

# Journal of Biological Research

Bollettino della Società Italiana di Biologia Sperimentale



**96<sup>th</sup> National Congress of the  
Italian Society for Experimental Biology**

L'Aquila, Italy, 25-28 April 2024

ABSTRACT BOOK

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Bollettino della Società Italiana di Biologia Sperimentale

eISSN 2284-0230

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PAGEPress Publications  
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 27100 Pavia, Italy  
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# 96<sup>th</sup> National Congress of the Italian Society for Experimental Biology

L'Aquila, Italy, 25-28 April 2024

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## ANTHROPOLOGY

### THE CLOSE RELATIONSHIP BETWEEN COMPARATIVE ANTHROPOLOGY AND URBAN BIODIVERSITY IN FORENSIC PRACTICE

Paolo BONIVENTO

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The close relationship between Comparative Anthropology and Urban Biodiversity in forensic practice is to be considered a very important factor, in particular following the application of Italian law 27 September 2021, n. 134 (aka "Cartabia Law"). This had, as its first consequence, the establishment of national registers of experts, who, until this operation, were included in local, paper registers, following a simple request to the president of the relevant territorial court. The old practice meant that many categories of professionals, including anthropologists, naturalists and ballistics experts, subjects without a formal professional association of reference, found themselves included in an "other" category together with many people with unattested skills. To enter the "new" categories, the new legislation requires not only the verification of certifications and experiences but also the obligation of continuous training. Therefore it has finally been possible to promote the close relationship, in the forensic field, of comparative anthropology with urban biodiversity, as they are foundations for the description, for example, of the scene of a crime that occurred in an urban area, where the forensic doctor, even if helped by a biologist, he cannot see the interactions between the anthropological field and the field of urban biodiversity. This work intends to pave the way for a series of studies that take both aspects into consideration in a correlated way, exploiting not only naturalistic and anthropological knowledge, which should be considered basic, but exploiting all the historical series to obtain parameters and algorithms so that any observation and deduction can be correctly inserted in space and time.

### WHAT CAN INFRARED SPECTROSCOPY TELL US ABOUT THE PRESERVATION STATE OF ANTHROPOLOGICAL REMAINS OF ARCHAEOLOGICAL INTEREST?

Maria Grazia BRIDELLI

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During the last decades, Fourier Transform Infrared (FTIR) Spectroscopy has grown from research laboratories to a well-established approach that is increasingly often used in archaeometry and conservation science. It is in fact one of the most interesting analytical technique, due to its non-destructive utilization and its sensitivity, for extracting most of the inorganic and organic information in ancient remains. When looking at this research fields, some novel trends can be detected with respect to the traditional applications of the technique.

The aim of this paper is to review the technical aspects of this kind of spectroscopy when applied to the bioarchaeological field of research and to present some applications and results for the characterization of organic and inorganic components of ancient remains by identifying the change in the macromolecular properties with respect to the modern ones. In particular, in the IR spectrum of the investigated tissues, the main absorption bands of the biological components

such as proteins, lipids, nucleic acids, and carbohydrates are detected and characterized. The recorded biochemical modifications reveal a partial alteration of the ancient tissues which have been changed and afterward stabilized by the chemical-physical environmental conditions that preserved them for hundreds of years. The whole IR spectrum of the tissues reveals traces of these processes: 1) the water OH-stretching band ( $\sim 3400\text{ cm}^{-1}$ ) features are indicative of the twofold role of the dehydration process, as a result of air temperatures and humidity, at the same time responsible for the protein structure modifications and/or determining the preservation; 2) conformational features of the proteins can be extracted by monitoring Amide I and Amide II bands ( $1500\text{-}1700\text{ cm}^{-1}$ ); 3) in the glucides specific spectral range ( $950\text{-}1150\text{ cm}^{-1}$ ), an increase was measured in the glucides/proteins ratio, a spectroscopic marker for the AGE compounds formation as a consequence of the collagen binding to sugars in tissues; 4) the amount of adipocere formation ( $2916\text{-}2849\text{ cm}^{-1}$ ,  $1700\text{ cm}^{-1}$ ) can be correlated with the ambient conditions where the remains were maintained until today. Furthermore the technique provides the change in organic and inorganic content of bones due to aging processes and the bone diagenesis was monitored by means of the mineralization parameters.

### THE PRIMATOLOGICAL COLLECTION OF THE MUSEUM OF ANTHROPOLOGY OF TURIN: DENTAL MORPHOLOGY AND ITS INCIDENCE IN PERIODONTAL DISEASE

Alberto PELUSO, Emma RABINO MASSA

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Animal dentitions possess the ability to self-cleanse, preserving them from periodontal disease, an ability that is similarly recognized in the human deciduous dentition, whereas it is lacking in the permanent dentition. Morphological differences in Primate dentitions were investigated in order to identify anatomical peculiarities that facilitate self-cleansing. Molars and premolars of 49 monkeys (28 species), 30 children and 30 adults from ancient Egypt were measured. The anatomical peculiarities affecting the self-cleansing process are located along the buccal and lingual walls of the teeth. They are: the distance of the equatorial line from the collar, the depth of the cervical convexity, and the taper of the walls. In non-Human primates and children, the shape of the teeth is conical with the favorable effect of increasing the functional occlusal surface. The conical shape is the result of continuous evolutionary adaptation in non-Human primates.

### *Brucella lymphadenitis* IN THE BLESSED SANTE FROM MONTEFABBRI (1343-1394). ADVANCED MORPHOLOGICAL AND ANALYTICAL INVESTIGATION

Martina POLIDORI<sup>1</sup>, Cinzia MERCURIO<sup>1</sup>, Lorenzo ARRIZZA<sup>2</sup>, Maria Grazia PALMERINI<sup>2</sup>, Antonio FORNACIARI<sup>3</sup>, Gino FORNACIARI<sup>4</sup>, Giancarlo MANDOLINI<sup>5</sup>, Luca VENTURA<sup>1</sup>

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Giansante Brancorsini was born to a noble family in Montefabbri, Urbino. He joined the Franciscan Order after killing an aggressor by sword, in order to live a life of penance for his sin. Hagiographic sources reported that he suffered for many years a large and deep sore in the right groin and recurrent fever, until he died in summer 1394. Recently, shotgun metagenomics allowed to identify *Brucella melitensis* DNA in his remains. In 1994, his partially skeletonized remains underwent Canonical Recognition by external inspection, on site radiographic survey, and tissue sampling for additional investigations. Several calcified polylobate nodules were found in the abdomino-pelvic cavity near the lumbar vertebral tract. Some of them underwent advanced morphologic imaging and analytical investigations. One nodule was cut and its surfaces were investigated by binocular stereomicroscopy (BSM) and scanning electron microscopy (SEM) also with energy dispersive X-ray analysis (EDX). Different portions of another nodule underwent histology/histochemistry X-ray diffraction (XRD) analysis, and Fourier's transformed infrared (FTIR) spectrometry. BSM, SEM, and histology/histochemistry showed the presence of an external fibrous capsule containing inner amorphous material, corresponding to necrotic debris. SEM-EDX and XRD highlighted the presence of apatite in the nodule, whereas FTIR demonstrated DNA and human serum proteins as organic compounds. Moreover, high resolution SEM with back scattered electrons allowed to visualize several micrococci measuring 1-3 micrometers in largest diameter in the inner portions of the nodule. Advanced morphologic and analytical investigation allowed to establish that the nodules found in the abdominal cavity of the Blessed were lymph nodes affected by granulomatous necrotizing lymphadenitis caused by *B. melitensis*. The micrococci observed by SEM examination morphologically corresponded to *Brucellae*. These findings are in agreement with multifocal periostitis and spondylodiscitis recognized in the skeleton as well as with the clinical history referred by hagiography. In conclusion, this represents the most ancient case of a morphologically documented *Brucella* lymphadenopathy, also with ultrastructural evidence of bacteria.

