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European Journal of Histochemistry
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The *European Journal of Histochemistry* was founded in 1954 by Maffo Vialli and published till 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 now published under the auspices of the University of Pavia, Italy.
The *European Journal of Histochemistry* is the official organ of the Italian Society of Histochemistry and a member of the journal subcommittee of the International Federation of Societies for Histochemistry and Cytochemistry (IFSHC), and has been an influential cytology journal for over 60 years, publishing research articles on functional cytology and histology in animals and plants.

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Coverage extends to:
- functional cell and tissue biology in animals and plants;
- cell differentiation and death;
- cell-cell interaction and molecular trafficking;
- biology of cell development and senescence;
- nerve and muscle cell biology;
- cellular basis of diseases.

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MAIN LECTURES

MODELS FOR DEVELOPMENTAL TOXICITY RISK ASSESSMENT

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Risk assessment of health effects of chemicals is traditionally based on *in vivo* studies in different animals' species and the observation of apical effects. These can be based on clinical-chemistry and hematological parameters, gross pathological observations, clinical/behavioural observations, pathological and immunocytochemical analysis, evaluation of reproductive parameters, including malformations. The demand of safety assessment of an increasing number of chemicals requires the development and application of less time-consuming and resource-intensive methods and assays. These include *in vitro* and computational (*in silico*) approaches. The application of these approaches relies on the understanding of the mode of action (MoA)/Adverse Outcome Pathway (AOP) leading to the apical effect. In fact, an AOP is a model that identifies the sequence of molecular and cellular events (Key Events, KE) required to produce a toxic effect. AOPs can be linked together by common key events to form AOP networks, which can inform on more complex toxicity endpoints such as cancer or developmental defects. Once an AOP is defined for an adverse outcome, specific cell- or biochemical-based tests can be identified that represent the molecular initiating events, key events, and key event relationships for that pathway. Hence, these biochemical or cell-based or *in silico* assays can be developed and used for the assessment of a substance. Depending on the sensitivity and specificity of the test and on the definition of a quantitative (*vs* qualitative) relationship, the results of these tests can be used for screening, prioritization, or quantitative risk assessment. The process of defining AOPs and AOP networks can also help researchers and test method developers identify areas needing improved characterization. This is particularly relevant in complex situations like reproduction/developmental toxicity where non-animal methods would improve the speed and quality of the assessment.

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CONTROL OF GENE EXPRESSION IN NOTOCHORD DEVELOPMENT AND EVOLUTION: LESSONS FROM A SEA SQUIRT

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The notochord is an embryonic structure of mesodermal origin that functions as a crucial signaling center and structural support for the surrounding embryonic tissues in all chordates. During development, the notochord provides indispensable structural support for the embryo, patterns the nervous system[1,2] and controls the segmentation of the paraxial mesoderm[3]. The notochord also controls the development of several organs, including liver and pancreas[4], and influences the formation of heart and blood vessels[5,6]. In humans, defects in notochord formation lead to severe embryonic abnormalities. Despite the central role of notochord in development and evolution, the information regarding the identity of notochord transcription factors (TFs) and the molecular mechanisms through which evolutionarily conserved TFs activate *cis*-regulatory modules (CRMs, or enhancers) to coordinate notochord gene expression are still largely uncharacterized.

Our lab uses an invertebrate chordate, *Ciona robusta* (sea squirt) to unravel the gene regulatory network underlying notochord morphogenesis and to characterize structure and function of notochord CRMs. In particular, we have identified evolutionarily conserved TFs expressed in the notochord of *Ciona*[7,8,9] and we have isolated and analyzed over 50 notochord CRMs[10-13]. These studies have elucidated different molecular strategies that enable control of gene expression in the notochord, and have highlighted similarities between the *Ciona* notochord and the more complex notochord of vertebrates.

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ABSTRACTS

EFFECT OF EXTRACTS FROM LEAVES AND RHIZOMES OF THE SEAGRASS *POSIDONIA OCEANICA* ON HEPG2 HEPATOCARCINOMA (HCC) CELLS

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Bioactive compounds produced by aquatic species exhibit a wide range of therapeutic effects, thus representing promising prevention and treatment agents and beneficial supplements for functional food and food-packaging material[1-4]. Aqueous extracts from green (GLE) and beached leaves (BLE) and rhizomes (RE) of *P. oceanica* were tested on HepG2 HCC cells to study cell viability/proliferation, cell cycle, apoptosis and autophagy modulation, mitochondrial function and redox state, as already reported.[5-7] Short-term cell viability was affected in a dose-response manner by GLE and RE and the IC_{50} at 24 h was calculated and used in the subsequent assays. Analyses of cell cycle and annexin-V binding indicated the apoptosis-promoting effect of both extracts, as also proven by the detection of a panel of activated caspases. The intracellular accumulation of acidic vesicular organelles, hallmarks of the autophagic process, decreased, more drastically after exposure to RE which also induced the loss of mitochondrial transmembrane potential. Differently from GLE, viability inhibition was not reverted by co-treatment of RE with the autophagy-promoter rapamycin, suggesting the occurrence of a more extensive cell damage. Moreover, the sole GLE treatment determined the steady downregulation of intracellular ROS, putative redox-active signaling messengers necessary for cell functions. RE selectively caused the inhibition of long-term replicating capacity, as shown by the *in vitro* clonogenic assay. The results obtained suggest the potential and diversified anti-HCC ability of the extracts which merits further investigation to identify the substance(s) responsible for cytotoxicity and opens new scenarios for future biomedical and nutraceutical applications.

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DEVELOPMENT AND CHARACTERIZATION OF *IN VITRO* MODELS FOR THE INVESTIGATION OF DIABETIC RETINOPATHY

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Retina is a layered structure of the eye, composed of different cellular components working together to produce a complex visual output[1]. Because of its crucial role in visual function, pathologies such as diabetic retinopathy (DR), generally represent the main causes of blindness. Among retinal layers, under DR conditions is observed a loss of integrity of the blood brain barrier (BRB), constituted by the choroid and retinal pigmented epithelium (RPE), accompanied by Müller cells gliosis and neurodegeneration[2]. For these reasons, it is important to develop *in vitro* models of retinal diseases to use them in first drug screenings before translating in *in vivo* experiments and clinics. In this study, different high glucose-injured *in vitro* models mimicking the effect of diabetes were developed. ARPE-19 RPE cell line was used to study the alteration of barrier function induced by high glucose conditions and to built-up a 3D BRB model[4]. The barrier function of the epithelium has been assessed by transepithelial electrical resistance measurements and immunofluorescence staining for ZO-1.
Moreover, rMC-1 Müller cell line and R28 cell line (modelling neural retina) underwent high glucose conditions to induce the injury. Using these models, live cell assays were performed to evaluate cytotoxicity. The protein levels involved in pro-survival pathways were analyzed using Western Blotting analysis. Furthermore, dicarbonyl stress products and oxidative stress levels were investigated with ELISA assays. The data obtained dissected at cellular levels the detrimental effects of hyperglicemia on retinal cells developing suitable models for drug screening.

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MICROCEPHALY-ASSOCIATED WDR62 MUTATIONS HAMPER GOLGI TO SPINDLE POLE SHUTTLING IN HUMAN NEURAL PROGENITORS

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WDR62 is a spindle pole-associated scaffold protein involved in diverse molecular mechanisms crucial for corticogenesis[1]. Recessive mutations in *WDR62* are associated with structural brain abnormalities and account for the second most common cause of autosomal recessive primary microcephaly (MCPH), indicating WDR62 as a critical hub for human brain development. WDR62 is highly expressed in the primary germinal zone and implicated in maintaining the neural progenitor pool during corticogenesis[2].

Here, we investigated a C-terminal truncating mutation (D955AfsX112) in WDR62 using induced pluripotent stem cells (iPSCs) obtained from a patient with MCPH2[3]. We generated neuroepithelial stem (NES) cells from patient-derived and isogenic retro-mutated iPSC lines in order to analyze *WDR62* mutation impact on this relevant population. We observed alterations in cell cycle progression in mutant NES cells and we report WDR62 localization at the Golgi apparatus during interphase, both in human neural progenitors *in vitro* and in human fetal brain tissue. We observed that WDR62 shuttling from the Golgi apparatus to spindle poles is dynamic and microtubule-dependent. We provide new evidence that impairment of WDR62 function and localization results in severe neurodevelopmental abnormalities, thus delineating new mechanisms in MCPH etiology.

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THE COST ACTION 16203 MARISTEM (STEM CELLS OF MARINE/AQUATIC INVERTEBRATES: FROM BASIC RESEARCH TO INNOVATIVE APPLICATIONS): A NETWORK OF SUCCESS

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The stem-cells discipline represents one of the most dynamic areas in biology and biomedicine. The vast majority of research on stem cells is currently being conducted in vertebrate models, the use of which is being increasingly restricted and regulated. However, stem cell research has already greatly benefited from discoveries in invertebrates. Yet, the study of these organisms has not been pursued vigorously. In this context, marine and aquatic invertebrate stem cell (MISC) biology is of prime research and medical interest.

The COST Action 16203 MARISTEM fostered the collaboration among groups previously isolated from each other. Tools like training school for young researchers and short-term scientific missions increased the exchange of protocols and ideas. The collaborations led to innovative ideas and important concept papers describing some unique properties of aquatic invertebrate stem cells[1-4], published in "high impact factor" journals, and the collection of other contributes in a recently-edited scientific book[5].

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MESENCHYMAL STEM CELL CONDITIONED MEDIUM PROMOTES VASCULARIZATION OF NANOSTRUCTURED SCAFFOLD TRANSPLANTED INTO NUDE MICE

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Human adult mesenchymal stem cells (hMSCs) have been largely studied over the past decades, for regenerative medicine applications, due to their multilineage differentiation and their potential use in several cell-based therapies. However, in the last few years there has been a growing body of evidence suggesting that the role of hMSCs in tissue regeneration is mainly due to their secretion of pro-angiogenic, anti-apoptotic and anti-inflammatory factors, known as paracrine effect. The increasing evidence showing the potential of hMSC secretome has led to the acknowledgement that the use of hMSC conditioned medium may represent a valid alternative to the use of stem cells, overcoming the main obstacles related to cell samples handling, survival, and rejection.

Accordingly, this study focuses on the characterization and *in vivo* application of hMSCs conditioned medium (CM). To this aim, hMSCs have been isolated from two different sources, adipose tissue (hASCs) and dental pulp (hDPSCs). Although hASCs have been largely studied, very few is known about hDPSCs, therefore hDPSCs have been characterized by FACS, qPCR and immunofluorescence up to their 30th passage to confirm their stemness maintenance over long culture. hASCs and hDPSCs CMs, obtained after 72h of starvation in both normoxic and hypoxic condition, have been concentrated and characterized by ELISA to evaluate the effect of hypoxia on the release of pro-angiogenic factors. To compare the pro-angiogenic potential of hMSC secretome *vs* the cells, the hASCs and hDPSCs CMs, obtained in normoxic condition, have been mixed with a collagen scaffold, INTEGRA® Flowable Wound Matrix, and grafted in BALB-C nude athymic mice for 28 days.

Even though, an exhaustive characterization of the conditioned culture medium, which also includes the microvesicle fraction, is still in progress, the data obtained demonstrated that Integra® FWM associated with CM showed the same efficiency of Integra® FWM associated with cells in promoting cellular invasion and capillary growth. This encourages the cell-free approach for damaged tissue regeneration.

STAGE-DEPENDENT EFFECTS OF BISPHENOL B (BPB) ON XENOPUS LAEVIS DEVELOPMENT

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Among analogues of bisphenol A (BPA, a plastic additive banned by EU member countries in baby feeding bottles, food contact materials and thermal papers), BPB is used as alternative in different not-EU countries but seems to share with BPA its endocrine disruptor properties[1]. Aim of the present work is the evaluation of the effects of BPB exposure (0-5-7.5-10 M) in *X. laevis* development. R-FETAX[2] methodology with a windowed exposure covering different developmental phases (gastrulation-early morphogenesis (NF 10-26), late morphogenesis (NF 26-38), tadpole (NF 38-46, corresponding to the spontaneous swimming acquisition period)) was applied. Samples were monitored during the full six-day test period and evaluated for lethal effects, external morphology, deglutition and swimming functional tests[2], and cartilage morphology.

Effects were observed in groups exposed to BPB 7.5-10 M. At the highest concentration exposure resulted in: *i)* 100% embryolethality after exposure at the early morphogenesis stages (NF 10-26), *ii)* lethal (73%) and teratogenic effects (head and tail defects associated to negative deglutition test and abnormal head cartilages) in embryos exposed at late morphogenesis stages (NF 26-38), *iii)* neurobehavioral deficits (as detected by swimming evaluation) in samples exposed at tadpole stages (NF 38-46). BPB 7.5 M induced effects both after gastrulation-early morphogenesis and late morphogenesis exposure in about 50% samples (lethal in about 30% samples and teratogenic nearly in 20%).

BPB-related lethality always occurred later in respect to the end of exposures (at about NF 42-44 stages) and resulted related to head and branchial abnormalities and apoptosis, detected at NF 38 and confirmed also by scanning electron microscopy.

In conclusion, our results show severe stage-dependent developmental effects induced by BPB and highlight the need of more detailed studies on BPB toxicological profile.

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DOSE-AND STAGE-RELATED CRANIOFACIAL DEFECTS IN *XENOPUS LAEVIS* EMBRYOS EXPOSED TO THE FUNGICIDE TRIADIMEFON (FON): IDENTIFICATION OF PREDICTIVE ASSAYS

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Among human malformations, craniofacial defects are one of the most frequent. The experimental detection of skeletal alterations requires complex procedures.

Aim of this work is the comparison of different assays to identify dose-and stage-related craniofacial malformations using R-FETAX[1]. The full cartilage evaluation (including flat mount technique) was the gold standard for skeletal defect detection. Different methods (external morphological evaluation of fresh samples, deglutition test[1], whole mount cartilage evaluation and Meckel-PalatoQuadrate angle measurements) were applied. FON was selected as causative molecule for its known potential to induce craniofacial defects in different animal models including the amphibian *X. laevis*[2].

FON exposure (0-31.25 M) covered the whole test (NF stages 8-46) or was limited to crucial developmental phases: gastrula (NF 8-13), early morphogenesis (NF 13-26), late morphogenesis (NF 26-38), tadpole (NF 38-46). Clear dose-dependent effects were evident for groups exposed during the morphogenetic periods; gastrula was insensitive to the tested concentrations, tadpole group showed malformations only at 31.25 M.

