humans,<sup>1</sup> however a systematic study in dogs has not yet been established. The aim of this study was to compare RBC membrane lipidome profiles between healthy dogs (HD, n=17), dogs with diabetes mellitus (DM, n=7) and dogs with chronic signs (i.e.>3 weeks) of enteropathy (CE, n=6).

RBC were isolated from EDTA-treated blood and fatty acid analyses were carried out by gas chromatography of the corresponding methyl esters (FAME).

In HD, saturated and monounsaturated fatty acids (SFA and MUFA) and 6 levels were similar, while the 3 values showed a wider variability (mean 1.67%; SD 0.91%) that can be probably due to the individual dietary variations.

When compared to HD, the CE dogs had decreased levels of palmitic (p<0.01) and higher stearic acid (p<0.01). In DM dogs lower levels of 6 were observed (p<0.05) while 3 levels were increased (p<0.05).

The MUFA levels were diverse in the two pathological conditions: higher palmitoleic and oleic in DM (p<0.01), while lower palmitoleic (p<0.05) and vaccenic (p<0.01) in CE.

It can be observed that the SFA-MUFA pathway shows significant involvement in canine diabetes mellitus, with a higher palmitic-palmitoleic and palmitic-oleic transformations due to an accelerated delta-9 desaturase enzymatic activity. On the other hand, the increased levels of stearic and decreased palmitoleic and vaccenic on CE dogs suggest an activation of elongation pathway, leading to profound changes of membrane fluidity and permeability properties.

In conclusion, our preliminary data indicate that erythrocyte membrane lipidome of dogs may be successfully applied in veterinary medicine, providing important information of different profiles under normal and pathological conditions.

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## IDENTIFICATION OF PRE-CLINICAL DRUG CANDIDATES AGAINST SCHISTOSOMIASIS

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Schistosomiasis is a major parasitic disease afflicting more than 200 million people worldwide. Its treatment relies on a single drug, praziquantel; less sensitive strains are emerging due to its massive use and, thus, identification of new cures is a necessity. Targeting the thiol redox pathway of the parasite is a promising strategy for finding new drugs, because (i) schistosomes are subjected to the reactive oxygen species produced by both the host immune response and its own metabolism and (ii) the thiol redox pathway of the parasite is different from the human one.

Thioredoxin glutathione reductase (TGR) heads the glutathione and the thioredoxin pathways in schistosomes and is one of the most promising drug targets against several parasitosis [1]. Taking advantage of both X-ray crystallography and hits selected from a quantitative high-throughput screening we undertook a structure-based drug-design study on TGR. We identify a novel secondary and druggable pocket in TGR demonstrating its biological relevance; indeed, small molecules, bound therein, are capable of disrupting the structural transition associated with

NADPH reduction. We demonstrate that these compounds are active against cultured worms at low micromolar concentrations and display selectivity for SmTGR. The new secondary site is present in several members of the NADPH-dependent flavoreductase family but its amino acid composition is not conserved. These differences potentially present an avenue for development of selective inhibitors.

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