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#### EFFECTS OF URBAN VS. EXTRA-URBAN ENVIRONMENTS ON GROWTH AND PHYSICAL DEVELOPMENT: A STUDY ON A 11-YEARS-OLD PIEDMONT SAMPLE

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It is globally known that there is a trend towards an increase, particularly among the youths, in overweight conditions, as well as a decrease in motor skills and difficulties in developing and maintaining physical and functional abilities (Masanovic *et al.*, 2020). These trends are usually attributed to the lifestyle, related to dietary habits, to a lack of physical activity, to the increasing of sedentary behavior, and the excessive usage of technology. Additionally, the extrinsic factor defined by the environment plays a crucial role in the physical development

of children (Mishra *et al.*, 2023). In order to explore the influence of the environment on growth, weight conditions, and neuromuscular development, this study compared urban and extra-urban children living in the western Piedmont region in terms of anthropometric measures and functional abilities. The sample consisted of 255 11-years-old subjects (decimal age range from 10.50 to 11.49 years), including 126 from urban environments (70 boys and 56 girls) and 129 from extra-urban areas (77 boys and 52 girls). Height, weight and waist circumference were collected according to international standards for anthropometric data collection (ISO 7250/2017); Handgrip Strength (HGS) was measured (three trials for each hand) using a digital dynamometer (Baseline Smedley). Subsequently, subjects were categorized based on body mass index (BMI), using reference cut-off values for Italian samples (Cacciari *et al.*, 2006), and on waist-to-height ratio (WHtR according to Eslami *et al.*, 2023). Statistical analysis, conducted using IBM SPSS Statistics (version 28), aimed at evaluating differences between sexes and/or living environments, with significance set at  $p < 0.05$ . Results highlighted that, regarding sex comparison, waist circumference was significantly higher in males in both urban and extra-urban groups. Only in the urban sample females resulted significantly taller than males. Moreover, in the urban sample, boys resulted significantly stronger than girls, but only for the dominant hand. Concerning the comparison between the two environment groups, the urban males sample showed a higher prevalence of obesity, although no significant differences were found in BMI or WHtR ratio between the two groups for both sexes. Regarding anthropometric variables extra-urban males were significantly taller than urban males. The most interesting significant difference was found in HGS values as the extra-urban sample showed significantly higher values, both in male and female samples, in both hands ( $p < 0.01$  for dominant hand,  $p < 0.05$  for non-dominant hand). These results pointed out how growing up in an urban or extra-urban environment could significantly affects growth, either in terms of anthropometric measurements or functional abilities such as strength, even within a delimited area like Piedmont. In conclusion, this study could indicate that, at least at the age of 11, the environment could have a greater influence even before the appearance of the specific sex-related traits due to puberty. Following these results, we are expanding the sample to observe if these differences, attributed to the environment, are also evident in younger age groups.

#### THE "WHITE PLAGUE" IN THE MONASTERY OF THE CLARISE HERMITS IN FARA IN SABINA: PALEOPATHOLOGICAL AND METAGENOMIC ASPECTS

Mirko TRAVERSARI<sup>1</sup>, Elisabetta CILLI<sup>2</sup>, Federico BRUNO<sup>3</sup>, Alessia RISDONNA<sup>3</sup>, Enrico PETRELLA<sup>4</sup>, Gianandrea PASQUINELLI<sup>1</sup>, Luca VENTURA<sup>5</sup>

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The mummified bodies preserved in the monastery of the Poor Clares Hermits from Fara in Sabina have been known for some time, but only occasionally mentioned in paleopathological literature. This monastic community of

Franciscan nuns, dating back to the 17<sup>th</sup> century, followed the first Rule of Saint Clare combined with strict seclusion and absolute loyalty to ecclesiastical hierarchies. The 17 perfectly mummified bodies, according to archive documents, are believed to be the founders of the 17<sup>th</sup> century monastery, behind which there is the shadow of relevant Roman families, such as Farnese or Barberini. At the beginning of 2022 a preliminary inspection was carried out to plan the study. The conservative investigations include external examination and anthropometric and paleopathological studies, carried out by computerized tomography, followed by minimally invasive sampling aimed at paleonutritional and paleogenomic analyses. An interesting aspect of the study of mummified bodies concerns the diseases that affected this monastic community. Diseases have been found such as tuberculosis, present in at least 6 individuals, and gallstones, in 3 individuals, probably linked to nutrition and genetic predisposition. Particular forms of arthrosis affecting the knee and foot were also frequent, certainly attributable to the daily activities of prayer and meditation. In particular, on ID-Fara 9, a CT-guided biopsy was carried out inside the right lung, which allowed the recovery of a calcific nodule on which a metagenomic analysis was applied, which involved the sequencing and isolation of the aDNA of the entire microbial community, present within it.

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#### CHOLELITHIASIS IN THE MUMMIES OF THE POOR CLARES HERMITS' MONASTERY OF FARA IN SABINA (17<sup>th</sup> CENTURY)

Luca VENTURA<sup>1</sup>, Federico BRUNO<sup>2</sup>, Anna AVITABILE<sup>2</sup>, Alessia RISDONNA<sup>2</sup>, Irene FAENZA<sup>3</sup>, Enrico PETRELLA<sup>4</sup>, Gianandrea PASQUINELLI<sup>5</sup>, Mirko TRAVERSARI<sup>5</sup>

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The mummified bodies in the monastery of the Poor Clares Hermits in Fara in Sabina, Rieti, were recently studied by our group. This monastic community of Franciscan nuns, dating back to the 17<sup>th</sup> century, followed the first Rule of Saint Clare combined with strict seclusion and absolute loyalty to ecclesiastical hierarchies. According to tradition, these 17 perfectly mummified bodies belonged to the founders of the first monastery, with most of them coming from relevant Roman families as Farnese and Barberini. Gallstones represent a relatively rare finding in ancient human remains, almost always related to high social classes. Aim of the present study is to investigate the presence of gallstones in these mummies. The mummies were conservatively investigated by external examination, computed tomography (CT) scanning, and minimally invasive sampling. 3D rendering and densitometry allowed to reconstruct the exact stone morphology and establish chemical composition. Written sources hosted in the monastery's current library were also examined in order to find information about the Sisters who lived there in past times. Cholesterol-based gallstones were found in 3 individuals (17.5%) and were probably linked to dietary factors and genetic predisposition. The anthropological age at death of the subjects ranged from 40 to >50 years old. The Book of the Dead held in the monastery allowed to recognize 2 sisters with symptoms ascribed to gallstones (Sr. Maria Francesca Romana, who died in 1730 at the age of 75; Sr. Maria Giovanna Romana, who died in 1718 at the age of 82) and 1 sister affected by sudden fever and an undefined deep lateral chest pain (Sr. Maria Isabella, who died in 1698 at the age of 50). Ancient cholelithiasis was described in mummified or skeletal remains of 21 subjects from Sweden, Greece, Egypt, China, Chile, Ohio, Italy, Spain, and Colombia, dating back from the 2000 b. C. to XVI A. D. The age at death of the subjects was comprised between 25 and 60 years old, with a female predominance, and a 3:1 ratio of cholesterol:pigmented stones. In conclusion, gallstones may be easily recognized in natural mummies through CT scanning, whereas densitometry helps to establish their chemical composition. As its modern counterpart, ancient cholelithiasis was most frequently due to cholesterol-based stones and may thus represent a good bioanthropological marker of high social class. The presence of gallstones may also represent a valid clue for the identification of the subject, when information recorded in textual sources are available.