Cartilages were affected either showing fusions visible only after flat mount or visible at whole mount examination; the most extended fusions were visible in whole FETAX and in the early morphogenetic exposure windows. FON stage-dependent embryotoxicity was evaluated modelling data, considering stage as covariate. The comparison of different applied assay potencies showed deglutition as the only assay comparable with the full cartilage evaluation.
In conclusion, we suggest i) R-FETAX windowed approach for xenobiotic dose- and stage-dependent hazard evaluation; ii) deglutition test (a rapid, inexpensive and vital test allowing to preserve samples for the application of further techniques) for a rapid screening of craniofacial abnormalities in *X. laevis*.

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CREATION OF A STABLE TRANSGENIC LINE FOR ALEXANDER DISEASE MODELING IN ZEBRAFISH

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Alexander disease (AxD) is a rare leukodystrophy occurring as type I or type II, characterized by different clinical manifestations such as seizures, intellectual disability, developmental delay, ataxia and spasticity. AxD is caused by heterozygous mutations in the glial fibrillary acidic protein (GFAP) gene[1], encoding the astrocytes intermediate filament GFAP. The hallmark of AxD is the presence of the so-called Rosenthal fibers in astrocytes, aggregates composed by GFAP, Heat Shock Protein 27 (HSP27), B-crystallin, ubiquitin and proteasome subunits. At present, there is no cure for this disease. As both *in vitro* and *in vivo* mice models of AxD suffer from limitations, the aim of this study has been the creation of a transient transgenic zebrafish model for AxD, based on Tol2 transposon approach. Zebrafish embryos were microinjected with pTol2-GFAPwt-GFP and pTol-GFAP(R239C)-GFP plasmids, whose expression in glial cells was driven by the promoter of the zebrafish *gfap* gene, demonstrating that the mutant well reproduces the main features of AxD such as glial localization of aggregates[2]. By microelectrode array platform, we further studied the electrophysiological response, and we observed a significant decrease in the head network burst duration and rate in mutants compared with control. Stated the validity of the transient zebrafish line for AxD, we managed to create a stable transgenic line, and we performed a transcriptomic analysis to further investigate and better characterize this disease.

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ADVERSE OUTCOME PATHWAYS-ORIENTED TOXICOLOGY FOR IMPLEMENTING SAFE AND SUSTAINALBE-BY-DESIGN NANOMATERIALS: CASE STUDY ON SILVER NANOPARTICLES

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The implementation of Safe and Sustainable-by-Design (SSbD) strategy for the development of new products, including nanomaterials (NMs), is a hot topic nowadays and lot of efforts are being performed to define methodologies for guaranteeing environmental and health safety, industrial relevance and regulatory preparedness[1]. Among metal-based NMs, antimicrobial silver nanoparticles (AgNPs) are the most used. The aim of this work is to characterize the hazard of new AgNPs, designed accordingly to a SSbD approach, using different biological models, according to estimated exposure scenarios. By relying onto an adverse outcome pathway (AOPs)-oriented testing strategy[2], the aim was to connect the NPs physico-chemical (p-chem) properties and the mechanistic aspects of their biological effects, using A549 human lung cells and zebrafish embryos as target systems. Different AgNPs, including the newly developed Ag hydroxyethylcellulose-coated NPs (AgHEC), were characterized for their p-chem properties. Comparative cyto- and embryotoxicity tests were performed to retrieve concentration-response curves and effective concentrations, as well as to measure biomarkers representative of initiating and key events along possible AgNPs-driven AOPs (*i.e.,* oxidative stress, inflammatory and genotoxic responses). AgNPs induced cytotoxicity, increased interleukin-8 release, ROS production and γH2AX, a marker of DNA-damage, expression in A549 cells, with positively charged and small sized NPs being more reactive. In zebrafish embryos, a concentration-dependent effect on lethality and malformations for all AgNPs was observed. However, LC_{50} and EC_{50} values indicate that AgHEC have a higher potential in inducing embryotoxicity and impacting embryos hatching, cardiac failure and skeletal muscle weakness. Overall, the results in both target systems support the relevance of p-chem properties on AgNPs toxicity.
Funding: EU-H2020 project ASINA, GA n.862444.

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PRESENCE AND CONTRIBUTION OF MOTILE CELLS DURING SENSORY ORGAN REGENERATION IN ADULT POMACEA CANICULATA AND *NEMATOSTELLA VECTENSIS*

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The contribution of innate immune components to tissue and organ regeneration is a fascinating and promising topic, still in need of suitable research organisms. The snail *Pomacea canaliculata* and the sea anemone *Nematostella vectensis* possess different immune components and they both regenerate tentacles in adult life. Cephalic tentacles of *P. canaliculata* are a pair of sensory components used for food search, co-specific recognition, and orienting[1]. In *N. vectensis*, the oral tentacles (4-18) are extensions of the diploblastic body, that feed, defend and expand the surface area of the gastric cavity[2]. A Matlab®-based semi-automated quantification of *P. canaliculata* tissue-interspersed hemocytes, combined with FISH- and qPCR-based approaches, showed hemocyte accumulation and the expression of hemocyte-derived and cell-proliferation markers (*i.e.*, *Pc*-Hemocyanin, *Pc*-AIF-1, *Pc*-TGA2 and *Pc*-RUNT) within the blastema of regenerating tentacles at 12 h post-amputation (hpa). The injection of *P. canaliculata* with Clophosome®, a phagocyte-targeting drug, transiently reduced both the circulating and the tissue-interspersed hemocytes; concomitantly, the onset of tentacle regeneration was delayed, suggesting a pro-regenerating role for snail phagocytic hemocytes.
In *N. vectensis*, a diploblastic cnidarian with no specialized hemocytes, a highly motile population of cells (mPC) was identified. Transgenic polyps with fluorescent mPC were developed. Fluorescent mPC movements were tracked after oral tentacle amputation, revealing mPC accumulation to the wound site at 6 hpa. Immunocytochemical experiments revealed that mPC share their origin with neural cells, and RNA SMART-sequencing is ongoing for further characterization.
In all, despite their diverse developmental origin, snail hemocytes and sea anemone mPC, immediately accumulate to the wound site, where they could play a proactive role in the early phases of sensory organ regeneration.

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IDENTIFICATION OF COMPOUNDS INVOLVED IN HABENULAR CIRCUIT DEVELOPMENT AND FUNCTION

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Bilateral clusters of habenular neurons in the forebrain of vertebrates' relay cognitive information into the interpeduncular nucleus and the median raphe in the ventral mid- and hindbrain, respectively. This neurotransmitter system has been implicated in behaviours from fear and social behaviour to reward responses and addiction. It is also linked to pathophysiological syndromes such as major depression disorder, autism and schizophrenia[1]. Our studies in zebrafish have revealed that the Wnt/beta-catenin signalling pathway gene Tcf7l2 and the precise temporal regulation of the pathway *via* Wif1 (Wnt inhibitory factor i) is pivotal for correct habenular neuron differentiation and laterotopic segregation of habenular efferent axons in the IPN target[2,3]. This knowledge enables us now to generate fish with defined aberrations in the habenulae for analysing the impact on behavior. Intriguingly, Tcf7l2 and Wif1, have been linked to schizophrenia and autism paving the path for further exploring the link between molecule, neural circuit and pathophysiological syndrome[4,5]. We apply *in vivo* high-throughput screening (HTS) to identify candidate compounds i) impacting habenula development and habenular neuron differentiation and ii) having an ameliorating effect on habenular neuron malformation and malfunction.
Up to now we screened 160 compounds from 3 different libraries and identified 6 promising candidates all showing an effect on habenula development. We will present a detailed analysis of one of the identified compounds. Transient treatment with this particular compound leads to an abolishment of habenular markers, an abundant reduction of habenular neurogenesis and axonogenesis and the induction of apoptosis of a specific subset of habenular neurons. Our current work aims at elucidating the underlying mechanism, the signaling pathway affected and the behavioral consequences of this specific phenotype.

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PROGNOSTIC AND FUNCTIONAL SIGNIFICANT OF MMP2 AND MMP9 IN BREAST CANCER UNVEILED BY PROTEOMIC ANALYSIS

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Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases involved in the degradation the extracellular matrix (ECM). Based on their substrate specificity, MMPs are classified into collagenases (MMP1, MMP8, and MMP13), gelatinases (MMP2 and MMP9), stromelysins (MMP3, MMP10, and MMP11), matrilysins (MMP7 and MMP26), membrane-bound/associated MMPs (MMP14 to MMP17, MMP24, and MMP25) and others (MMP12, MMP19 to MMP23, MMP27, and MMP28)[1]. The proteolytic activity of MMPs have been found to play key roles in cancer invasion and metastasis, angiogenesis and tumorigenesis. MMPs are deregulated in almost every type of human cancer, including breast cancer[2], and correlate with an advanced tumor stage, increased invasion, metastasis, and prognosis[3]. Here, we performed a multi-

omics analysis of MMP2 and MMP9 expression in breast cancer and investigated their correlation with prognosis. Moreover, the activity levels of both MMP2 and MMP9 were investigated by zymography in a large sample set of breast cancer (BC) tissues and paired normal tissues, as well as in the corresponding sera. Both gelatinases were significantly upregulated in BC tissues compared to normal adjacent tissues. No correlation was found between MMP expression in the tissues and sera, indicating their complex regulation in BC. Interestingly the expression levels of MMP2 and not MMP9 in sera of a cohort of breast cancer patients was able to predict their prognosis.
Collectively, our results showed that MMPs could have a high potency as target in breast cancer and might serve as novel biomarkers. However, further studies are needed to explore the molecular mechanisms through MMPs activity impact tumor progression

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EARLY-LIFE EXPOSURE TO TRICLOCARBAN INDUCES ZEBRAFISH OCULAR DEVELOPMENTAL TOXICITY

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Triclocarban (TCC) belongs to the category of emerging pollutants, and its presence in the aquatic environment depends on its wide use as an antimicrobial agent in personal care products[1]. This chemical is recently classified as an endocrine-disrupting chemical because it can affect the hormonal balance in exposed organisms. The TCC induces hormonal imbalance and could be responsible for neurotoxicological effects[2]. The present study aimed to evaluate the developmental and long-term consequences of TCC exposure on the visual function of zebrafish. We tested two concentrations of TCC on the zebrafish model, a sublethal and an environmentally relevant concentration. We investigate the effect of TCC on zebrafish larvae and juvenile eyes by evaluating the expression levels of two genes involved in eyes development, *mitfb* and *pax6a*, and by histological and behavioral analysis. The present study results confirmed the ocular developmental toxicity of TCC in the zebrafish model. TCC downregulated the expression of *mitfb* and *pax6a* genes in zebrafish larvae, and it induced increased anxiety compared to controls. The histological analyses revealed alterations in the thickness of retinal layers. Zebrafish juveniles exposed during development to TCC showed impaired visual discrimination abilities and increased sociability. Overall, the results of the present research highlight the negative developmental and long-term consequences of TCC exposure on the visual function of the zebrafish model.

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EXTRACELLULAR VESICLES FROM NSC-34 MN-LIKE CELLS TRANSFECTED WITH MUTANT SOD1 DRIVE MODULATION OF INFLAMMATORY STATUS OF MACROPHAGES

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Extracellular vesicles (EVs) can mediate communication between tissues, affecting the physiological conditions of recipient cells. They are increasingly investigated in Amyotrophic Lateral Sclerosis (ALS), the most common form of Motor Neuron Disease, as transporters of misfolded proteins, including SOD1, FUS, TDP43, or other neurotoxic elements, contributing to disease dissemination. Neuroinflammation is a common pathological feature of ALS and even if it cannot trigger ALS, its potential role in exacerbating the degeneration of motor neurons and the progression of the disease is attracting the researchers interest. Activated CNS microglia and astroglia, proinflammatory peripheral and infiltrated T lymphocytes, and monocytes/macrophages as well as the immunoreactive molecules they released represent the active players for immune dysregulation enhancing the neuroinflammation. In the current study, EVs were obtained from NSC-34 motoneuron-like cells transfected with mutant SOD1 (G93A, A4V, G85R, G37R) cell culture medium. Large and small were isolated at 20,000 or 100,000 g respectively, and their effects on the activation and polarization of the recipient Raw 264.7 macrophages were investigated. Several markers were used to distinguish isolated EVs: CD63 and calnexin, which are typical of exosomes, while Annexin A1 is a specific marker of microvesicles. The EVs from NSC-34 expressing WT and mutant SOD1, induced a persistent NF-kB activation and an upregulation of inflammatory gene expression, suggesting the switch to mixed M1 and M2 subpopulations. In conclusion, modulation of the inflammatory-associated markers, in Raw 264.7 macrophages reveals that EVs impact physiological and behavioral macrophages processes and are of potential relevance to MN degeneration.

THE EFFECTS OF GADOLINIUM ON XENOPUS LAEVIS EMBRYONIC DEVELOPMENT

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Lanthanides are present everywhere on the earth's crust with relatively wide concentration ranges and a toxicity considered low. Gadolinium (Gd) is one of the most widespread; it is used in technological devices and in contrast media for diagnostic imaging. Its concentration in the rivers of industrialized, densely populated areas is often anomalous; in Germany, for example, it is up to three orders of magnitude higher than the expected natural values[1]. Many aquatic organisms are vulnerable to lanthanides[2]. Few studies have analyzed their potential long-term effects in freshwater. We used *Xenopus laevis* as a model system and the FETAX assay[3,4], the expression of genes involved in early embryonic development and the ROS production to evaluate the possible toxic effects of Gd. We also verified whether it could activate

the multixenobiotic resistance mechanisms (MXMR) and induce an immune response. The embryos were exposed to Gd ions ranging from 0.2µg/L to 80µg/L considering the environmental concentrations. The FETAX assay showed that Gd is not lethal to *Xenopus* embryos; between 40 and 80µg/L, the treated embryos reach longer lengths than the controls and at 40µg/L 15% are malformed. As concentration increases, embryos show tachycardia and total ROS production increases. Finally, Gd modifies the expression of early embryonic genes, stimulates the production of cytokines at highest concentrations and activates the MXMR pump at the lowest. These data indicate that Gd, although not lethal for embryos, modifies their growth and exerts a slight toxic/teratogenic effect; it also induces tachycardia and activates the immune response. The observed phenotypic modifications are ascribable to modifications in the expression of genes responsible for the "construction" of the tadpole. This may be due to the production of ROS, which is known to affect gene expression, but also to the difficulty of embryos to activate MXMR. However, the most interesting results are visible at the highest concentrations used.

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TRANSCRIPTIONAL CONTRIBUTION OF TRASPOSABLE ELEMENTS IN RELATION TO SALINITY CONDITIONS IN TELEOSTS: ARE TE SILENCING MECHANISMS INVOLVED?