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## AGING

**EVALUATION OF THE REPARATIVE EFFECTS OF PROBIOTIC *Streptococcus thermophilus* ON HYDROGEN PEROXIDE-INDUCED AGING IN HUMAN DERMAL FIBROBLASTS**

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Skin aging is a complex process that implies numerous biological and structural changes in the skin. It is known that the most relevant cellular and molecular underlying mechanisms are associated with the aging of dermal fibroblasts and impaired extracellular matrix homeostasis (Shin *et al.*, *Front Physiol.* 2023, doi: 10.3389/fphys. 2023.1195272). An increasing body of evidence supports the use of topical probiotics in treating various skin conditions, including skin aging (Habeebuddin *et al.*; *Pharmaceutics.* 2022, doi:10.3390/pharmaceutics 14030557). In particular, positive effects of *Streptococcus thermophilus* on skin aging models have been reported *in vitro* and *in vivo*, (Lombardi *et al.*, *Biomolecules.* 2019, doi: 10.3390/biom9120756; Lombardi *et al.*, *J Inflamm (Lond).* 2022, doi: 10.1186/s12950-022-00324-9; Di Marzio *et al.*, *Int J Immunopathol Pharmacol.* 2008, doi: 10.1177/03946320080210011). This study aims to deep the biomolecular mechanisms underlying these anti-aging effects, evaluating the reparative ability of *S. thermophilus* lysate in a hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced aging model of human dermal fibroblasts. The effects of *S. thermophilus* used at different concentrations were assessed on cell proliferation and the expression of aging markers, matrix metalloproteinases (MMPs), collagen I, and prolyl 4-hydroxylase (P4HA1), an essential protein in collagen biosynthesis. Moreover, the principal biomarkers of oxidative stress and inflammation were also evaluated. *S. thermophilus* lysate exposure led to a significant and dose-dependent increase in the proliferation and improved the changes in cell morphology of the aged fibroblasts. Notably, probiotic lysate treatment counteracted the H<sub>2</sub>O<sub>2</sub>-induced aging by reducing β-galactosidase activity and p21 expression levels. In addition, *S. thermophilus* significantly promoted collagen I synthesis and reduced the MMP expression, thus affecting the collagen degradation in aged fibroblasts and maintaining the ECM homeostasis to achieve anti-aging effects. Interestingly, *S. thermophilus* lysate alleviated H<sub>2</sub>O<sub>2</sub>-induced oxidative damage by effectively decreasing reactive oxygen species and malondialdehyde levels, as well as increasing antioxidant enzyme activities through the activation of the nuclear factor E2-related factor 2 (NRF2) in aged fibroblasts. Moreover, probiotic lysate also inhibited the nuclear factor kappa B (NF-κB) pathway, leading to the downregulation of pro-inflammatory markers. Overall, our results show evidence that the *S. thermophilus* lysate is efficacious in suppressing the biomolecular events associated with H<sub>2</sub>O<sub>2</sub>-induced cellular aging, thus supporting the reparative action of the *S. thermophilus*, helpful in treating skin aging.

**GENOTOXIC STRESS INDUCED BY MECHANICAL FORCE IN CELLULAR MODELS OF PARKINSON'S DISEASE**

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Increasingly, studies are shedding light on the correlation between changes in brain age-related stiffness and the onset of Parkinson's disease (PD). It is hypothesized that these alterations of cell mechanobiology perturb brain parenchyma homeostasis and contribute to neuronal degeneration. However, the molecular mechanisms that regulate these phenomena are rudimentarily understood. The purpose of this work is to elucidate how perturbations in brain stiffness mechanistically alters cytoskeleton organization and nuclear morphology, which directly reflect cell mechanobiology properties. Here we examined how these processes relate to DNA damage, a recognized hallmark of aging and neurodegeneration. We leveraged on an experimental setup based on differentiated neuroblastoma cells grown on substrate with different stiffness, which therefore influence the mechanical properties of the cell. We exposed these cell lines to stressors that are relevant for PD pathology, that is 6-OHDA, rotenone, and preformed fibrils of alpha-synuclein. As readout measures, we monitored a nuclear shape parameter (roundness and circularity) and aberrations (blebbing and honeycomb structures) using the anti-Lamin A/C antibody. We also observed chromatin organization under different conditions with the anti-Histone H3k9me3 and cytoskeleton conformation with the F-Actin marker. Following these experiments, we evaluated sensitivity to DNA damage through analysis based on 53bp1/γH2AX colocalization. We found that the effect of exogenous stressors on indirect measures of mechanical stress such as nuclear shape, chromatin and cytoskeleton architecture depend on the substrate stiffness. Moreover, harder substrates increase cell sensitivity to exogenous insults, particularly at the level of DNA damage. These findings are very relevant given that natural aging, *i.e.* PD main risk factor, is parallel by changes in brain stiffness. Mechanistically, we demonstrated that the effects rely on the YAP pathway, and more specifically on YAP localization within the cell, consistently with YAP role as central regulator of mechano-transduction. In conclusion, our results offer new mechanistic insights into how cellular mechanical alterations, a poorly recognized age-related factor in neurological diseases, contribute to neurotoxicity in Parkinson's disease.

**CACO-2/HT-29 CELL CO-CULTURE MIMICKING THE INTESTINAL BARRIER IS A TUNABLE MODEL FOR GUT AGING**

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Considering the physiological role played by the intestinal epithelial barrier (IEB), the research related to modifications due to the microbiota and intestinal cell alterations with aging

is attracting more and more attention. The necessity to standardize the appropriate experimental models is still unmet and is accompanied by a critical need to develop an *in vitro* study model of the IEB reproducing the interactions between the absorptive and the secreting cells related to aging. The present study aimed at characterizing the morphology and the physiology of the aged IEB through an *in vitro* model constituted by a co-culture of the two cell lines Caco-2 and HT-29 that we previously differentiated and characterized in absorptive and mucus-secreting cells respectively<sup>1,2</sup>. The co-culture was set up by plating a 70/30 ratio mixture of differentiated Caco2 cells from the 24<sup>th</sup> to 50<sup>th</sup> passage and HT-29 cells from the 8<sup>th</sup> to 35<sup>th</sup> passages for inducing "physiological" aging, in the absence of any exogenous stimulus. In the aged co-culture set up by plating Caco-cells which had reached at least 40<sup>th</sup> sub cultivation passage and HT-29 the 21<sup>st</sup> passage, we observed relevant morphofunctional impairments as i) a diminished epithelial electrical resistance (TEER); ii) an increased paracellular permeability; iii) a slight decrease in cell proliferation; and iv) a less homogeneous distribution of the membrane-associated claudin-1 immunostaining. Transmission electron microscopy (TEM) analysis revealed that the intracellular mucus and desmosomes were less represented in the aged co-culture, together with underdeveloped apical microvilli. These results suggest that this experimental setting can reproduce some of the main morphofunctional modifications of IEB reported in clinics in the leaky/aged gut. Future experiments could ascertain the use of the aged *in vitro* Caco-2 and HT-29 cell co-culture as a useful model for studying the molecular process and testing potential drug/nutraceutical treatments to ameliorate gut aging.

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#### MICROGRAVITY-INDUCED A-SYNUCLEIN AGGREGATION: UNRAVELING PATHWAYS, OXIDATIVE STRESS, AND LYSOSOMAL DYNAMICS IN NEUROBLASTOMA CELLS AND GBA-KO MODELS

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Parkinson's disease (PD), an advancing neurodegenerative condition prevalent among individuals aged 60 and above, manifests with distinctive Lewy Bodies containing  $\alpha$ -synuclein ( $\alpha$ -syn) aggregates in post-mortem brain tissue [1]. These aggregates serve as promising indicators and therapeutic targets for PD. Exploring microgravity in research provides a unique avenue to simulate accelerated aging, thereby amplifying the physiological relevance of PD *in vitro*. Our study delves into the repercussions of microgravity on  $\alpha$ -synuclein levels in SH-SY5Y and PD-mutated 3K-SNCA cell models, unraveling an expedited progression of cellular pathology and aging within 24 hours. Employing confocal imaging and Proteomic analysis, our study uncovers an augmentation in

total  $\alpha$ -syn levels, encompassing both monomeric and aggregated forms. Microgravity conditions induce compromised protein clearance mechanisms, leading to GBA1 protein (GCase) accumulation, heightened oxidative stress, and reactive oxygen species (ROS) production, culminating in endoplasmic reticulum (ER) stress monitored by the ATF6 marker [2,3]. The findings propose that microgravity and  $\alpha$ -syn accumulation collectively impede ER activity, foster  $\alpha$ -syn aggregation, and potentially induce GCase misfolding in the ER. Additionally, an elevation in phosphorylated synuclein, linked to PD severity, substantiates the efficacy of this method in replicating PD pathology. Furthermore, a detailed examination into microgravity's impact on  $\alpha$ -syn aggregation in SH-SY5Y, featuring a mutant 3K-SNCA clone, was conducted. Cells exposed to microgravity via a clinostat displayed a notable time-dependent surge in  $\alpha$ -syn aggregation, peaking at 48 hours. Complementary investigations into oxidative stress and lysosomal activity contribute to a comprehensive understanding of cellular responses to microgravity. These results propose that microgravity serves as a valuable model for PD and aging-related studies, offering insights into potential therapeutic avenues.

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#### LRRK2, A KINASE IMPLICATED IN CANCER AND NEURODEGENERATION, PARTICIPATES IN DNA REPAIR

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The Leucine-rich repeat kinase 2 (LRRK2) is a large multidomain protein with both serine-threonine kinase activity and GTPase activity<sup>1</sup>. At present, LRRK2 function has been predominantly related to endocytosis regulation via Rab proteins; the role of this kinase, however, is incompletely understood and some evidence points to the involvement in alternative processes such as maintenance of mitochondrial DNA integrity. A more in depth understanding of LRRK2 biological function is highly relevant given that its variants have been correlated with neoplastic progression in mammary carcinoma, lung tumorigenesis, and renal cell carcinoma, as well as with the development of neurodegenerative disorders such as Parkinson's disease<sup>1,2,3,4</sup>. Here we interrogated whether LRRK2 may participate in nuclear DNA repair given that accu-

mulation of DNA damage is a hallmark of aging, that is the principal risk factor for both cancer and neurodegeneration. Canonical pathway analysis on a phosphor-proteomic data set from LRRK2 mutant cells revealed that several proteins involved in DNA repair are LRRK2 substrates. Consistently, we observed that X-ray induced DNA damage increases LRRK2 transcription via an ATM mediated mechanism. Bioinformatics and cell biology studies revealed that LRRK2 transcription upon damage is mediated by the ATM substrate TRIM28. We also found that LRRK2 participates to DNA damage because its depletion via silencing experiments significantly affects DNA damage induced recruitment of different DDR key players such as ATM, MDC1, RNF168 and 53BP1. Consistently, dysregulated DDR is also observed in cells harbouring LRRK2 mutations causing constitutive activation of the its kinase activity. Taken together, our data demonstrate for the first time that LRRK2 transcription is augmented upon DNA damage and that the protein participated in DDR efficiency.

We thank Dr. Arjan Theil, Dr. Hannes Lans, Dr. Alex Pines and Prof. Dr. Wim Vermeulen for shared cell lines and reagents.

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#### ANTI-AGING ACTIVITY OF *Citrus bergamia* EXTRACT IN HUMAN ERYTHROCYTES

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Aging is a dynamic and progressive process characterized by increased oxidative stress and glycation processes. Functional foods rich in phytochemicals, especially polyphenols, are good candidates to counteract age-related changes. This work aimed to investigate the potential protective role of bergamot (*Citrus bergamia*, cultivar *Femminello*) peel and juice extract in a model of aging represented by human erythrocytes exposed to D-galactose (D-Gal, 100 mM, 24 h). Both peel and juice extracts were subjected to RP-HPLC/PDA/MS to determine their composition in bioactive compounds. Markers of oxidative stress, including ROS production, thiobarbituric acid reactive species (TBARS) levels, total protein sulfhydryl groups oxidation, as well as expression and anion exchange capability of Band 3 protein and glycated hemoglobin (A1c) production were analysed in D-Gal-treated erythrocytes, with or without preincubation (15 min) with 5 µg/ml of peel or juice extract. In addition, the activity of the endogenous antioxidant system, including catalase (CAT) and superoxide dismutase (SOD) was examined, as well as the deviation of erythrocyte metabolism from glycolysis to the pentose phosphate pathway, as indicated by the activation of glucose-6-phosphate dehydrogenase (G6PDH). Results suggest that both bergamot extracts: i) prevented increased oxidative stress markers and CAT, SOD, and G6PDH over-activation; ii) reduced A1c production; iii) restored D-Gal-induced alterations in the distribution and ion transport kinetics of Band 3 protein. These findings help to elucidate aging mechanisms in human erythrocytes and identify bergamot as a functional food useful to prevent oxidative changes linked to pathological states during aging.

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#### INVESTIGATING THE SYNERGISTIC EFFECTS OF FUNCTIONAL FOODS ON SARCOPENIA AND INFLAMMAGING: A PROMISING STRATEGY FOR PROMOTING HEALTHY AGING

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Aging is characterized by a state of low-grade systemic inflammation called "inflammaging", which plays an important role in age-related diseases as well as sarcopenia and osteoporosis. Sarcopenia is a disorder characterized by a generalized reduction in muscle mass and strength and related to important negative clinical outcomes, with a series of delicate economic and social implications, including impaired mobility, loss of quality of life, hospitalization and death. Therefore, sarcopenia markers, are urgently needed especially related to musculoskeletal, hormonal and biochemical status. Several papers, agreed on evaluating anthropometric indices, hormonal markers including cortisol, inflammatory markers including C-reactive protein (CRP) and interleukin-6 (IL-6), and vitamin D concentration, which deficiency of which correlates with increased inflammation and sarcopenia. Increasing scientific evidence shows that improved lifestyle, reduced sedentary lifestyle and proper nutrition can be effective in improving everyone's health in the short and long term. The present study discusses whether, in the nutritional context, supplementation with brown algae of the *Ecklonia* species within functional foods can improve the condition of inflammation and sarcopenia. Brown algae are rich in natural compounds such as vitamins A, B, C, E, fatty acids, phenolic compounds, sterols, essential amino acids and polysaccharides. They possess therapeutic properties that are antimicrobial, antiviral, hepatoprotective, cardioprotective, neuroprotective, anticarcinogenic, immunomodulatory, hypolipidemic, antidiabetic, antioxidant, and anti-inflammatory. We have conducted a blindly nutritional intervention for four weeks on a court of 48 volunteer subjects divided into two experimental groups, control lettuce group and lettuce group biostimulated with *Ecklonia* species brown seaweed. The subjects consumed 100 grams of lettuce with or without biostimulation for 4 weeks. Blood samples and anthropometric and nutritional analysis were conducted at the beginning of recruitment and after 4 weeks. The results showed changes in biochemical and anthropometric markers of sarcopenia.