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The evolutionary success of organisms is strictly linked to the genome composition and in particular transposable elements (TEs) represent one of the most intriguing components. Fish are an interesting taxon comprising species adapted to a wide range of environments. In this work, we analysed the transcriptional contribution of TEs in the gill transcriptomes of three fish species exposed to different salinity conditions. We considered the giant marbled eel *Anguilla marmorata* and the chum salmon *Oncorhynchus keta*, both diadromous, that have to face changes in salinity in a defined stage of their life cycle and the marine medaka *Oryzias melastigma*, an euryhaline organism *sensu stricto*. Our analyses revealed an interesting activity of TEs in the case of juvenile eels, commonly adapted to salty water, when exposed to brackish and freshwater conditions. Moreover, the evaluation of the expression of genes involved in TE silencing mechanisms (six in heterochromatin formation, 14 known to be part of the NuRD complex, and four of the Argonaute subfamily) unveiled that they are active. Combining gene expression with molecular modelling analyses we predicted the three-dimensional (3D) assembly of the TRIM33/KRAB-like complex obtaining specific structural insights. Intriguingly, our results evidenced for the first time a KRAB-like domain specific of actinopterygians that together with TRIM33 might allow the functioning of NuRD complex. Therefore, this complex, so far known to be mainly active in tetrapod TE silencing, acts also in fish species and might be responsible for the observed TE transcription variation.

FOLLICULOGENESIS STUDIES OF *CARETTA CARETTA*: NEW INSIGHT THROUGH FOURIER TRANSFORM INFRARED MICROSPECTROSCOPY IMAGING (FTIRI) AND HISTOLOGICAL ANALYSES

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C. caretta is evaluated as a vulnerable species according to the Red List of IUCN[1]; therefore, it is very important to prevent the collapse of their populations and its indirect consequences on the marine environment. Despite this species plays a key role in marine ecosystem, some aspects of its reproductive biology need to be clarified. The maturation stages associated with macroscopic gonadic characterization in both sexes have already been determined in *C. caretta*[2]. However, the sequence of follicular developmental stages and the oocytes' biochemical composition at different maturation phases is missing. The aim of the present study was to study the morphological and macromolecular characterization of *C. caretta* folliculogenesis by coupling histological and FTIRI analyses. Ovaries of 23 *C. caretta* found stranded and examined by IZS AM in 2021 along the Abruzzo coasts were sampled and histologically processed to describe the folliculogenesis following the description proposed for the hawksbill sea turtle *Eretmochelys imbricata*[3]. Analyses were also performed considering both the period of stranding and the curved carapace length (CCL) which is normally used to identify the maturity stage in sea turtles. FTIR-imaging analysis was performed to determine changes on distribution of the main macromolecules in the follicles at each maturation stage focusing on follicular cells, ooplasm, zona radiata, and yolk vesicles in vitellogenic oocytes. Noteworthy, particular attention was given to atretic follicles and corpora lutea and albicans. The results obtained in the present study represent a clear and comprehensive picture of the *C. caretta* folliculogenesis and suggested that the CCL-based method applied to determine the sexual maturity of sea turtles is not representative of the real gonadal maturity

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FIRST EVIDENCE OF MICROPLASTICS IN THE YOLK OF LOGGERHEAD SEA TURTLES (CARETTA CARETTA) EMBRYOS AND THEIR CORRELATION WITH DEVELOPMENTAL IMPAIRMENT

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Microplastics (MPs) are ubiquitous and widespread in all the aquatic environments representing a major threat to the marine environment and wildlife[1].

The loggerhead sea turtle is particularly vulnerable considering its long life, trophic position, and habits, and can accumulate pollutants along the trophic chain and over wide areas at different aquatic compartments. MPs accidentally ingested by loggerhead turtles have been usually associated with health status impairment and gastrointestinal damages[2] in addition to reduced fecundity and fertility[3]. However, a possible mechanism of vertical MPs transmission from the female to the egg yolk and in turn to the embryo has not yet been proven. For the first time, the present study provided evidence of MPs occurrence in the yolk of loggerhead sea turtles embryos collected from two monitored nests along the coast of the Tuscany region in August 2020, with a 100% mortality registered. For each embryo biometric parameters including total, carapace and fin length, total and yolk weight were recorded. Concomitantly each yolk was digested separately to investigate the abundance, size, colour and polymer type of MPs by Raman spectroscopy. Finally, from each embryo, the liver was sampled and histologically investigated in order to characterize melanomacrophages and quantify the lipids content. This pilot study evidenced for the first time in sea turtles, how MPs could be transferred from the mother to the embryo through the yolk and their effects on liver health as well as lipid metabolism, affecting embryonic development and survival.

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THE EFFECTS OF CAULERPA RACEMOSA ON THE GAMETOGENESIS OF *MYTILUS GALLOPROVINCIALIS*

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Caulerpa racemosa is a tropical marine Chlorophyta introduced into the Mediterranean Sea from south-western Australia that is raising ecological problems, including changes in the physical and chemical conditions of the invaded habitat (water movement, sediment deposition, substrate characteristics)[1,2]. In addition to environmental changes, it is known from the literature that *C. racemosa*, as well as other species defined as invasive killers (*Caulerpa taxifolia*), can be very dangerous for the health of aquatic organisms, causing systemic damage, including the reproductive system [1,3-5]. Here we present our studies on the effects of *C. racemosa* on gametogenesis in *Mytilus galloprovincialis*, a sessile, filter-feeding aquatic species widespread in the Mediterranean Sea. From the histological analysis it emerged that acute and chronic treatment with high concentrations of Caulerpa caused an emptying of ovarian follicles and spermatic cysts compared to controls, with an increase in degenerating cells. We also recorded the presence of immune cell infiltrates in the connective tissue in the treated samples. These data were supported by molecular and biochemical investigations, which showed, in animals treated with Caulerpa at high concentrations, a variation within the male and female gonads in the levels of enzymes involved in oxidative stress such as Superoxide dismutase (SOD), Catalase and Glutathione-S-transferase (Gst), suggesting an increase in oxidative stress induced by the alga. Taken together, all these data, although preliminary, suggested that *C. racemosa* may have a toxic effect on the reproductive fitness of mussels.

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VANADIUM MODULATES METAL RELATED ENZYMATIC ACTIVITIES DURING DEVELOPMENT OF SEA URCHIN EMBRYOS

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Vanadium (V) toxicology represents a relevant topic as several V-compounds are released into the environment. Some of them have also a clinical interest due to their application as metallodrugs in numerous diseases (*e.g.,* obesity, diabetes, cancer, neurodegenerative and heart disorders)[1]. Since wastewater treatment plants do not sufficiently remove these compounds, pharmaceutical residues could represent emerging pollutants of aquatic environments[2].

Paracentrotus lividus embryo exposure with different V-concentrations, starting from fertilization to pluteus stage, induces altered embryos phenotypes, skeletal absence or malformations, cellular stress response mediated by HSPs, autophagy and apoptosis, and the modulation of metal related proteolytic activities[3,4].

We have extended our studies on V-toxicity to determine the embryonic response to two different V-doses (1 mM and 500 µM), analyzing metal related proteolytic activities through gel zimography.

These enzymatic activities could represent important markers of V-toxicity, since it generates a cellular imbalance in several metal ions. This would disturb the catalytic mechanism of gelatinolytic enzymes as they require ions as cofactors.

During embryo development we observed a total of 9 gelatinases with apparent molecular masses ranging from 309 to 22 kDa. Control and V-treated embryos showed a high level of low molecular weight gelatinases (from 34 to 22 kDa), from 12 h to 24 h of development.

After 30 h of V exposure, in control embryos there was an

increased level of low molecular weight gelatinases and started the expression of other 5 high molecular weight gelatinases (from 309 to 59 kDa). V-treated embryos did not show any activity of low molecular weight gelatinases, suggesting a drastic interference between V-exposure and these enzymes. In conclusion, metal related proteolytic activities could represent a reliable method to test V-toxicity, using the sea urchin as a sensitive model system.

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PROTECTIVE EFFECT OF NGF IN THE DIABETIC RETINOPATHY

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Diabetic retinopathy (DR) is the most common complication of diabetes and one of the major causes of blindness in working-age people worldwide[1]. DR initially was considered as a microvascular complication of diabetes and classified as either nonproliferative or proliferative based on the presence of neovascularization[2,3]. However, there is increasing evidence indicating that all cells housed in the retina are affected. Therefore, DR is also considered a neurodegenerative disease other than a microvascular complication[4].

NGF is one of the main actors of the neurotrophic family, with well-recognized effects including increased neuronal survival *in vitro* and in neurodegenerative diseases. In the visual system, NGF modulates retina and optic nerve development/differentiation and promotes the survival and recovery of retinal ganglion cells[5]. Above all, *in vitro* and *in vivo* studies showed that NGF exerts a protective action also on retinal photoreceptors, Müller cell, and vascular pericyte[6].

In this study, we tested NGF treatment in *in vitro* and *in vivo* models of DR. In particular, to study the potential therapeutic effect of NGF and dissect the underlying biological and molecular mechanisms, three retinal cell lines were used: ARPE-19 (retinal pigmented epithelium), R28 (model of neuronal retinal cells) and rMC-1 (Müller cell line).

Subsequently, the protective effect of intraocular administration of NGF was analyzed in Streptozotocin-induced diabetic mice. Electroretinogram, morphological analysis and protein expression evaluation indicated a significant protective effect of this neurotrophin in DR.

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EFFECTS OF THE HOMEOBOX GENE DBX2 ON ASTROCYTE FUNCTION AND ON THEIR CROSS TALK WITH NEURAL STEM CELLS

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Adult neural stem cells (NSCs) reside in the subventricular zone (SVZ) of the lateral ventricles and in the subgranular zone (SGZ) of the dentate gyrus. These brain areas act as neurogenic niches thanks to a heterogeneous and specialized micro-environment that promotes and sustains adult neurogenesis[1]. During aging, changes to the niche micro- environment progressively reduce NSC ability to generate neurons. Our group identified *Dbx2* (*Developing brain homeobox gene 2*) as a candidate regulator of age-associated neurogenic decline in the mouse SVZ[2].

Of note, *Dbx2* is also expressed in astrocytes and regulates the expression of some genes important for their maturation[3], but the role of *Dbx2* in astrocytes functions remains to be fully elucidated. Astrocytes are an important component of the NSC niche, contributing to the establishment and maintenance of a permissive neurogenic environment. However, during physiological aging processes, astrocytes undergo age-associated changes[4] that might impair NSC properties and neuron generation. In this study we intend to investigate the role of *Dbx2* in the regulation of astrocyte functions.

NSCs derived from the murine adult SVZ were engineered with an inducible expression cassette, allowing for overexpression of *Dbx2* by the administration of doxycycline (Dox). NSCs were first differentiated into astrocyte-like cells and then treated with or without Dox for 24h or 48h. We have been carrying out gene expression analyses to define the identity and proprieties of *Dbx2*-overexpressing astrocytes at molecular level. Furthermore, to assess astrocyte functional properties, we have been collecting conditioned media (CM) from control (-Dox) or *Dbx2*-overexpressing (+Dox) astrocyte cultures and testing their effects on undifferentiated NSCs. CM collected from astrocytes +Dox inhibits NSC differentiation into neurons when compared with CM -Dox. These preliminary data suggest that increased expression levels of Dbx2 change astrocyte properties, shaping their function toward an anti-neurogenic phenotype.

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PACLITAXEL BINDS AND ACTIVATES C5AR1: A NEW POTENTIAL THERAPEUTIC TARGET FOR THE PREVENTION OF CHEMOTHERAPY-INDUCED PERIPHERAL NEUROPATHY AND HYPERSENSITIVITY REACTIONS

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Chemotherapy-induced peripheral neuropathy (CIPN) and hypersensitivity reactions (HSRs) are among the most frequent and impairing side effects of the antineoplastic agent paclitaxel. The administration of chemotherapeutic treatments is often followed up by sensory illness and hypersensitization, directly related to the specific drug or combination or anti-cancer agents used, dosing regimen, and clinical conditions.[1] This is due to the interaction of molecular components triggering pro-inflammation mechanisms.[2,3] Here, we demonstrated that paclitaxel can bind and activate complement component 5a receptor 1 (C5aR1) and that this binding is crucial in the etiology of paclitaxel-induced CIPN and anaphylaxis. By *in vitro* studies, we confirmed the specific and competitive nature of the C5aR1-paclitaxel binding and found that it triggers intracellularly the NFkB/P38 pathway and c-Fos. In F11 neuronal cells and rat dorsal root ganglia,[4] C5aR1 inhibition protected from paclitaxel-induced neuropathological effects, while in paclitaxel-treated mice, the absence (knock out mice) or the inhibition of C5aR1 significantly ameliorated CIPN symptoms and reduced the chronic pathological state in the paw. Finally, we found that C5aR1 inhibition can counteract paclitaxel-induced anaphylactic cytokine release in macrophages *in vitro*, as well as the onset of HSRs in mice. Altogether these data identified C5aR1 as a key mediator and a new potential pharmacological target for the prevention and treatment of CIPN and HSRs induced by paclitaxel.

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CAFFEINE AND SALICYLIC ACID: THREAT FOR AQUATIC BIOTA? A CASE STUDY OF MUSSELS UNDER REALISTIC SCENARIOS

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The discharge of micropollutants (ng/L-µg/L) like pharmaceutical active compounds (PhACs) in coastal environments constitutes an emerging concern for aquatic biota. Hence, an assessment of the biological impact of PhACs at realistic conditions (environmental concentrations and combined exposures) is essential for defining new guidelines and enhancing more effective recovery strategies. In this work, marine mussels *Mytilus galloprovincialis* were exposed for 12 days to environmental concentrations of caffeine (CAF) and salicylic acid (SA), two of the most persistent PhACs in the aquatic environment, both individually (CAF: 5 ng/L to 10 µg/L; SA: 0.05 µg/L to 100 µg/L) and in combination (CAF+SA; 5 ng/L+0.05 µg/L to 10 µg/L+100 µg/L). Histological and biochemical analyses were conducted on the gills at different sampling points (T0; T3: 3 days; T5: 5 days; T12: 12 days) to assess tissue organization (H&E staining) and PhACs effect on the antioxidant system (catalase, superoxide dismutase, glutathione *S*-transferase, lipid peroxidation) and neurotransmission (acetylcholinesterase). A moderate haemocyte infiltration, more marked for the combined CAF+SA exposure, was observed in the gills. A more prominent pro-oxidant effect induced by CAF was observed compared to SA, likely attributable to a mitochondrial damage triggered by this salicylate. However, in the combined exposure counterbalanced patterns occurred between the two PhACs effects on the antioxidant system. The biochemical analysis revealed neurotoxicity at T12 caused by the two single compounds, observed also after their combined exposure at T5. Overall, findings of this work provide new insights into the elucidation of the real health status of non-target organisms exposed to pharmaceutical micropollutants, thus representing a preliminary phase for the implementation of recovery and mitigation approaches towards these emerging pollutants.

COUNTERACTING INFLAMMATION IN DUCHENNE MUSCULAR DYSTROPHY BY PROMOTING PRO-REGENERATIVE MACROPHAGE POLARIZATION THROUGH A METABOLIC APPROACH

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The compensatory regenerative potential of skeletal muscle declines at later stages of Duchenne Muscular Dystrophy (DMD) progression. A key role in allowing proper regeneration is played by macrophages (M) whose number greatly increases in damaged muscles[1,2]. The early steps of skeletal muscle regeneration are associated with a robust recruitment of pro-inflammatory M , whereas intermediate-late stage anti-inflammatory M are needed to dampen inflammation and to promote muscle remodeling. A prolonged M pro-inflammatory activation exacerbates muscle injury[3]. Since pro- and anti-inflammatory M subsets are distinguished by metabolic differences[4], we established to stimulate the anti-inflammatory/pro-regenerative M reprogramming and to counteract the pro-inflammatory one by using molecules able to promote the mitochondrial metabolism.
First, we evaluated *in vitro* the anti-inflammatory effect of riboflavin, idebenone, ranolazine, trimetazidine[5] and mildronate. Subsequently, we administered riboflavin and idebenone to *mdx* mice as model of DMD[6]. We evaluated the muscle functionality, the percentage of oxidative myofibers, the extent of fibrosis, and the expression level of collagens and of M pro-inflammatory and anti-inflammatory markers. Our results showed that some compounds acting on cell metabolism are able to modulate M maturation *in vitro* having an anti-inflammatory effect. Notably, some of them also have a beneficial effect *in vivo* being able to ameliorate muscle functionality and to preserve its cyto-architecture in *mdx* mice. Our data support the possibility of using molecules able to stimulate mitochondrial metabolism, and already approved for their use in humans, for the improvement of the quality of life of DMD patients.

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RESCUE OF CELL SURFACE TRAFFICKING OF R451C NEUROLIGIN3, AN AUTISM-LINKED MUTATION

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Autism spectrum disorders (ASDs) are neurodevelopmental syndromes characterized by deficits in social behavior and neurotransmission. Cell-adhesion synaptic proteins of the Neuroligin family are among the risk genes associated with ASDs. The human autism-linked substitution, R451C in Neuroligin3 (NLGN3), affects folding of the extracellular domain and causes defective trafficking of mutant protein to the cell surface and its partial retention in the Endoplasmic Reticulum (ER). The accumulation of the misfolded protein in the ER, activates the Unfolded Protein Response (UPR) *in vitro* and *in vivo*[1,2]. The knock-in (KI) mouse expressing the R451C substitution in the NLGN3 gene presents reduced protein levels, autistic-like behaviors and changes in synaptic transmission[3,4]. By screening an FDA-approved library, we have selected dexamethasone (DEX) for its effect in increasing R451C protein levels, favoring the exit of the mutant protein from the ER and improving its trafficking to the cell surface. The treatment with DEX also diminishes ER stress caused by the mutation, both in over-expression in HEK-293 cells and in physiological conditions, in neural progenitor cells (NPCs) derived from the adult hippocampus of the R451C KI mice. The effect of the DEX *in vivo* will evaluate whether the improved trafficking of NLGN3 R451C to the cell surface by DEX is accompanied by a rescue of social behaviors and functional changes described for the NLGN3 R451C KI mouse.

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ETHANOL EXTRACT OF *GANODERMA PFEIFFERI* INHIBITS SURVIVAL AND EPITHELIAL-MESENCHYMAL TRANSITION OF HEPG2 CELLS

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Ganoderma pfeifferi Bres is an uncommon lignicolous species, found almost exclusively in Europe. This mushroom is a source of several bioactive compounds, including sesquiterpenes, such as ganomycin A, B, K, and triterpenoids, such as Ganoderone A, B, C, and Lucialdehyde D[1]. *G. pfeifferi* exhibited antimicrobial activity, antiviral properties and UV protection on human keratinocytes, while the anti-tumour effects have never been studied.

In this research, we investigated the *in vitro* effects of an ethanol extract of *G. pfeifferi* on growth, survival and migration of the hepatocarcinoma cell line HepG2. The tested extract significantly inhibited proliferation of HepG2 cells in a time and dose-dependent manner. The extract also decreased the expression of cyclin D1 and increased the expression of p53, p21 and p27 protein levels. In addition, the extract decreased the levels of the anti-apoptotic protein Bcl-2, while increasing those of the pro-apoptotic proteins Bax and cleaved caspase-9 and 3. We also found that *G. pfeifferi* extract was able to inhibit HGF-induced Epithelial-Mesenchymal Transition (EMT) and migration of HepG2 cells. This was paralleled by a significant reduction in Twist protein expression and by an increase in E-cadherin and -catenin levels. Immunofluorescence experiments showed that the increased expression of E-cadherin and -catenin was paralleled by a re-localization of these proteins on cell membranes, which could be indicative of improved function of the adherent junctions in these cells. Thus *G. pfeifferi* extract besides inhibiting cell proliferation and invasiveness, improves cell-cell adhesion, supporting the expression of a more differentiated phenotype.

In conclusion, although further studies are needed to clarify the molecular mechanisms underlying the anti-cancer effects of this fungal species, we believe it could be of interest not only in the prevention of hepatocellular carcinoma, but also as adjuvant, thus allowing a reduction of the concentrations of drugs currently used, and therefore their toxicity.

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MICRO-COMPUTED TOMOGRAPHY 3D RECONSTRUCTION OF THE MOUSE OVARY FOLLOWING GONADOTROPINS TREATMENT

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Gonadotropins regulate mouse folliculogenesis during follicles recruitment (type 4-5, T4-5) and selection for growth or elimination (T6-7). By using micro-Computed Tomography (microCT), the only technique with high isotropic resolution allowing organs 3D in-silico reconstruction[1], we recently demonstrated that in the mouse ovary physiological follicles recruitment occurs simultaneously all-over the cortex and folliculogenesis is completed within the same region[2]. Here, using microCT, we studied the impact of a superovulation treatment with PMSG and hCG (FSH- and LH-like gonadotropins, respectively) on the number of the different follicle types, their 3D localisation, recruitment and selection dynamics inside the ovary.

Compared to untreated ovaries, 48hr after PMSG, the number of preantral follicles was the same, suggesting that FSH-dependent recruitment of T4-5 follicles is balanced by an equivalent number of follicles growing from the preceding primordial pool. An evident change in PMSG ovaries was a 5-fold decrease ($p=0.002$) of antral T7 follicles, those mainly involved in follicle selection. Also, in the medulla region, we observed many atretic-like follicles, characterised by a more intense microCT contrast associated with a collapsed antrum, de-structured granulosa-cell layers and

fragmented oocytes.
In PMSG+hCG ovaries, follicles number was analogous to that shown in PMSG, although atretic-like follicles almost disappeared, suggesting their elimination sometime prior to ovulation.
To understand whether follicle recruitment and selection have a territoriality, ovaries were virtually divided into eight dorsal (D-I/II/III/IV) and ventral (V-I/II/III/IV) sectors. PMSG injection increased follicle selection in sectors V-I (p=0.03), V-II (p=0.01) and V-IV (p=0.02), whereas PMSG+hCG displayed a higher follicle recruitment in V-IV (p=0.0007). Overall, these data suggest a spatial differential effect of the gonadotropins treatment inside the mouse ovary.

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PLANARIAN ASEXUAL REPRODUCTION AS A TOOL TO STUDY HOX FUNCTIONS

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Hox genes are conserved transcription factors whose combinatorial expression along the A-P axis specifies body patterning and diverse cell lineage commitment[1]. Unfortunately, the majority of our knowledge of Hox genes is extrapolated from their embryonic functions in a limited number of animal models. In these systems, Hox gene functions in specific cell types or later developmental stages are masked by severity of early embryonic phenotypes and functional redundancy[2]. One possible strategy to expand our understanding of Hox gene mechanisms is to take advantage of alternative animal models with inherent biology that circumvents these issues. The planaria *Schmidtea mediterranea* is a rising model to study animal regeneration[3]. These flatworms are obligate adults, circumventing embryogenesis by asexually reproducing *via* transverse fission. During this process, planaria adhere their posterior tissue to a substrate, mechanically separate it, and then regenerate a clonal individual from that fragment[4]. We recently uncovered roles for Hox genes in planarian asexual reproduction[5]. In particular, planarian Hox gene Post2b is required for the structure and function of the Marginal Adhesive Gland (MAG), a secretory organ required for substrate attachment during fission. Although the MAG is present on the entire lateral edge of the flatworm, only the posterior-most tissue is fissioned[4,5]. We hypothesized that Hox gene activity localizes MAG secretions to the posterior. Using serial block face electron microscopy (EM) and 3-D reconstruction, we resolved the structure of the MAG and its components: anchor, releasing, and viscid cells. Hairpin Chain Reaction (HCR) *in situ* analysis revealed a posterior-biased enrichment of the Post2b effectors Synaptotagmin 1 (Syt1) and Reticulocalbin-1 (Rcn-1) within the viscid cells of the MAG. Additionally, EM analysis of the MAG following RNAi interference (RNAi) of Syt1 and Rcn-1 revealed a robust reduction of MAG surface secretions. In conclusion, we resolved the cellular structure of the MAG and identified posteriorly localized Post2b effectors with roles in secretion. In the future, we aim to use this system to resolve how Hox genes can integrate positional information with tissue functions.

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PSYCHOTROPIC DELORAZEPAM INDUCES EPIGENETIC CHANGES AND RETINAL DISORDERS IN *XENOPUS LAEVIS* EMBRYOS

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Delorazepam, a derivate of diazepam, is a psychotropic drug belonging to the benzodiazepine class. Used as a nervous-system inhibitor, it treats anxiety, insomnia, and epilepsy, but is also associated with misuse and abuse[1]. Nowadays benzodiazepines are considered emerging pollutants[2]: conventional wastewater treatment plants indeed are unable to completely eliminate these compounds[3]. Consequently, they persist in the environment and bioaccumulate in *non-target* aquatic organisms[4]. The impact and consequences are still not fully clear. To collect information, we tested the effects of three increasing concentrations of delorazepam (1, 5, and 10 g/L) on *Xenopus laevis* embryos, a representative model of amphibians, a particularly endangered class of aquatic organisms. Results demonstrate that, besides being sedative, delorazepam is teratogenic and able to induce relevant changes in gene expression and in the oxidative state. New analysis highlighted an epigenetic effect of delorazepam, which induces an increase in methylation of the genomic DNA of the tadpoles and a differential methylation of the promotors of some early developmental genes. Parallel histological investigation highlighted retinal disorganization, indicative of interference on the developing nervous system, and suggestive of visual defects. Results are alarming considering the growing trend of benzodiazepines concentrations in superficial waters[5] and the fact that benzodiazepines receptors are present in all aquatic organisms.

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YOLK CONSUMPTION: A NOVEL TARGET FOR MICROPLASTIC TOXICITY?

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Microplastic represents a class of contaminants of emerging concern due to the numerous interferences exerted on biological processes[1]. Among these, developmental processes should be included[2] and the effects of microplastic on zebrafish and sea urchins' embryos are now well documented[3]. In particular,

microplastics accumulate in the yolk[4] but no much attention has been dedicated to the potential effects they exert on yolk degradation during early embryogenesis. For this reason, we carried out a preliminary investigation in an oviparous model, *Artemia salina*. Cysts were hatched and nauplii grew for 5 days in presence of virgin and non-virgin polystyrene fragments (mixture, max diameter 10 µm) and, for control, in presence of polystyrene microbeads (3 µm; Sigma-Aldrich). Histological analyses confirm[5] that in control cysts and in nauplii at stages L1 and L2 yolk platelets are very numerous. They reduce significantly in nauplii at stage L3 and disappear in nauplii at stage L4. After microplastic exposure, no matter the type, not only cysts and nauplii at stages L1 and L2, but also a significant percentage of nauplii at stages L3 and L4 contain a vast number of yolk platelets. These are occasionally located in the gut cells, but most often are dispersed in intracellular spaces. Persisting platelets have a different glycan composition as indicated by staining with a panel of lectins (WGA, DBA, and PNA). Though preliminary, results open up an interesting new field of research. Microplastic, in fact, represents worldwide a serious emerging environmental threat to health being a proven vehicle for contaminants[6]. Our evidence suggests a much more direct impact on water organisms and recruitment mechanisms.

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HOW CAN WE DEFINE "SELF"? COMPLEMENT SYSTEM AND MISSING-SELF THEORY

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The Complement System, with its main protein C3, is the main humoral system of the innate immunity, that type of immunity possessed by almost all the animal kingdom. In the absence of regulatory proteins, uncontrolled activation of the system can also result in disruption of host cells, resulting in immune-mediated tissue damage[1]. We know that vertebrate immune system is able to discriminate between self tissues from non-self as well as invertebrates. Furthermore, the former are able to have a rejection reaction against allogeneic transplants thanks to the adaptive part of their immune system. The latter, that totally lack of adaptive immune system, are able to discriminate between self body and body parts derived from conspecific organisms very similarly to vertebrates[2]. How is it possible if they lack of adaptive immunity? Now we know allorecognition machineries in invertebrate animals has nothing to do with those of Vertebrates partially responding to the question if allorecognition in the animal kingdom are of monophyletic origin or evolved independently[3].
Cnidarian, Annelids and Protochordates, *e.g.*, are able to allograft rejection with molecular machineries that has nothing in common with the MHC-based histocompatibility reactions of vertebrates, but (some of them) involved highly variable complement receptor-like protein[4]. Now I'm exploring the hypothesis that proteins controlling complement system might have an evolutionary ancient role not only in immune defense but also in histocompatibility. Hypothesis that associates well with the so called "missing-self theory".[5]

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EPIGENETIC NANOTOXICOLOGY OF NEWLY SYNTHETIZED IRON-BASED NANOPARTICLES

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By means of an alternate magnetic field, we can remotely control the catalytic activity of enzyme-nanoparticle (NP) systems[1]. We expect to tune temperature gradients at the nanoscale to precisely and locally reach the optimal temperature of each immobilized enzyme. To this aim, the HOTZYMES consortium (https://www.hotzymes.eu) is producing several new iron- based NPs with a large spectrum of magnetic characteristics to obtain the desired local temperature and with different surface modifications to optimize enzyme binding. These enzyme-NP systems can be applied in different fields, but, to ensure their safe utilization, it is mandatory to investigate their potential toxicological impact. This task, however, is not so straightforward as it may appear. In fact, our current knowledge of the toxicology of nanomaterials is still poor and the existing evidences on the factors that affect uptake, translocation to other tissues, intracellular trafficking and nanotoxicity of metal-based NPs reveals more uncertainties than certainties. Moreover, since there is increasing evidence on the hereditability of nanotoxicity that could be explained by epigenetic modifications[2], we have decided to analyze the genomic distribution of the main histone modifications marks (H3K27ac, H3K4me, H3K27me3 and H3K9Me2) in NIH-3T3 cells treated with newly synthetized NPs. Epigenetic toxicity is the capacity of a substance to negatively affect the epigenome, that is to modify the status of expression of genes causing undesirable effects on organisms such as cancer, neuronal or cardiovascular diseases. In order to this, the distribution of histone marks was defined with ChIP-sequencing (ChIP-seq). Then, the impact of epigenetic changes on gene expression was determined through cross-comparison ChIp-seq data with the expression profile obtained from the same cells by RNA-sequencing (RNA-seq). The preliminary results suggested that although several NPs promote gene expression change, only few promote these changes by an epigenetic mechanism.