Subjects who consumed for four weeks biostimulated lettuce showed a reduction in levels of cortisol, inflammatory biomarkers such as c-reactive protein (CRP) and IL-6. With respect to vitamin D, the biostimulated cohort showed increase concentration after four weeks. In the context of anthropometric analysis, there was an increase in muscle mass and decrease in fat mass in the subjects who consumed biostimulated lettuce. These preliminary data suggest that brown seaweed supplementation within functional foods may be a functional choice that can be proposed to improve sarcopenia associated with aging and perhaps age associated disease.

## PROTECTIVE ROLE OF AÇAÍ BERRIES AGAINST AGE-RELATED OXIDATIVE STRESS IN HUMAN ERYTHROCYTES

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Aging is a dynamic chronological process that results in a general structural and functional decline. Increased reactive oxygen species (ROS) play a pivotal role in the development and progression of aging. Considering the close relationship between aging and oxidative stress (OS), functional foods rich in polyphenols are promising candidates to counteract age-related alterations. This study aimed to investigate the protective role of flavonoid-rich Açai extract in a D-galactose (D-Gal)-induced aging model in human erythrocytes. Markers of OS (ROS production, thiobarbituric acid reactive substances (TBARS) levels, protein sulfhydryl group oxidation), markers of aging (CD47 and Band 3 protein (B3p) distribution), as well as anion exchange capability through B3p and glycated hemoglobin (A1c) levels have been assayed in erythrocytes treated with 100 mM D-Gal for 24 hours, with or without preincubation with 0.5-10 µg/ml of Açai extract for 1 hour. Results show that Açai extract avoided acanthocyte and leptocyte formation, prevented the increase in OS and aging markers, and restored B3p anion exchange capability observed after exposure to D-Gal. These findings shed light on the aging mechanisms of human erythrocytes and propose dietary supplementation of flavonoid-rich functional foods as a possible preventive tool in OS-related diseases.

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## ELECTROSPUN NANOFIBERS AND NATURAL COMPOUNDS: A NEW DEVICE TO COUNTERACT SKIN AGING

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Skin aging is a biological process increased by extrinsic factors, as photodamage. Indeed, solar radiation causes oxidative stress, compromising the function of the epithelial barrier. Natural extracts, as *Helichrysum italicum* oil (HO), containing notable bioactive compounds, are recognized for their antioxidant properties. The advent of nanotechnology, allowed the combination of nanoformulations with traditional treatments, improving the passive penetration of substances in the skin layers. Skin treatments for rejuvenation and repairing processes depend on the type of molecule enrolled and on the way of administration. Electrospinning is an effective method for encapsulating bioactive compounds into multifunctional nanofibers that can be used in cosmesis and biomedical fields. Within this context, we studied the biological properties of electrospun nanofibers, including Polyvinyl acetate (PVA) and Polyvinylpyrrolidone (PVP) encapsulated with HO, also by scanning electron microscope (SEM)<sup>1</sup>. The activity of nanofibers was tested *in vitro* on Skin Stem Cells (SSCs) and BJ fibroblasts. Our results show a positive trend in cell proliferation and viability, counteracting aging triggered by UV stress for both SSCs and BJ fibroblasts. The molecular senescence program activated after UV exposure was counteracted by pretreatment with PVA and PVP nanofibers in stressed cells<sup>1</sup>. Moreover, the β-galactosidase assay, performed on stem cells, highlights the protective effect of both nanofibers on aging induced by UV irradiation<sup>1</sup>. Our results suggest that these biocompatible nanofibers, which are safe for human health, could potentially be used as a medical device, making them excellent candidates for topical application.

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## AGING-RELATED OXIDATIVE STRESS AFFECTS KIR2.1 FUNCTION: PROTECTIVE ROLE OF MELATONIN

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Aging is a pathophysiological process that causes increased susceptibility to oxidative stress-related brain diseases, including epilepsy. Epileptic hyperexcitability is related to spatial K<sup>+</sup> buffering mechanism alteration, which is mediated by glial inwardly rectifying K<sup>+</sup> channels (Kir). Increased aging-related oxidative stress could play a pivotal role in the functional impairment of these channels, thus promoting epileptogenesis. To verify this hypothesis, a human glioblastoma cell line (U-87 MG cells) was exposed to D-galactose (D-Gal), which mimics a natural aging condition caused by increased markers of oxidative stress, lipid peroxidation, and protein oxidation. As demonstrated by RT-qPCR analysis, Kir2.1 is the predominant Kir transcript in U-87 MG cells. Exposure to D-Gal dramatically reduced Kir2.1 current density, monitored through the patch-clamp technique in whole-cell configuration. The loss of Kir2.1 current is mainly provoked by the reduction of protein abundance, rather than a

channel direct oxidation and/or alterations in transcript levels or trafficking to the cell surface. Pre-exposure to melatonin (Mel), an antioxidant molecule with neuroprotective properties, prevented oxidative changes and restored Kir2.1 current density. All the obtained results have been reproduced in different heterologous expression systems (NIH/3T3, HEK293T, HeLa, and *Xenopus laevis* oocytes). In conclusion, oxidative inhibition of Kir2.1 activity in neuronal glia could affect the extracellular K<sup>+</sup> buffering mechanism and contribute to the epileptogenesis process during aging. In this context, Mel is a useful tool to elucidate the molecular mechanisms underlying epilepsy and may contribute to formulate novel therapeutic strategies to counteract epileptogenesis and astrogliosis processes.

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#### ROLE OF NAD METABOLISM IN THE OVARIAN AGING: INSIGHTS FROM HUMAN GRANULOSA CELLS

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The progressive decline of ovarian function, with advancing age, together with the reduction of quality and number of oocytes, is defined as ovarian aging. With the increase in lifespan expectancy, ovarian aging has gradually become a key health problem for women. When these processes occur earlier or accelerate, their clinical correlation is the diminished ovarian reserve and/or triggers POI. In the last few years, NAD<sup>+</sup>, a coenzyme involved in cellular redox reactions, has emerged as a potent regulator in reducing func-

tional decline and age-related illnesses. The relationship between NAD<sup>+</sup> levels and ovarian aging has gradually become clearer, with studies showing that strategies to raise NAD<sup>+</sup> levels may delay ovarian aging, improve ovarian quality, and increase fertility in animal models. The cells require a high level of NAD<sup>+</sup> biosynthesis through pathways which begin with precursors derived from the food, specifically vitamin B3 in the form of nicotinic acid (NA), nicotinamide (NAM), nicotinamide mononucleotide (NMN), and nicotinamide riboside (NR). The integration of NAD<sup>+</sup> precursors has demonstrated preventive and therapeutic effects in phase I clinical trials across many pathologies. In order to support the validity of these interventions in improving the fertility of women of advanced reproductive age, it is necessary to combine *in vitro* studies on human cells with animal models. The aims of this study were to develop an *in vitro* senescence model in human granulosa cells; investigate the changes associated with senescence in human granulosa cells at the level of NAD<sup>+</sup> metabolism; and assess the impact of various concentrations of NAD<sup>+</sup> precursors (NA, NR, and NAM) and NAMPT, the enzyme involved in NAD<sup>+</sup> production (P7C3) on the bioavailability of NAD<sup>+</sup> and redox state changes in senescent human granulosa cells. We have realized a senescent model of human granulosa cellules (KGN cell line) based on replicative senescence and exposure to oxidative stress (H<sub>2</sub>O<sub>2</sub>) which has been confirmed by SA-β-Gal (Senescence-Associated β-Galactosidase Staining Kit) and by protein expression of p21, marker of senescence. The senescent cells were treated with non-toxic concentrations of NAM (0,3-300 μM), NR (3-3000 μM), and P7C3 (5-30 μM). The effect on NAD<sup>+</sup> production has been measured using the NAD<sup>+</sup>/NADH bioluminescence assay (NAD<sup>+</sup>/NADH-GloTM Assay, Promega). The results showed that NAD<sup>+</sup> levels decreased in senescent granulosa cells and that NAD<sup>+</sup> levels were restored by 300 μM NAM, 300 μM, and 3000 μM NR. The expression of SIRT1, a key activator of the adaptive response to stress, was upregulated in senescent granulosa cells, and treatment with NAD<sup>+</sup> boosters restored it to comparable levels of non-senescent granulosa cells. This study lays the groundwork for future research that elucidates the efficacy of these supplements in order to promote the use of NAD boosters in the clinical setting as oral therapy and as an additive to culture media to improve the quality of the follicular environment in patients with advanced maternal age.