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A WINDOW OF VULNERABILITY: CHRONIC ENVIRONMENTAL STRESS DOES NOT IMPAIR REPRODUCTION IN THE SWORDFISH XIPHIAS GLADIUS

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The swordfish (*Xiphias gladius*) is a cosmopolitan, highly migratory teleost species and an important fishing resource. Since the Mediterranean stock is considered in overfishing and declining[1], the evaluation of the crosstalk among metabolism, stress response, immune system and reproduction in immature and mature females could be of great importance.
For these reasons, in the present study we applied transcriptomic and histological approaches on the liver of immature and mature females to shed light on the crosstalk among these aspects. A total of 750 genes were differentially expressed between the liver of mature and immature females. The fatty acid metabolism, steroid biosynthesis, complement system and metabolism of xenobiotics by cytochrome P450 were among the enriched KEGG pathways. Instead, lipid transport, response to estrogen, egg coat formation and response to polycyclic arene were among the enriched GO Biological Processes. At histological level, lipid metabolism shifted from hepatic accumulation before puberty onset to mobilization toward the ovary during the reproductive period to sustain vitellogenesis, a finding which well reflects a capital breeding strategy. Furthermore, mature females showed a greater exposure to chronic environmental stress as evidenced by the higher size and number of melanomacrophage centres (MMCs) in the liver. Noteworthy this exposure to environmental stressors was not able to impair reproduction, since no histological alterations were evidenced at gonadal level. The estrogen- and Ahr-Receptor signalling pathways, up-regulated in mature females, responsible of the inhibition of *cyp450* detoxification pathway making the organism more vulnerable at this stage of the life cycle. The present findings unveil the crosstalk among metabolism, response to environmental stressors and reproduction highlighting that mature females invest most of energy in reproduction instead of detoxification and immune response.

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A NEW CRISPR/CAS9 DISEASE MODEL: FROM THE GENERATION TO THE CHARACTERIZATION OF A ZEBRAFISH STABLE KNOCKOUT FOR SPASTIN (SPG4) TO STUDY *IN VIVO* HEREDITARY SPASTIC PARAPLEGIA

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Hereditary spastic paraplegia's (HSP's) are a heterogeneous group of neurodegenerative disorders involving upper motor neurons and showing spasticity and weakness of the lower extremities. The most common form of HSP is associated to mutations in Spastin gene (SPG4) and accounts for 40% of all cases. The disease's aetiology is poorly understood, but different cellular activities might be disrupted such as axonal transport, endoplasmic reticulum modeling, lipid metabolism or BMP (Bone Morphogenetic Protein) signaling pathway[1]. In view of this, we generated by CRISPR/Cas9 a stable spastin KO zebrafish mutant named Spast^{ins11}, marked by 11 bp insertion in exon 2 of the Spastin gene that introduces a premature stop codon p.(Glu120AspfsTer56) in the resulting predicted protein. In order to characterize this new animal model, Spast^{ins11} KO embryos were collected at different time-points to assess the presence of peculiar morphological features but development was not significantly affected, as well as survival rate compared to wild-type (WT). In basal condition, WT and mutant spastin fish exhibited similar swimming capacity, while after the administration of tunicamycin, homozygous KO larvae showed a reduced mobility, demonstrating a major sensibility to ER stress than controls. Tg(BRE-AAVmlp:eGFP)^{mw29} zebrafish reporter line (responsive to SMAD-mediated BMP signaling)[2] was outcrossed with SPG4 mutants to investigate pathway alteration associated to the mutation. Interestingly, Spast^{ins11} mutants showed a dysregulation of the BMP signaling pathway that resulted overexpressed during the embryonic development. To deepen these data, motor neurons integrity was assessed by specific antibody and the presence of malformations analyzed by conventional and confocal microscopy. Altogether our preliminary results highlight the possibility to obtain a valid tool toward the exploration of the Hereditary Spastic Paraplegia's molecular scenario, as well as a suitable zebrafish model for application in large-scale pharmacological screenings.

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EFFECTS MEDIATED BY 7 NICOTINIC RECEPTORS IN PERIPHERAL NERVE REGENERATION

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The peripheral nervous system can regenerate due to the remarkable plasticity of Schwann cells (SCs). In response to damage, demyelinating pathology or peripheral nerve injury, SCs de-differentiate and acquire phenotype responsible of supporting neurons survival and axonal regeneration. The new SC phenotype is called *Repair SCs*. Inflammation is the first response to nerve damage because the fast removal of cellular and myelin debris is essential in preventing the persistence of the local inflammation that may negatively affect nerve regeneration. Acetylcholine (ACh) is one of the neurotransmitters involved in the modulation of inflammation through the activity of its receptors. In particular, the activation of 7 nicotinic receptor (7 nAChR) mediates the so-called anti-inflammatory cholinergic pathway, capable of inhibiting the production of pro-inflammatory cytokines. 7 nAChR is absent in rat sciatic nerve immediately after dissection, but its expression is significantly enhanced in SCs after 24 h in cultured sciatic nerve segments or in cultured SCs maintained in presence of the pro-inflammatory neuropeptide Bradykinin. We found that the activation of 7 nAChR with the selective partial agonist ICH3 inhibits IL-6 transcript level expression as well as

the cytokine release. Moreover, its activation causes an upregulation of plasminogen activator urokinase, metalloproteinases 2 and 9 activity. These results suggest that ACh, probably released from regenerating axons or by SC themselves, may actively promotes, through 7 nAChRs activation, an anti-inflammatory microenvironment and an active remodeling of extracellular matrix that may contribute to better improving the peripheral nerve regeneration. The analysis of signal transduction pathway downstream 7 nAChRs has highlighted the activation of a metabotropic pathway involving the PI3K/AKT/mTORC1, relevant for the acquisition of the *Repair* phenotype. The effects mediated by 7 nAChRs on SCs proliferation and migration have been also investigated.

EFFECTS OF ALUMINUM CHLORIDE ON THE MUCOUS SECRETION IN THE STRIPED VENUS CLAM, CHAMELEA GALLINA

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Al is toxic for many organisms[1,2] and is responsible for neurodegenerative diseases in humans[1]. Although it is naturally occurring in aluminosilicate and oxide minerals[3], anthropogenic activities determine a surplus of this metal in the environment. We estimated the alterations of mucous secretions in the Venus striped clam, *Chamelea gallina*, a marine bottom filter-feeder common in the Mediterranean and Eastern Atlantic, exploited in shell fisheries.
Four groups, 20 individuals each, were housed for three days in 10L seawater aerated tanks with daily replacement of water. Animals were fed daily with 50cc of algae. Three groups were treated for three days with $AlCl_3$ at 25 M, 50 M, and 200 M, respectively, the fourth being used as a control. Three individuals died in the treatment at 25 M, 14 in the 50 and 14 in the 200. The survivors were sedated, and tissue samples of gills and feet were processed for routinely paraffin inclusion. Sections of 5 m were analysed by histochemical methods such as PAS, AB pH 2.5, HID-AB pH 2.5 and FITC-lectins (PNA, desolation-PNA, SBA, desulfation-SBA, WGA, AAA, ConA, UEA I, MAA II, LTA, SNA).
Controls were positive for PAS, AB pH 2.5, and stained brown with HID-AB pH 2.5, indicating the presence of sulfated glycans. ConA positivity revealed that the deep mucous cells of the foot produce a secretion with mannosylated and/or glycosylated residuals. The glycocalyx of the epidermal layer of the foot was rich in fucosylated residuals and poor in glycosaminylated/sialylated ones, as revealed by AAA and WGA binding, respectively. Gill filaments strongly linked AAA and WGA. A general reduction in staining/binding was observed in the high doses treated. From the previous, we can preliminarily conclude that high dosages of $AlCl_3$ affect the quali-quantitative expression of glycans in the mucous secretion.

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IMAGING THE MOUSE DEVELOPING AUDITORY CORTEX

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During postnatal development, sensory experiences shape neural connections and individuals acquire memory of these sensory cues[1,2]. Among the variety of sensory stimuli at which newborn mice are exposed during development, paternal ultrasound vocalizations (USVs) play an important role in forming memories of conspecifics that are recalled in adulthood females to influence mate choice[3-5]. However, how paternal USVs influence the functional development of cortical auditory circuits is unknown. Here, we performed *in vivo* two-photon functional imaging and characterized spontaneous activity of layer 2/3 (L2/3) neurons in the primary auditory areas of anaesthetized mice at different postnatal ages, in animals raised with or without their father. P0 mice were injected in auditory cortex with adeno-associated viral vectors expressing GCaMP7 and spontaneous activity was characterized at three developmental time windows: i) P8-P11: when the external auditory canal is closed; ii) P12- P14: when the onset of hearing occurs; iii) P15-P21: during the critical period for hearing. At P8-P11, L2/3 neurons showed spontaneous synchronous transients. From hearing onset, a switch from synchronous to asynchronous activity occurred and, at the end of the critical period, most cells displayed asynchronous largely decorrelated neuronal activity. Interestingly, during the critical period, animals raised in the absence of their father showed significant more correlated activity compared to those raised in the presence of the father. Overall, these results suggest a relevant role of sensory stimuli linked to the developmental presence of the father in shaping auditory circuits.

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AUTOPHAGY AND MITOCHONDRIAL FUNCTIONALITY IN THE HARDERIAN GLAND OF HIGH-FAT DIET-FED RATS

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The Harderian gland (HG) of the rat (*Rattus norvegicus*) is a large orbital gland secreting lipids, which accumulate in excess under condition of increased lipid metabolism[1]. In order to study the response elicitated by the lipid overload in rat HG we have housed the animals in thermoneutral conditions (28-30 °C) in association to high fat diet (HFD). The thermoneutrality exacerbates lipid accumulation in topic and ectopic tissues when com-

pared to standard housing temperature (22-24°C). In HFD rat the altered blood lipid levels (increased cholesterol and triglycerides) determine lipid accumulation in HG as demonstrated by the increased gland weight and by ultrastructural analysis. The results show that high fat diet-caused oxidative stress forces the gland to trigger antioxidant defence mechanisms and autophagic processes. Lipophagy and mitophagy were induced to eliminate excessive lipid droplets and damaged mitochondria, respectively. Furthermore, a strong induction of mtDNA damage and activation of repair mechanisms was even more pronounced in the HG of HFD rats. In the same time, an increase in the expression levels of markers of mitochondrial biogenesis, and fission and fusion occurred to counteract the reduction of mtDNA copy number and mitophagy process. Thus, the accumulation of lipid droplets is deleterious to cell health by damaging particularly the mitochondrial compartment. The results provide further evidence for high-fat diet-induced changes on the mitochondrial compartment and support the hypothesis of the role of mitophagy and membrane dynamics in the mitochondrial adaptive response.

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INSIGHTS ON THE GENDER SPECIFIC REPRODUCTIVE TOXICITY OF GLYPHOSATE IN ZEBRAFISH

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The herbicide Glyphosate and its formulation Roundup® are commonly used for weed control in crops, gardens and municipal parks. Despite its wide environmental application, few studies have been carried out so far showing its toxicity on wildlife and humans[1,2]. In this study, male and female adult zebrafish were exposed to a 700 µg/L concentration of Glyphosate, the Maximum Concentration Level (MCL) assessed by EPA in drinking water. A second group was exposed to Roundup® containing an equivalent concentration of glyphosate (700 µg/L) and the results were compared to those of a control group.
Fish exposed to Roundup® died shortly after the addition of the herbicide, while those exposed to glyphosate were sacrificed after a 28 day-trial and tissue were sampled. The first and recently published results obtained by metabolomic analysis evidenced a disruption of hepatic metabolism, an increased incidence of steatosis in both male and female, an increased stress inflammatory response in female and the disruption of oxidative stress response in male, suggesting a sex-specific toxicity[3]. Thus, considering the hepatic alterations, the effects at gonadal levels were herein analyzed, hypothesizing a possible impairment at the reproductive level. Results clearly showed the alteration of gametogenesis. In particular in male, testis of fish exposed to glyphosate presented an increase of Spermatogonia A, B and spermatids and a decrease in spermatozoa. On the contrary, no differences were found regarding the abundance of previtellogenic-, vitellogenic and mature follicles between control and treated ovaries. A deeper investigation was made to gain evidence regarding glyphosate hormonal behavior, thus the expression of genes involved in gonadal steroidogenesis, differentiation and apoptosis was analyzed, suggesting an antiandrogenic and estrogenic effect, as well as a sex-specific effect of this widely used pollutant.

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$PM_{2.5}$ AND SARS-COV-2: BIO-INTERACTIONS AND MOLECULAR MECHANISMS UNDERLYING THE EFFECTS AT PULMONARY LEVEL

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The hypothesis that particulate matter (PM) may promote SARS-CoV-2 infection is of great interest. PM has been suggested to act either as a "carrier" of SARS-CoV-2 or by reducing the cellular defenses and making people more susceptible to infections. PM ability to induce a state of chronic lung inflammation, characterized by the over-expression of the angiotensin 2 converting enzyme (ACE2), for which SARS-CoV-2 has a high affinity, is currently under evaluation[1,2]. Thus, understanding how ACE2-related pathways in pulmonary epithelial cells could be perturbed by PM and exploited by SARS-CoV-2 infection can help in suggesting new strategies to prevent/mitigate the effects of COVID-19 pandemic. This project proposes to evaluate the *in vitro* interactions between SARS-CoV-2 and $PM_{2.5,}$ using human alveolar epithelial cells (A549) to define if and how PM facilitates virus entry and to identify biological markers of exposure and risk. $PM_{2.5}$ was collected in Milan during winter 2021. Cells were firstly treated for 72h with $PM_{2.5}$ in combination or not with SARS-CoV-2 inactivated form. Successively cells were exposed only to PM for 72h, afterward SARS-CoV-2 was added for additional 24h to mimic a sub-chronic exposure to air pollution. The activation of the inflammatory response was studied at biochemical and molecular level. The endosomal pathway was also explored as parameter for the internalization of viral particles. Results demonstrated that exposure to $PM_{2.5}$ induced an ACE2 over-expression in A549 cells, even in absence of SARS-CoV-2. Moreover, PM/SARS-CoV-2 co-exposure promoted an increase of the pro-inflammatory cytokine IL-6 release. Preliminary data suggested that co-exposure might facilitate the virus entry within the endosomal route. Our data support the hypothesis that the mechanism by which PM exposure can contribute to enhancing SARS-CoV-2 infection and consequent COVID-19 severity is related to an increase in the basal level of the ACE2 receptor in lung epithelial cells and in the inflammatory status.

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IMPACT OF POLYSTYRENE MICROBEADS ON THE EMBRYONIC DEVELOPMENT OF THE SEA URCHIN *PARACENTROTUS LIVIDUS*

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The unstoppable use of plastic materials in daily life and their release into the environment has reached alert levels both for the environment and for human health, thus raising debates in the scientific community[1]. Runoff and weathering breakdown of plastics abandoned in the environment lead to their fragmentation down to lower scales, the so-called nano/microplastics. The sea represents the destination of the nano/microplastics dragged there by the movements of terrestrial waters and rivers. The small size favors their ingestion especially by aquatic living organisms. The effects that ingestion can have on marine organisms have not yet been fully clarified. Microplastics in marine environment differ in chemical properties, degradation, size, and shape. These factors, together with the chemicals and/or microorganisms adsorbed on the microplastic, can impact on biota[3].
To examine the impact of microplastics on the development of marine organisms, we set up the *in vitro* development of the sea urchin *P. lividus* in presence of different concentrations of 40nm and 200nm fluorescent polystyrene microbeads, from fertilization to pluteus stage (72 hours). Modifications of the normal development in terms of morphology and rate were assayed at 2-6-24 and 72 hours from fertilization. The presence of the microbeads has determined the onset of extensive morphological modifications, the incidence of which is concentration dependent, and it is paralleled by skeletogenesis genes (*msp130, SM50, SM30* and *PM27* genes) modulation.
In summary, even though polystyrene microbeads are just a simplified model of microplastics compared to those ingested by organisms in the environment, they play a crucial role in *P. lividus* abnormal development. Further research to highlight the other developmental patterns involved in the organism response are needed.

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INDUCED PLURIPOTENT STEM CELLS AND DERIVED MOTOR NEURONS AS *IN VITRO* MODEL FOR NEURODEGENERATIVE DISEASES: NEW INSIGHTS INTO RIBOFLAVIN TRANSPORTER DEFICIENCY

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Induced Pluripotent Stem cells (iPSCs) are generated by reprogramming somatic cells, using Yamanaka's method[1]. Their properties of self-renewal and pluripotency allow to obtain different cell types, including neurons, to be used as *in vitro* model to explore pathomechanisms of rare diseases. Among these, Riboflavin Transporter Deficiency (RTD) is a childhood-onset motor neuron disease, caused by mutations in *SLC52A2/3* genes, resulting in riboflavin deficit. As this disorder lacks dependable *in vivo* models, we took advantage of iPSCs technology to recapitulate cellular aspects of RTD. We previously demonstrated altered energy metabolism pathways depending on mitochondria and peroxisomes, accompanied by impaired differentiation and cytoskeletal derangement[2-4]. In the present work, we aimed at characterizing pathomechanisms associated to RTD, using patient-specific iPSC-derived motor neurons (MNs). Our ultrastructural data, obtained by Focused Ion Beam/Scanning Electron Microscopy (FIB/SEM) and conventional SEM, demonstrate profound alterations in RTD cells. Poor differentiation features are accompanied by typical neurodegeneration hallmarks, namely neurite swellings, suggesting impaired intracellular trafficking. Ongoing apoptotic process, suggested by the presence of vesicles and blebs budding from the plasma membrane, cell shrinkage, and nuclear dysmorphism, is confirmed by Immunofluorescence using specific markers and TUNEL assay. Aberrant mitochondrial features are also present in RTD MNs, compatible with the concept that energy metabolism is affected. Thus, the understanding of these pathogenic pathways, ranging from organelle dysfunction to apoptotic cell death, may represent the starting point for the identification of novel targets for the treatment of this devastating pathology.

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EFFECTS OF ACCESSIONS OF CHICKPEAS IN THE DIET ON THE EXPRESSION OF MURINE INTESTINAL MUCINS

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Inadequate nutrition and wrong lifestyles contribute to the onset of various diseases including metabolic syndromes. In mice, dietary fibers intake can improve the intestinal mucosal barrier function, enhance the differentiation process of goblet cells, and increase acidic mucin production[1,2]. *In vitro* and *in vivo* studies revealed that chickpeas accessions have significant antioxidant abilities[3]. We investigated the effects of a fatty diet (HFD) and a fatty diet associated with two accessions of chickpeas (HFD + MG_13; HFD + PI358934) on the mucin expression in murine colon of mice. By conventional histochemistry (PAS, AB pH 2.5, HID), lectin-histochemistry (AAA, UEA-I, SNA, WGA, PNA, SBA), and immunohistochemistry (anti-rabbit Muc2) we evaluated possible alterations of Muc2, the main mucin secreted by the mucous cells of the colon. In all the experimental conditions, a reduction of Muc2 expression was observed. Fucosylation and sialylation patterns tended to increase, while glycosaminylation

and sulfation decreased. In HFD + MG_13 these effects were reduced and resulted similar to the control.
Although preliminar, our results suggest that MG-13 has a protective role and can attenuate the effects on the secretion of colonic mucins induced by a high-fat diet.

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EFFECTS OF TWO PLASTIC ADDITIVES ON EMBRYONIC DEVELOPMENT AND PHOTOSYMBIOSIS ESTABLISHMENT IN THE ACOEL *SYMSAGITTIFERA ROSCOFFENSIS*

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Symsagittifera roscoffensis is a tidal acoel, which establishes an obligatory symbiosis with the green alga *Tetraselmis convolutae*. This unique photosymbiotic unit has emerged as a powerful model system allowing the study of key biological processes such as early embryogenesis and photosymbiosis induction in basal invertebrates. Indeed, *S. roscoffensis* acoels can be reared in controlled conditions: after hatching the aposymbiotic juveniles have to establish the symbiosis within few days in order to survive[1]. Nowadays, these processes are hampered seriously by anthropogenic pollution. Bisphenol A (BPA) and NonylPhenol (NP) are two plastic additives that have attracted considerable attention as established endocrine disrupters chemicals[2] and for their seemingly ubiquitous presence in the environment[3]. In the present study, we exploited the potentiality of *S. roscoffensis* to study the effects of these pollutants on acoel embryogenesis and neural development and on the establishment of the photosymbiosis. According to our results, both BPA and NP affected embryonic development and prevented juveniles hatching at high concentrations. Malformations appeared to be dose-dependent for both the chemicals but effects on nervous system were observed only after BPA exposure. Similarly, photosymbiosis induction was completely impaired at BPA high concentrations. Comparing BPA and NP, the latter affected *S. roscoffensis* development and proved to be lethal at lower concentrations as reported in other model systems[4] but neural development seemed to occur properly even in treated samples displaying a seriously compromised morphology.

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STRETCH-GROWTH AND CELL THERAPY: A NOVEL COMBINATORIAL APPROACH FOR TREATING SPINAL CORD INJURIES

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Spinal Cord Injury is a pathological condition with devastating physical and socio-psychological consequences[1] but effective treatments are lacking due to the complex pathophysiology. Recent investigations in the field of regenerative medicine show the therapeutic potential of neuroepithelial stem (NES) cells to treat this type of injury[2], while advancement in nanotechnologies enables the development of novel nanomedical tools. Previous studies investigated the use of magnetic nanoparticles (MNPs) to promote axonal stretch growth by applying an external magnetic field[3,4]. The purpose of this work is to validate a novel combinatorial approach to treat spinal cord injuries based on this nanotechnology applied on NES cell-derived neurons for better achieve axonal re-growth and regeneration. Indeed, the success of stem cell-based therapies depends on the capacity to promote axonal growth, which is necessary to reconstitute lost neural circuits. Our preliminary data concern mechanically induced stretch growth of MNP-labelled human-derived differentiating neurons both *in vitro* and microinjected in the *ex vivo* model of mouse spinal cord organotypic slices. The neurites of MNP-labelled cells subject to the external magnetic field were found to be longer compared to the control ones both *in vitro* and after the microinjection. We are also developing an innovative platform of cortico-spinal assembloids to test the effective regenerative potential in a 3D cytoarchitectural context.

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THE EFFECTS OF MIXTURES OF DIBUTYLPHTHALATE AND ENDOGENOUS HORMONES ON HUMAN PROSTATE CELLS

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Endocrine Disrupting Chemicals (EDCs) raise many concerns due to their chemical and physical features, such as high hydrophobicity and low water solubility. EDCs can persist in different environmental matrices; so, human population is predominantly exposed to them, through ingestion or skin contact. They can biomagnificate in food chain and bioaccumulate in adipose tissue[1]. EDCs mimic endogenous hormones, affecting the homeostasis of several organs, such as prostate gland.[2-4] Dibutylphtalate (DBP) belongs to a subclass of EDCs widely used as plasticizer in personal- care products, children's toy,

pharmaceuticals, food products. In the present work, we studied the effects of DBP on human prostate epithelial cells (PNT1a) with or without endogenous hormones, testosterone (T) and 17 estradiol (E2), in order to assess the potential sinergystic or antagonistic effects of DBP. First, we observed cell viability showing that all mixtures increased cell proliferation, with a potential antagonistic effect of DBP. Second, we observed the localization of estrogen (ERs) and androgen (AR) receptor and we showed that DBP, alone or in combination with T or E2, was able to interact with steroid hormones and to translocate them into the nucleus, mainly interacting with ER . Furthermore, we observed that cytokines and chemokines levels, such as IL-9 PDGF, TNF , MIP-1 , MIP- 1 , IL-1 , were altered after all the treatments. DBP involvement should play a key role in the onset of inflammation processes. In conclusion, we have pointed attention on dangerousness of the mixtures able to induce a strong imbalance of prostate cell physiology.

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HISTONE ACETYLTRANSFERASE P300 REGULATES THE METABOLIC REMODELING OF THE HEART DURING AGING

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During aging, a progressive deterioration of both the structure and function of the heart[1] is observed, as well as a metabolic remodeling within the myocardium. This metabolic shift, from mitochondrial oxidation to anaerobic glycolysis[2], causes an energy deficit that contributes to impairment of cardiac function in the elderly[3]. Preliminary data suggest that the increase in glycolytic pathways in the onset of aging is led by the activation of enhancers by the acetylation of H3K27.

To understand how enhancers of glycolytic genes are activated in the onset of aging, we are now focusing on the histone acetyltransferase p300, which is required for the activation of enhancers during heart development and for modulating the activity of MEF2C during cardiac hypertrophy[4]. In order to investigate the effect of p300 on cardiac function in old-age onset mice, we treated mice prior to the onset of aging (16-month-old C57BL/6J mice) with C646, a p300 inhibitor, for 2 months. Echocardiographic analysis revealed that the inhibition of p300 is able to preserve the cardiac function in mice at the onset of aging. Quantification of three key metabolites of glycolysis (glucocose-6-phosphate, pyruvate, and lactate) suggested that C646 interferes with the activation of anaerobic glycolysis mediated by histone acetyltransferase p300 in mice at the onset of aging. In support of this, we found that p300 inhibition is able to prevent the activation of anaerobic glycolysis in HL1 cells in hypoxia, a condition characterized by a shift from aerobic to anaerobic metabolism. Together, these results confirm our hypothesis that the activation of anaerobic glycolysis in cardiac aging is characterized by an increase in the expression of some key glycolytic genes dependent on the activation of a set of enhancers, mediated by the acetylation of H3K27 by p300.

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THE ALLOGRAFT INFLAMMATORY FACTOR 1 (AIF-1): EVIDENCE FOR ITS PRESENCE IN THE ADULT AND EMBRYONIC STAGES OF *PARACENTROTUS LIVIDUS*

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Allograft inflammatory factor-1 (AIF-1) is a 17 kDa calcium-binding protein involved in various aspects of inflammation. In human the AIF-1 gene is in the major histocompatibility complex class III region on chromosome 6p21.3, in an area densely clustered with inflammatory response genes[1,2]. In mammals, increased expression of AIF-1 was observed in several diseases including endometriosis, breast cancer and rheumatoid arthritis[3]. Literature data indicate that proteins of the AIF-1 superfamily are also present in invertebrate species from sponge to echinoderms. In invertebrates, the highest and regulated expression level of AIF-1 in hemocytes indicates its key role in immunity. However, the ubiquitous presence of AIF-1 transcripts in different tissues suggests its possible participation in various biological processes[4].

The first report on the presence of AIF-1 in echinoderms identified one gene in the purple sea urchin *S. purpuratus*[5]. But it was later found in different echinoderms species including the green sea urchin *P. lividus*[6]. Also among echinoderms, the highest expression level of AIF-1 has been observed in circulating cells, being very active in the host responses against pathogenic challenge.

Here we report about the presence of the AIF-1 protein in different developmental stages of *P. lividus*. A different localization was evidenced between male and female gonads. In male gonads the protein is localized in the cortical region, indicating the specific expression in spermatids. In female gonads the protein is visible only in the accessory cells.

The role of AIF-1 in germ cells remains largely unknown and data about the response during early development stages is still needed to better understand. Functions and involvement of AIF-1 in the ontogenesis and development of invertebrate immune system are under investigation.

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GERMLINE DIFFERENTIATION IN BIVALVES: TDRD7 AS A CANDIDATE FACTOR INVOLVED IN *RUDITAPES PHILIPPINARUM* GERM GRANULE ASSEMBLY

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The differentiation of the germline in animals is intimately associated to the formation of cytoplasmic ribonucleoproteic granules. Even if these structures - collectively called germ granules - are characterized by similar functions and molecular content, they differ in position, shape, and complexity both between species, and between germ cell stages[1,2]. Part of the inter-specific variability is represented by the molecular factors that drive their assembly, with cases of co-option or new evolution of genes involved in it.