## ENVIRONMENT AND HEALTH

### COMPARISON BETWEEN CONVENTIONAL AND BIODEGRADABLE PLASTICS: EFFECTS OF NANO- AND MICROPLASTICS ON DEVELOPING ZEBRAFISH LARVAE

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In recent years, improper plastic disposal has raised an increasing scientific and social concern. Plastic pollution is mainly caused by small plastic debris, categorized by the size in nano-plastics (NPs) and micro-plastics (MPs), released into the environment. The conventional plastics such as polystyrene, have been widely demonstrated to have a highly toxic effect on the biota and human health. Recently, bioplastics such as polylactic acid, have been used by industries as a green alternative to the non-degradable ones, with the purpose of reducing their toxic effects (1). However, to date, the effects of biodegradable plastic debris on biota and human health still remain to be elucidated. In recent years, zebrafish (*Danio rerio*) has been widely used as a successful model in biomedical and toxicological research because of several forceful features, *i.e.* its small size, short life cycle, ease of breeding and maintenance, high fecundity and genetic similarities with humans (2). Moreover, the transparency of zebrafish embryos and larvae provides an additional advantage in studying the localization of fluorescent-labeled contaminants. In the last few years, it has been reported that the exposure of zebrafish larvae to the conventional NPs/MPs causes several behavioral and morphological alterations along with the induction of high toxicity at different tissue levels (3). On the contrary, the impact of the biodegradable plastics exposure on zebrafish organisms still remains unclear and needs further investigation. For this reason, we exposed zebrafish embryos to biodegradable Polylactic acid nanoplastics (PLA-NPs) and to the non-biodegradable Polystyrene MPs (PMPs) in order to compare their effects on developing larvae. Zebrafish embryos, after collecting (T0), have been exposed to rhodamine-embedded PLA-NPs (size 250 nm) and to fluorescent PMPs (size 1 µm) at the concentrations of 100 and 1000 µg/L, up to 120 hours post fertilization (hpf) (T5). Zebrafish Embryo Toxicity was performed in accordance with OECD guidelines (OECD, Test No. 236: Fish Embryo Acute Toxicity (FET) test). The distribution and accumulation of both PLA-NPs and PMPs have been assessed at different time points (24, 48, 72, 96 and 120 hpf) by their *in vivo* observation at a fluorescence microscope. The heart

rate was measured at 96 and 120 hpf in both experimental groups and the effects of PLA-NPs and PMPs have been also evaluated through histological analysis. Finally, the cardiac, inflammation, apoptotic and cellular stress marker expression has been assessed by immunofluorescence and gene expression analysis. Here we report preliminary data on morphological and molecular alterations on zebrafish exposed to conventional and biodegradable plastic debris during its first stages of development, however, further investigations are necessary to understand how they may impact aquatic and human organisms.

We thank the research group of Professor Fabio Marino (UniMe) and Professor Tibullo Daniele (UniCt) for their precious collaboration.

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### UNVEILING NATURE'S HIDDEN ARSENAL: EVALUATION OF THE ANTIBACTERIAL POTENTIAL OF MICROALGAE AND CYANOBACTERIA EXTRACTS

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The past decade has witnessed a significant increase in the resistance to antibacterial, together with the associated increase in bacterial infections. The rising resistance to antimicrobial agents carries significant consequences for community health, including increased morbidity and mortality, as well as increased healthcare costs. Consequently, there's been a heightened emphasis on discovering new, safer, and more potent agents to tackle bacterial infections. In recent years, the pharmaceutical industry has directed its attention towards unicellular organisms as potential sources for natural compounds with antimicrobial properties. This approach underscores the urgent need to explore novel avenues in the fight against antimicrobial resistance. In this scenario, microalgae come into play, as they are ubiquitous, photosynthetic microorganisms that might lead to therapeutically useful agents. Microalgae represent a unique opportunity to discover novel compounds, or to produce already known ones at lower costs. Microalgae possess the additional advantage of a substantial metabolic plasticity, dependent on their physiological state (*i.e.* stressed vs. non stressed); in fact, specific environmental conditions can have an impact on the formation of compounds and their effectiveness. In the last decade microalgae have become the focus of extensive research efforts, aimed at finding novel bioactive compounds. Furthermore, a large number of microalgal extracts have been found to have proven antibacterial. The antimicrobial activity of microalgae has been attributed to compounds belonging to several chemical classes, including indoles, terpenes, phenols, peptides, polysaccharides and fatty acids. However, further efforts to identify the compounds directly responsible for those antimicrobial fea-

tures are still needed. In this work polysaccharide-containing aqueous extracts and fatty acid-containing ethanol extracts were prepared from the biomass of microalga *Chlorella* sp. and the cyanobacterium *Spirulina platensis* which were commercially available or cultivated under different growth conditions. The extracts were evaluated for their antioxidant activity and antibacterial activity against *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC V583E, *Escherichia coli* ATCC 15325, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028 through the determination of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) values by the microdilutions method.

### DISTRIBUTION OF TERMITES (DICTYOPTERA, ISOPTERA) IN TRIESTE (FRIULI VENEZIA GIULIA, ITALY): THE STUDY OF THE SPREAD ON THE URBAN TERRITORY IN CORRELATION TO PEST CONTROL ACTIVITIES AND CLIMATE CHANGE

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The results of the screening of termite infestations in Trieste, a coastal city in north-eastern Italy, may be of particular relevance if observed as a time series. The city and its surroundings can represent an ideal laboratory for studying and monitoring the effects of climate change on urban biodiversity, being located in the far north of the Mediterranean Sea, in an area that is a meeting point of different climates. The Isoptera present, as in the rest of Italy, are represented by two confirmed genera, *Kaloterмес* and *Reticuliterмес*, both known as responsible for significant infestations in European urban areas. This study documents the discovery of colonies of termites in Trieste, especially inside historic houses built between 1800 and 1949. From 1946 to today, collapses of wooden parts of buildings resulting from the xylophagous activity of termites have been documented in Trieste. The findings highlight the importance of continuous monitoring and control efforts to mitigate potential damage and contain termite populations. Furthermore, the study collected information on the increase in the presence of termite colonies over time for both genera identified in Trieste. The systematic collection of data is becoming a good basis for the application of multivariate analysis algorithms in order to understand the correlations with ongoing climate changes.