The aim of our analysis is to better characterize the steps of germline differentiation in the Manila clam *Ruditapes philippinarum,* with a focus on germ granule dynamics. This species, like many bivalves, is distinguished by the annual renewal of the gonads, with the reiteration of germline differentiation steps at every reproductive season. Previous works have identified, with the germline marker *vasa,* the germ cell differentiation steps[3,4], and, through electron microscopy, germ granules have been identified and associated to different stages in the different sexes[5]. In the present study, we characterized through immunofluorescence and immunohistochemistry the expression patterns in mature and immature gonads of the protein TDRD7, a candidate factor involved in germline differentiation and germ granule assembly. We could observe granular structures in previously identified germ cell stages, from the proposed germ stem cell repositories in the intestine/connective tissue[3,4], to the different stages of gamete differentiation. Crossing our data with those already obtained in the same species, we refined our understanding of bivalve germline, whose under-covered basic biology is an important source of knowledge to grasp the variability of germline differentiation mechanism in Metazoa.

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ABSENCE OF LARVAL REGENERATION IN THE HIGHLY REGENERATIVE CRINOID *ANTEDON MEDITERRANEA*

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Echinoderms are characterized by extraordinary regenerative abilities, both in adults and larval stages. Larval regeneration is well documented for all echinoderm classes, except Crinoidea, the most basal taxon. Therefore, the aim of this work was to assess if the larval stage (doliolaria) of the crinoid *Antedon mediterranea*, whose adults are perfectly able to regenerate almost any tissue, can regenerate as well. In normal conditions, free-swimming *A. mediterranea* doliolaria metamorphose in a temporary sessile and stalked stage (pentacrinoid) provided with an apical calix; this latter will eventually detach originating the free-swimming adult individual. Adult specimens of *A. mediterranea* were collected at Le Grazie (La Spezia Gulf). After hatching, doliolaria larvae were transversally bisected and the obtained anterior and posterior halves were monitored for 2-3 weeks. For each fragment type we defined different post-amputation "developmental" stages which were characterized by light and electron microscopy (SEM, TEM). Results showed that none of the surviving halves was able to completely regenerate. Rather, after a wound-healing phase each half continued its pre-determined development and the obtained post-metamorphic stage lacked structures deriving from the missing half: anterior fragments originated a stalk without a calix whereas the posterior halves produced a calix without a stalk. In terms of inner anatomy, each of the fragment developed the specific tissues normally present in the corresponding half of the larva. These data suggest that: doliolaria cells are strictly committed to their original fate; cellular plasticity/dedifferentiation is temporary blocked and/or "stem cells" are missing or in a "stand-by" state. This inability to regenerate is reverted upon the transition from pentacrinoid to free-swimming juvenile, as freshly "detached" individuals are perfectly able to regenerate their tissues/structures. Considering the basal phylogenetic position of Crinoidea these results are particularly significant to better understand the evolutionary trajectories which led to gain or loss (larval) regenerative abilities among echinoderms and metazoans.

ALTERED EXPRESSION OF PEROXISOMAL MARKERS IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Peroxisomes are dynamic cytoplasmic organelles, involved in numerous anabolic and catabolic functions. These include control of lipid and reactive oxygen species (ROS) metabolism, a role carried out in tight connection with mitochondria. Indeed, (dys)-functions in either of the two organelles determine altered metabolism of the other[1]. Peroxisomes are able to respond to physiological and pathological changes and extracellular stimuli, by altering their enzyme content, morphology, size and abundance. These dynamic features are modulated by peroxisome proliferator activated receptors (PPARs), a class of ligand-activated transcription factors[2], and their coactivator PGC1 , which also controls mitochondrial biogenesis.

Increased energy expenditure, lipid metabolism alterations and oxidative stress related to mitochondrial dysfunction, play crucial roles in amyotrophic lateral sclerosis (ALS) onset and progression. ALS is a neurodegenerative disorder affecting motor neurons and leading to skeletal muscle atrophy and weakness[3]. Skeletal muscle in turn contributes to defective energy metabolism and determines a derangement of basal metabolic rate, switching its metabolism towards an oxidative phenotype and to the preferential use of fatty acids as fuel[4]. The aim of this study is to address the role played by peroxisomes in ALS, by

analyzing the expression of membrane and matrix markers in the spinal cord and muscle of a transgenic mouse model overexpressing the human mutation SOD1-G93A.
The performed immunoblotting and qRT-PCR analyses of peroxisomal proteins and antioxidant enzymes, strongly suggest an involvement of these organelles in ALS onset and progression, possibly counteracting redox imbalance and mitochondrial dysfunction.

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FUNCTIONAL ANALYSIS OF THE EXTRACELLULAR PROTEIN TSUKUSHI IN NEURAL STEM/PROGENITOR CELLS FROM THE MOUSE EMBRYONIC CEREBRAL CORTEX

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Tsukushi (TSK) belongs to the small leucine-rich proteoglycan family of extra-cellular proteins[1]. It is expressed in the cerebral cortex and in the subventricular zone in the embryonic and postnatal mouse brain. TSK regulates essential signaling pathways, such as TGFß, Wnt, Notch, by interacting with their ligand or receptors at the extracellular level[1,2]. Knock-out mutant mice lacking TSK function display hydrocephalus and alterations in postnatal neurogenesis[3], but the underlying molecular mechanisms remain unclear. Using neural stem/progenitor cells (NSPCs) derived from the mouse embryonic cerebral cortex, we generated transgenic NSPC lines, which express the wild-type secreted form of TSK (TSK-S), or a membrane-tethered form of TSK (TSK-TM).These lines were compared to control NSPC lines expressing Green Fluorescent Protein (GFP). Preliminary gene expression analyses by microarray and real-time RT-PCR showed transcriptional changes in genes related to cell signaling and cell proliferation between TSK and GFP lines, but also between TSK-S and TSK-TM lines. TSK lines showed comparable cell viability and proliferation to GFP lines, based on the analysis of growth curves, flow cytometry of propidium iodide-stained cells and trypan blue exclusion assays. Our current results suggest that TSK overexpression modulates specific gene expression programmes in NSPCs, but it is not sufficient to alter their proliferation *in vitro*, possibly owing to exogenous growth factors compensating for TSK-dependent gene expression changes. They also suggest that TSK localization at the outer cell membrane or in the extracellular space may affect its function. Further experiments are underway to analyse the transcriptomic effects of TSK overexpression and functional inactivation in NSPCs by RNA-sequencing.

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BIOLOGICAL EFFECTS INDUCED BY NANO AND MICROPLASTIC IN *MYTILUS GALLOPROVINCIALIS* AFTER ACUTE AND CHRONIC EXPOSURE

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Microplastic presence in the marine environment is one of the most contemporary pollution problems. The high persistence of microplastics in the marine environment creates a new pathway for chemical contaminants in the water column[1]. Physical factors, temperature increase, wave action, and photo-oxidation drive the fragmentation process, reducing their size and increasing the particle's bioavailability[2]. The presence of microplastics in the marine environment has been registered in a large variety of organisms at different trophic levels.
In our work, we studied at histological levels the effects of nano and microplastics in *Mytilus galloprovincialis*, a model organism used in the last decades as a good bioindicator. Polystyrene at a dimension of 5 and 0.1 µm was used. Gills, gonads, digestive gland and byssus were fixed at 1, 3, and 11 days of treatment. Time points and concentrations were used according to the literature and a pilot study. Preliminary results show that both micro and nanoplastics interfere with tissue morphology at early and late time points. In deep, the attention has been focalized on the gills; investigation shows a significant alteration in the structural organization of the septum and lamellae, the presence of granules, infiltration, and alteration of mucus cells. Future investigation will clarify how the treatment could affect the reproductive process. We can conclude that polystyrene induces tissue damage in the gills of the mussels altering their function. This would represent a risk for their health, for the trophic chain, and indirectly for human consumers.

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EFFECTS OF THE HERBICIDE GLYPHOSATE ON TESTICULAR MORPHOLOGY, STEROIDOGENESIS AND ESTROGEN RECEPTORS EXPRESSION IN THE FIELD LIZARD *PODARCIS SICULUS*

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In the last two decades, human and animal health may be compromised by environmental pollution caused by the intensive use of herbicides, such as glyphosate[1,2]. Glyphosate-based herbicides (GBH) are broad-spectrum, non-selective, non-systemic herbicides widely used in agricultural and non-agricultural systems[2]. In a previous study carried out on the field lizard *Podarcis siculus*, glyphosate was shown to cause liver damage at both molecular and morphological levels[3]. Now, in this study, we evaluated in *P. siculus* the testicular histopathology, steroidogenesis, and expression/localization of estrogen receptors after three weeks of oral exposure to glyphosate at 0.05 and 0.5 g/kg body weight on alternate days. Our results showed for the first time that

glyphosate affected testicular morphology, reduced spermatogenesis, altered cell junctions by reducing the expression of Connexin 43, and changed the localization of estrogen receptors in germ cells, increasing their expression; the effects were mostly dose dependent. The result also showed that glyphosate, at least at these concentrations, did not affect steroidogenesis, indeed, no changes in the distribution of steroidogenic enzymes 3 -HSD, 17 -HSD and P450 cytochrome aromatase were recorded in the seminiferous epithelium. In the complex, the data indicate that this herbicide can disturb the morphophysiology of the male lizard reproductive system with obviously detrimental effects on their reproductive capacity. The effects of glyphosate must be considered biologically relevant and could endanger the survival of lizard populations; a more controlled and less intensive use of glyphosate in areas suitable for plant production would therefore be desirable.

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IMAGING OF ZEBRAFISH EARLY EMBRYONIC DEVELOPMENT USING AUTOFLUORESCENCE AND CALCIUM INDICATORS

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Owing to its external fertilisation and development and to its optical transparency and ease of manipulation, zebrafish has long become an important model for the study of vertebrate embryonic development[1]. Combination of fluorescent reporters of gene activation or of cellular activity (such as calcium indicators) with the observation of developmental dynamics represent a powerful tool for the study of embryo development, cell calcium signalling[2,3], cell lineage[4,5], tissues and organ differentiation. All these processes take place for the most part within the first 24 hours of development, thus allowing monitoring in real time and with cellular (or subcellular) resolution the phases of segmentation, gastrulation and neurulation up to the formation of a fully functional heart, skeletal muscles, central nervous system and all other organs.

Here, we present methods for imaging all cells of the developing embryo from the zygote stage on, employing cell autofluorescence. This method offers the advantage of being applicable in confocal and two-photon microscopy, providing means for the study of cellular dynamics during development and producing a complete cellular map of the embryo onto which fluorescence signals (for example from calcium indicators or specific gene reporters) can be overlaid to gain information on the location of the processes under investigation. Autofluorescence is a complex process mostly attributable to cellular pigments, among which mitochondrial cytochromes. Unfortunately, as any other fluorescence emission, it implies production of Reactive Oxygen Species (ROS) and, consequently, photodamage to the sample. We assessed the sensitivity of zebrafish embryos to photodamage in different experimental conditions and report parameters for optimal imaging of the embryo depending on the process of interest (for example, monitoring of cell division at early stages versus long-term imaging of the first 24 hours of development). Key aspects will be addressed, not only in terms of imaging parameters with confocal and two-photon microscopy but also in regards to sample preparation and mounting for proper orientation. We then employ these methods to monitor and follow calcium dynamics in neuroblasts and during the development of neural tube and central nervous system.

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EFFECT OF NON-PHYSIOLOGICAL LEVELS OF D-ASPARTATE ON SPERMATOGENESIS IN DDO KNOCKOUT AND DDO KNOCKIN MOUSE MODELS

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Experimental studies performed in mammals showed that D-Asp acts at all levels of the hypothalamic-pituitary-testis axis suggesting that this amino acid plays a key role in reproductive processes[1,2]. D-Asp regulates the synthesis and release of testosterone by promoting the release of gonadotropin-releasing hormone (GnRH) in the hypothalamus and luteinizing hormone (LH) in the pituitary. *In vitro* experiments carried out on isolated Leydig cells, GC-1 spermatogonia and GC-2 spermatocytes demonstrated that D-Asp also exerts a direct effect on both steroidogenesis and spermatogenesis. To elucidate the endocrine role of D-Asp in the reproductive processes, two models of mutant mice with targeted overexpression (Ddo^{ov}) or deletion ($Ddo^{-/-}$) of D-aspartate oxidase, a peroxisomal flavoprotein which catalyzes the deaminative oxidation of D-Asp, have been used. In one-month-old Ddo^{ov} mice a dramatic reduction of D-Asp levels and a significant decrease in serum testosterone levels occurred. Moreover, since histological and morphometrical data showed an altered testicular organization, to clarify if the lack of D-Asp may also produce effects also on cytoskeleton, prolyl endopeptidase (PREP) and disheveled associated activator of morphogenesis 1 (DAAM1) protein expression levels have been evaluated. The data showed that testis levels of DAAM1 decreased in Ddo^{ov} mutant mice, while those of PREP increased as compared to the wild type. These results were corroborated by immunofluorescent localization of DAAM1 and PREP, showing an altered distribution pattern particularly in spermatogonia and Leydig cells, respectively. Interestingly, preliminary studies showed that one-months-old $Ddo^{-/-}$ mice showed both higher testis D-Asp levels and increased serum/testis testosterone levels of as well as a more active spermatogenesis than wild type.

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ATOMIC FORCE MICROSCOPY: A NEW EXPERIMENTAL APPROACH FOR TOPOGRAPHICAL MAPPING OF SURFACE BUDDING

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Extracellular Vesicles (EVs) are membranous nanoparticles released by all cell types, carriers of bioactive cargoes and regulators of intercellular communication[1]. EVs trafficking is altered in several stress conditions, being considered a pathway involved in several pathological disorders[2]. Due to their heterogeneity and the lack of standardized protocols, it is hard to characterize EVs with a good reproducibility[3]. Our aim was to develop a new methodology to study EVs morphological features, spatial distribution over the cell surface, and alterations under different physiological conditions, exploiting Atomic Force Microscopy (AFM). Human U251MG, U373MG, T98G and U87MG glioblastoma multiforme cells, control or treated with Temozolomide (TMZ), and human neuroblastoma SH-SY5Y cells, control or treated with H2O2, were selected as platforms to perform our analysis. EVs-enriched fractions were isolated by ultracentrifugation or ultrafiltration combined to size exclusion chromatography, and their morphology, quantity and size distribution were quantified by TEM and AFM. AFM defined the EVs topographical distribution and size, and cell surface roughness after treatment. On both the cell types, the amount of EVs, the budding vesicles topographical distribution, and cell surface roughness resulted altered after treatment. These results may provide interesting perspectives to validate AFM for EVs morphological characterization, mapping of surface budding, and understanding the pathophysiological state of the cell.

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IMMUNE RESPONSE UPON CARBON IONS AND IMMUNE CHECKPOINT INHIBITORS THERAPY REDUCE THE METASTATIC BURDEN IN A MOUSE MODEL

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Radiation therapy is one of the standards of care for multiple malignancies aimed at direct tumor destruction. However, metastatic disease still remains a problem. The immune system plays a crucial role in eliminating primary tumors and preventing tumor recurrence.

Ionizing radiation is reported to be immunogenic, *i.e.*, it may induce immunogenic cell death in tumor cells leading to immune activation, in turn resulting in the shrinkage of out-of-field metastases, phenomenon known as abscopal effect. The right combination of radiotherapy with immunotherapy could be a strategy to induce durable responses and improve survival. Particle therapy, especially carbon ions, is assumed to increase the immunogenic effect further. The aim of this study was to investigate the efficacy of X-ray and high-energy carbon ion therapy in combination with immunotherapy.

In this work we investigated the combination of radiotherapy, with either carbon ions or X-rays, and immune checkpoint inhibitors (CPI) (anti-CTLA-4 and anti-PD-1), in a syngeneic osteosarcoma mouse model (C3H/He mice, LM8 cells). Tumor cells were injected into both hind limbs of the mice. Following an initial growth period, one of the tumors was irradiated while the other was kept out of the irradiation field, mimicking the abscopal tumor. Macroscopic analysis on out-of-field metastases was carried out to investigate spontaneously formed lung metastases. Immune responses were investigated by histological analyses on the abscopal tumors screening. The results show that a combination of carbon ion therapy and CPI leads to reduced growth of abscopal tumors and reduced formation of lung metastases. In addition, in the abscopal tumors treated with combining therapy, an immunogenic response resulting in increased of infiltration of CD8+ cells were observed. Combining CPI with high-energy carbon ion radiotherapy could be a promising strategy for treating tumors and metastasis.

INSULIN-RESISTANT M2-CD163+ MACROPHAGES RELEASE EXTRACELLULAR VESICLES AFFECTING LIPID METABOLISM AND EXTRACELLULAR MATRIX GENE EXPRESSION IN MUSCLE CELLS

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In this study, we investigated whether muscle-resident macrophages produce extracellular (EV) vesicles able to influence skeletal muscle (SkM) homeostasis in an obesogenic environment. For this purpose, small and large EVs (sEV and lEV) were collected from macrophages treated with palmitate+oleate (FFA) with or without 15 mM D-glucose (FFA/G15). Activation markers, lipid composition, and the metabolic signatures of treated macrophages were analyzed. We also investigated the effects of sEV and lEV on muscle lipid composition, gene expression, and insulin response in C2C12 myotubes. As a result of FFA treatment, macrophages polarized into M2-CD163^{+} cells, released pro-/anti- inflammatory cytokines, accumulated toxic lipid species, and had reduced insulin sensitivity. Furthermore, C2C12 treated with lEV-FFA accumulated triacylglycerols (TAG) and fatty acids and showed reduced insulin sensitivity when compared with untreated cells, reproducing the effect of FFA on macrophages. RNA sequencing of recipient myotubes revealed that lEV-FFA also induced the expression of extracellular matrix components. Conversely, sEV-FFA decreased the expression of genes involved in lipid oxidation and mitochondrial respiration, and increased TAG accumulation without affecting muscle insulin resistance. Therefore, macrophages release EVs during diet-induced obesity that maintain muscle integrity. In macrophages, FFA/G15 conditions decreased CD163+ and IL-10 while increasing IFN , IL-1 , and IL-8. Therefore, macrophage-FFA/G15-released EVs lacked the beneficial effect of macrophage-FFA and induced insulin resistance in C2C12. In conclusion, our data shown that EVs can mimic the phenotype plasticity in releasing macrophages, which may explain it the events leading to SkM insulin resistance associated with obesity-induced diabetes.

IMPACT OF AMINO-FUNCTIONALIZED MESOPOROUS SILICA NANOPARTICLES (NH2-MSINPS) ON THE EMBRYONIC DEVELOPMENT OF THE SEA URCHIN *PARACENTROTUS LIVIDUS*

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The growing application of amino-functionalized mesoporous silica nanoparticles (NH2-MSiNPs) in medicine requires, in addition to assessing safety for human use, consideration of potential indirect and direct dispersion in the environment and assessment of any harmful effects.[1,2] Here, we investigate both the chemical stability of NH2-MSiNPs depending on pH and assess their possible toxicity on the embryonic development of *P. lividus.* NH2-MSiNPs degrade at neutral pH when dispersed in synthetic seawater (SSW). In terms of embryonic development, our experiments carried out at neutral pH in SSW show that NH2-MSiNPs can limit fertilization at high doses (0.5 mg/L) while at low doses (0.1 mg/L), although fecundation is not affected, embryos exhibit morphological alterations in late-stage development. The latter are associated with the impairment of mitochondrial activity and a lack of time coordination in the expression of genes involved in the development of embryos (mainly linked to skeletogenesis). Furthermore, for the first time to our knowledge, we report a dysregulation of ribosomal mRNA expression during *P. lividus* embryo development due to NH2-MSiNPs treatment. In summary, even though NH2-MSiNPs degrade at physiological pH, they play a crucial role in *P. lividus* abnormal development. It is important to conduct additional research to highlight possible environmental and human toxicity, with a special focus on developing organisms.

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GLUCOSYL-STEROLS TRIGGER BOWEL INFLAMMATION AND COULD CONTRIBUTE TO NEURODEGENERATION THROUGH THE GUT-BRAIN AXIS

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Functions of glucosyl-sterols in cellular metabolism are poorly known, but alteration of their homeostasis has been associated with the onset of different neurodegenerative diseases[1]. In particular, the plant-derived ß-glucosyl-sitosterol was recognized as a contributor to the pathogenesis of a complex disorder[2], thus representing a critical neurodegeneration risk factor. Glucosyl-

sterols mode of action is still unknown. However, since they derive from sterols, they may interact with membrane and nuclear receptors of cells and neurons, possibly altering proper response in signaling pathways. In this work, we study the potential roles of glucosyl-sterols in nervous system biology using the zebrafish model. Larvae treated with ß-glucosyl-sitosterol develop normally without morphological changes. However, the presence of aggregates in the intestine of treated larvae suggests the occurrence of an initial local inflammation that may progress into a systemic inflammatory state. Indeed, RT-qPCR performed on whole larvae RNA shows an increasing trend in the expression of genes involved in inflammatory response (*il-1ß*, *mmp9*) and oxidative stress (*hmox1*). Moreover, the significant reduction of *atg5* and *lc3b*, two autophagy-related genes, suggests a possible alteration of the autophagic flux that can result in the occurrence of proteinopathy. Treated larvae also show a significant decrease in peristaltic activity that may be caused by some dysfunction of the enteric nervous system (ENS), and intestinal morphology is altered in chronically treated individuals. According to the gut-brain axis model[3], we aim to understand if the intestinal inflammation due to glucosyl-sterols treatment may trigger neuronal damage starting from the ENS and then spreading towards the central nervous system. In line with this, after treatment, larvae present a reduced locomotor activity and an impaired response to stress that may be ascribed to neuronal cues.

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THE SPATIO-TEMPORAL EXPRESSION PATTERN OF THE YAMANAKA FACTORS IN THE BUDDING TUNICATE *BOTRYLLUS SCHLOSSERI*

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Yamanaka Factors (YFs), *i.e.* the four transcription factors *Sox2*, *Klf4*, *Pou5f1* and *cMyc*, induce the de-differentiation of mature mouse cells *in vitro*, and control pluripotency and proliferation in early vertebrate embryogenesis. In invertebrates however, their expression in pluripotent stem cells is poorly characterized. In the colonial tunicate *Botryllus schlosseri* stem cells participate in bud development and regeneration, and in adults, they locate in stem cell niches. Here, we identified *B. schlosseri* orthologs of vertebrate YFs, investigated their evolution and analyzed, by whole-mount *in situ* hybridization, YF spatio-temporal expression pattern. *SoxB1* and *Myc* genes were identified as the orthologs of vertebrate *Sox2* and *cMyc*, respectively, while both *Pou2* and *Pou3* genes were analyzed as the most probable ancestors of the vertebrate-specific *Pou5f1* gene. The large size of *Klf* gene family, associated with the reliable sequence conservation only in the very short zinc finger domain, has prevented the identification of *Klf4* ortholog in *B. schlosseri*. *Myc* is expressed in all developing tissues except for central nervous system, with diminishing expression as buds progresses in development and disappearing in adult tissues. *SoxB1* is detectable in developing branchial epithelium, esophagus, nervous system, and endostyle. *Pou3* shows expression in germline, nervous system, heart, and digestive system primordia. *Pou2* expression is limited to the first phases of development and to oocytes. All genes are co-expressed in developing oocytes. In adults, at least 20% of candidate stem cells co-express *Myc*, *Pou3* and *SoxB1* in stem cell niches. In conclusion, our work suggests that in *B. schlosseri*, as in vertebrates, the single YFs have distinct roles in a variety of developmental processes, while together, *Myc*, *SoxB1*, and *Pou3* may have a function in the maintenance of pluripotency. These results show that YFs spatio-temporal expression pattern is shared in chordates development.

NEW INSIGHT ON THE *IN VITRO* EFFECTS OF MELATONIN IN PRESERVING HUMAN SPERM QUALITY

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Male fertility depends on high-quality spermatozoa (SPZ) in terms of morphology, motility, and DNA integrity[1]. Growing evidence is demonstrating a worldwide decrease of fertility, proportionally to an increased deterioration of gamete quality[1]. SPZ are extremely specialized cells but, at the same time, they are particularly delicate and sensitive to most kind of physical, chemical and biological stressors. Between them, ROS-generating oxidative stress seems to be particularly effective in influencing SPZ physiology[3]. In this regard, many efforts have been made to develop methods, as the addition of antioxidant molecules to cryopreserving agents, with the aim of achieving the best environment for to preserve, as much as possible, SPZ quality[4]. Among these, melatonin (MLT) is one of the most studied molecules, due to its well-known antioxidant and antiapoptotic properties[5]. The *in vitro* effects of MLT treatment on sperm parameters using cadmium (Cd) to induce oxidative stress and, consequently, SPZ alterations were investigated. Twelve semen samples, coming from nonsmoker patients between 27 and 35 years, were divided into: 1) control, 2) 10µM $CdCl_2$, 3) 2mM MLT and 4) $CdCl_2$+MLT and analyzed after 30min, 6h and 24h of exposure. Results showed a time-dependent decrease of SPZ motility, DNA integrity and increased apoptosis, counteracted by MLT co-treatment. Based on these data, other parameters were further explored at 24h. Cd increased lipid peroxidation and altered the levels and localization of motility (PREP and RSPH6A) and morphology (DAAM1) protein markers. Moreover, Cd reduced the % of SPZ able to undertake acrosome reaction, as well impaired protein levels and localization of two markers of acrosome membrane integrity: PTMA and IAM38. Co-treatment with MLT partially counteracted the harmful effects posed by Cd on all the parameters. Collectively, data encourage MLT use as an integrative molecule to ameliorate SPZ quality when compromised by stressful condition.

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WHOLE BRAIN REPRESENTATION OF IMPRINTED CUES

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Establishing sensory-related memories during infancy is a shared process among several animal species and it is fundamental in a wide spectrum of vital animal behaviours. One among many is sexual imprinting, a process of instinctive learning that happens early in life, when individuals acquire memories of odours, vocalizations, and other characteristics of their relatives, and then utilize this information for mate choice as adults[1-2]. Despite sexual imprinting has evolved in many taxa, almost nothing is known about the underlying neuronal mechanisms. Past works in inbred mice showed females display an innate preference for novel unfamiliar males of a different strain, but only if reared with their own father[3]. By using an interdisciplinary approach, we investigate how imprinted (familiar) or novel (unfamiliar) sensory cues are represented in the adult female brain. We perform whole brain mapping of immediate early genes (IEGs) after acute exposure of adult females to familiar or unfamiliar odours (urine). We combine iDISCO[4] tissue clearing with light-sheet fluorescence microscopy to image IEGs stained neurons across the whole brain. We use ClearMap[5], an open-source software for single cell segmentation and atlas alignment, to evaluate the number of IEGs positive cells for all brain areas. Our data show that both familiar and unfamiliar olfactory cues recruit brain areas known to guide mating and social interaction such as hypothalamic and amygdalar areas. Despite these areas were activated in both conditions, unfamiliar odours tend to elicit stronger activation. Importantly, we identified a small subset of hypothalamic regions which were recruited by unfamiliar, but not familiar, odours. These preliminary results indicate that hypothalamic areas might play a prominent role in discriminating imprinted odours, contributing to male preference during mate selection.

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EXTRACELLULAR VESICLES FROM MESOANGIOBLASTS MODULATE MACROPHAGE BEHAVIOUR

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It is now well known that stem cells release large amount of extracellular vesicles (EV) that are involved in tissue regeneration. Inflammation also plays an important role in tissue repair and regeneration.

The aim of our work was to determine the effect of EV released by mesoangioblast stem cells (C57) on macrophages (Raw264.7), as they play a central role in all stages of the inflammatory response. In damaged tissue macrophages migrate to carry out their function of identifying and removing dead cells, debris, and foreign particles *via* phagocytosis.

To this aim we have proved that EV treatment influenced negatively Raw264.7 cells cell proliferation index, and positively their migratory phenotype, when compared to untreated cells. We also proved, that this enhanced migration is due to an elevated expression and activity of MMP2/9. Moreover, *in vitro* phagocytosis index calculation highlighted that EV treatment is able to improve Raw264.7 phagocytic ability, which is important at early stages of tissue repair. As C57-EV contain Hsp70 as a transmembrane protein to elucidate whether it is involved in phagocytosis modulation, we performed phagocytosis assays also in the presence of neutralizing antibodies (*i.e.*, anti-Hsp70, anti-TLR2, anti-TLR4 antibodies), or in the presence of exogenous Hsp70. The obtained results demonstrated that C57-EVs are able to increase Raw264.7 phagocytosis through Hsp70 and its surface receptors.

The healing process consists of three overlapping phases: inflammation, tissue regeneration, and tissue remodelling. It is known that "classically activated" M1 macrophages dominate at early times after injury, while M2 macrophages dominate at later stages, although they are also present at initial time points. To point out whether EV modulate macrophage phenotype we explored the presence of M1/M2 markers (*i.e.*, iNOS and arginase), and NO and cytokine synthesis. At the initial stage after EV treatment Raw264.7 synthesized both iNOS and NO, whereas no arginase mRNA was detected. These data suggest an M1 phenotype. After a recovery following EV treatment, was observed an increased release of anti-inflammatory cytokines, typical of M2 macrophages.

These data suggest that C57-EV could positively influence tissue repair through macrophage modulation.