### PHYTOCHEMICAL PROFILING AND INVESTIGATION OF FUNCTIONAL PROPERTIES IN SPONTANEOUSLY GROWN SICILIAN SUMAC (*Rhus coriaria* L.) FRUITS

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The importance of dietary phytochemicals in promoting health is well recognized. Beyond health benefits, phyto-

chemicals are also utilized to improve the safety and functional properties of food products. This has led to the incorporation of plant-derived products in food processing to increase antioxidant content and reduce the negative impacts of microbial contamination (1). The *Rhus* genus, commonly known as Sumac, is part of the Anacardiaceae family. Despite extensive studies on the phytochemical profile and biological activity of different *Rhus* species, much remains unknown about the functional value of *Rhus coriaria*, the typical sumac of the Mediterranean area. Moreover, studies emphasize significant biodiversity in the phytochemical profile and associated biological activity within *R. coriaria*, influenced by geographical origin (2). In Sicily, "Sicilian sumac" was historically used as a colorant for tanning leather, with its dried and ground leaves. Today, it grows wild and is primarily utilized in culinary applications. While some scientific studies have been conducted, there remains a notable gap in the literature regarding the functional properties of Sicilian sumac. This study aimed to assess the phytochemical profile and associated functional properties of sumac (*Rhus coriaria* L.) fruits harvested from wild plants in Sicily. Chemical characterization unveiled that Sicilian sumac fruits contained exceptionally high levels of polyphenolic antioxidants (TPC: 10.99±0.06 g GAE/100 g DW), including substantial quantities of proanthocyanidins (61.577±5.729 mg PACE/100 g DW). In particular, HPLC-DAD-ESI-MS/MS analysis identified 82 phytochemicals belonging to various flavonoid classes. In terms of functional properties, the hydroalcoholic extract from Sicilian sumac fruits exhibited remarkable radical scavenging and metal-reducing activities, providing antioxidant protection in a cell-based model of lipid peroxidation (CAA50 1.116±0.098 µg/mL cell medium). Additionally, the extract displayed significant antiproliferative activity against four human epithelial cell lines (HeLa, Caco-2, HepG2, and MCF-7), with GI50 values ranging from 31.08±2.25 to 149.74±8.72 µg/mL cell medium. Finally, specific investigation into the antibacterial activity of the extract revealed a robust inhibitory spectrum against major food-borne pathogenic bacteria, with a minimum inhibitory concentration (MIC) in the range of 12.5-25.0 mg/mL. Our findings highlight Sicilian sumac fruit as a rich source of phytochemicals positively contributing to the cellular redox state even when consumed in small quantities, making it compatible with culinary use as a spice. Additionally, its diverse bioactivities indicate potential applications across food and non-food sectors.

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### QUINOXYFEN 1995-2019 IS THE STORY'S END? EVALUATION OF ITS ADVERSE EFFECTS ON HEAD SIZE AND NERVOUS SYSTEM GENES INVOLVED IN SYNAPTIC MATURATION

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Quinoxifen (QXY) is a fungicide belonging to the quinoline family, introduced for the control of powdery mildew. In 2013 QXY was included in the list of priority hazard pollutants of the European Water Framework Directive due to its toxicity to aquatic organisms. The use of products formulated containing QXY was banned from all commercials starting from 27th June of 2019. Nevertheless, the QXY is an organic pollutant with potential persistence and bioaccumulation and its effects on neurological developmental are not still investigated. The aim of the work is to evaluate the toxicity of this compound using a zebrafish (*Danio rerio*) early-life stage focusing in particular on neurological defects. The study was conducted in 2 steps: in the first one, using the Fish Embryo Acute Toxicity test (FET), the toxicological endpoints as sublethal alterations and abnormal behaviors were investigated and in the second one two sublethal concentrations (0.4 and 0.8 mg/L) were selected to performed molecular investigations and Alcian blue staining. The *cyp19a1b*, *shank3a*, *gad1b*, *neurexin1a* genes, involved in the development of the nervous system and in the regulation of synaptic transmission, were evaluated. Furthermore, to investigate the craniofacial alterations, morphometric analyses were performed. The results of FET tests showed as the tested concentrations allowed to calculate the lethal concentration 10 (LC<sub>10</sub>) of 0.9 mg/L, while it was not possible to reach LC<sub>20</sub> and LC<sub>50</sub> values. Moreover, several sublethal alterations as yolk sac edema, pericardial edema, reduced blood circulation, blood stasis, reduced head size and altered mouth morphology were observed. Additionally, at 72 hours post fertilization the larvae exhibited a particular phenotype characterized by abnormal mouth's gape. Morphometric analysis revealed a decrease head length, an increase of Meckel and palatoquadrate cartilage angle and an increase of Meckel and Ceratohyals cartilage distance. These alterations can be linked to the brain malformations as reported by Raterman *et al.*, 2020. This phenotype was supported by the changes in the expression of crucial genes influencing the normal development of the central nervous system and during the zebrafish embryonic phase, such as *cyp19a1b*, *shank3a*, *gad1b*, and *neurexin1a*. At both concentrations QXY led to a decrease in *cyp19a1b* gene expression suggesting an altered production of estradiol in the brain and a potential role as endocrine disruptor but an increase in *gad1b*, *shank3a* and *neurexin1a*. These results could represent a starting point to study the potential role of QXY on neurodevelopment and possible link with birth defect induced by organism exposure by bioaccumulation effects. Ethical statement: treatments were performed in non-feeding embryos and all experiments were carried out following the Italian law for the protection of research animals D.L. n. 26, 4 March 2014 and the European regulation directive 2010/63/U for animal experiments.

*Acknowledgment: this research was funded by the European Union – Next Generation EU. Project Code: ECS00000041; Project CUP: C43C22000380007; Project Title: Innovation, digitalization and sustainability for the diffused economy in Central Italy – VITALITY.*

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## EFFECTS OF FLUID EXTRACT OF *Boswellia sacra* GUMMI-RESIN (BosLiq®) ON EPCs' FUNCTIONS

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Endothelial Colony Forming Cells (ECFCs) are descendants of endothelial progenitor cells (EPCs) found in both Cord Blood (CB-ECFCs) and Adult Peripheral Blood (APB-ECFCs). They express endothelial markers while retaining progenitor cell characteristics, including robust proliferation. These cells play a crucial role in endothelial regeneration and angiogenesis, rendering them promising candidates for revascularization strategies. CB-ECFCs, with their stable culture passages and undifferentiated endothelial phenotypes, provide a suitable model for assessing the vascular effects of various compounds. Recent scientific studies have highlighted the therapeutic potential of several phytochemicals in vascular regenerative medicine, particularly polyphenols and terpene derivatives, known for enhancing endothelial cell functions (1). *Boswellia* spp. is renowned for its aromatic resin frankincense, which has been used for centuries in religious ceremonies and traditional medicine due to its healing and anti-inflammatory properties. Furthermore, numerous studies have revealed that boswellic acids and cembrenes, terpenic compounds found in *Boswellia* resin, exhibit diverse biological activities (2). BosLiq®, a fluid extract of *Boswellia sacra* frankincense marketed by Abel Nutraceuticals, contains a significant concentration of boswellic acids and derivatives (25% w/w). However, the functional potential of this extract remains poorly characterized to date. This study aimed to evaluate the effects of BosLiq® on the proliferation, migration, and angiogenic potential of CB-ECFCs. While BosLiq® did not enhance the proliferation of CB-ECFCs across the tested concentration range, migration assays revealed its favorable impact on regenerative activity. In wound healing experiments, BosLiq® accelerated lesion closure in a dose-dependent manner compared to controls. Additionally, Transwell migration assays demonstrated increased cell migration in response to BosLiq®, comparable to VEGF stimulation. Furthermore, BosLiq® exhibited dose-dependent modulation of nitric oxide production and showed potential pro-angiogenic effects in *in vitro* vascular structure formation assays. Overall, these findings suggest that BosLiq® may augment the regenerative capabilities of *ex vivo* expanded human endothelial progenitor cells, presenting promising avenues for treating vascular disorders.

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## ENVIRONMENTAL BIOSENSOR SYSTEM: ORGAN-ON-A-CHIP FOR THE ANALYSIS OF POLLUTANTS ON THE RESPIRATORY MUCOSA (BIORESYSTEM)

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Monitoring air quality and understanding the impact of airborne components on the human respiratory mucosa is a crucial priority for today's scientific community. Environmental pollution poses significant risks to respiratory health, but obtaining data on the effects of such exposure is often complex and requires an extensive analysis. In response to this need, our team developed the BioRESsystem project, which consists of an Organ-on-Chip (OoC) system for real-time and long-term monitoring of airborne pollutants on an *ex vivo* respiratory mucosa model. The biosensor development fuses biomedical and engineering expertise based on an established bioengineering model. To optimize the protocols, the team uses an *ex vivo* three-dimensional model of respiratory mucosa, which closely replicates the cytoarchitecture present *in vivo*. The mucosa is integrated within an OoC equipped with an electrospun biopolymer membranes for the development of an air-liquid interface. The electrospinning technique permits easy control of the final scaffolds' fibre diameter, porosity, and mechanical properties by changing the processing parameters and materials used. Electrospun membranes are often engineered for advanced applications, including controlled drug release, bioprocess intensification, and biosensing. Beyond its versatility in material selection, which can include both natural and synthetic polymers, electrospinning also provides the possibility to include nanoparticles in the polymeric fibres. By considering the unique properties of nanometric size and high specific surface, incorporating functional nanoparticles into an electrospun polymer matrix can provide substantial property enhancements, even at low nanoparticle content. The OoC/environmental sensor is fabricated by using rapid prototyping equipment in additive (3D printing) and subtractive (laser cutting) manufacturing. This iterative approach enables swift progress from design conception to the final product, allowing rapid device optimization and enhancement of its ultimate properties. The OoC design is implemented using CAD software, while the fluid dynamics is modelled using CFD software according to parameters (for airflow and culture medium) based on human physiological data. Overall, this organ-on-a-chip system enables long-term monitoring of the effects of environmental exposure on the respiratory mucosa, morphological and biomolecular evaluation, reliable data collection, and the possibility of validating its functionality in various relevant environments. Engineering tests, including testing of the microfluidic system, validated the biosensor at the Technology Readiness Level (TRL) 5. The biosensor has a patent pending, and a university spin-off (University of Palermo) is being set up between the BiND (Biomedicine, Neuroscience and Advanced Diagnostics) and Engineering Departments.

#### IMPACT OF BETANIN DEGRADATION PRODUCTS ON ROS SIGNALING, PROLINE ACCUMULATION, AND PHYTOHORMONE LEVELS IN GERMINATING *Arabidopsis*

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Seed germination, a crucial stage in the plant life cycle, undergoes regulation through a complex interplay of internal and external factors. While the established roles of mitochondrial activity, ROS balance, and phytohormones are well-acknowledged, ongoing research suggests an additional layer of involvement from bioactive molecules, including those contained in biostimulant formulation derived from agri-food waste. This study delves into the potential of betanin degradation products (BDPs), sourced from pitaya fruit processing waste, to influence *Arabidopsis thaliana* germination and seedling development. The investigation spans diverse physiological processes, including ROS signaling, proline accumulation, and phytohormone homeostasis. Notably, lower BDP concentrations (0.02 – 0.20 mg L<sup>-1</sup>) significantly enhanced germination rates and seedling biomass compared to controls, while higher dosages (>1.00 mg L<sup>-1</sup>) exhibited adverse effects on morphological traits. Through an assessment of mitochondrial activity using the MTT assay on both seeds and purified organelle fractions, we affirm that the distinct compounds within BDPs, characterized via HPLC-DAD-MS/MS, neither affect mitochondrial activity nor compromise its integrity. This underscores the independence of both positive and cytotoxic effects from mitochondrial performance. Mechanistically, BDPs modulate ROS signaling, diminishing free H<sub>2</sub>O<sub>2</sub> content by amplifying antioxidant activity and regulating the gene expression of the ROS scavenging system. Gene expression analyses pinpoint the primary cellular locations where detoxification occurs, monitoring the expression of various isoforms of SOD, CAT, GPX, and GR. Furthermore, BDPs exhibit an ability to influence proline accumulation, indicative of heightened tolerance. Intriguingly, the rise in proline contents correlates with alterations in its metabolism and catabolism, as evidenced by monitoring the expression of genes involved in these pathways. In addition to these findings, BDPs alter phytohormone homeostasis, favorably balancing seedling establishment. Specifically, the ABA/ABA-glu, tZea/tZea-rib, and tZea/IAA balances strongly suggest enhanced germination performance and seedling development at lower concentration ranges (0.02 – 0.20 mg L<sup>-1</sup>) and inhibition at higher doses. The increase in the content of GA4 and GA7 compared to other GAs implies an involvement of GA13ox, a pivotal enzyme in the biosynthetic switch, a hypothesis supported by the evaluation of the gene expression of this protein. In summary, this study underscores the potential of BDPs as sustainable promoters of plant growth, influencing critical regulatory pathways during germination. Further research is essential to fully comprehend and explore their extensive applications in agricultural practices, especially under diverse abiotic and biotic stresses.

#### HARMFUL EFFECTS OF TWO PHACS AT REALISTIC DOSES ON *Mytilus galloprovincialis* (LAMARCK, 1819)

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In recent years, within the wide range of xenobiotics consistently found in the marine environment, pharmaceutically active compounds (PhACs) are the most worrying pollutants. This is due to the current inability of wastewater treatment plants to effectively retain these compounds that are commonly detected in the environment within concentration in the range from ng/L to µg/L. Caffeine (CAF) and salicylic acid (SA) are among the most PhACs released, therefore their effects at realistic dosages have been assessed on marine mussel *Mytilus galloprovincialis*, which plays a crucial ecological role into the coastal environment. A set of assays (histological, biochemical, and molecular) was carried out on digestive gland of samples after a 12 day-exposure of the two contaminants (CAF, SA) individually and in combination (CAF+SA). While the histological observation showed a general haemocyte infiltration for the two tested compounds coupled with thinning and thickening at the digestive tubule level, the biochemical and molecular results revealed how differently CAF and SA behave. Indeed, CAF revealed a prominent pro-oxidant activity, whereas SA triggered an inhibitory action on the antioxidant system probably related to the disruption of mitochondrial activity. In CAF+SA exposure, enhanced and counterbalanced effects were recorded, demonstrating the relevance of investigating the impact of these contaminants at realistic conditions. The current findings extend the knowledge on the effects of PhACs on non-target aquatic organisms and emphasize the necessity of accurately evaluating their environmental impact, endorsing further investigations and bio-monitoring actions to preserve the biodiversity of coastal marine areas.

*Acknowledgements: this work was supported by the National Biodiversity Future Center (European Grant #0592055538).*

#### EXPOSURE TO PER- AND POLYFLUOROALKYL SUBSTANCES IN BELGIAN FLANDERS: A CASE STUDY OF *Sander lucioperca*

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Per- and polyfluoroalkyl substances (PFAS) are a heterogeneous group of synthetic chemicals containing carbon-fluor bonds, including perfluorinated carboxylates and sulfonates. Due to their many properties, including resistance to water and dirt, PFAS have been used since the 1950s in a variety of applications, including packaging, cookware, clothing, carpets, fire-fighting foams, and the development of medical and personal care products. PFAS are known to persist in the environment and to bioaccumulate in the food chain. Furthermore,

various adverse health effects are associated with PFAS exposure. For the sum of 4 PFAS (PFOS, PFOA, PFNA and PFHxS), a tolerable weekly intake (TWI) of 4.4 ng/kg bw/week was set by EFSA in 2020. The aim of this study was to investigate the presence of PFAS in recreationally caught Pikeperch (*Sander lucioperca*) used for human consumption by anglers. Fifty-nine samples of pikeperch from 14 different areas in Flanders, Belgium were collected between 2022 and 2023. Muscle tissue was dissected and analysed for 25 PFAS using liquid chromatography coupled to mass spectrometry (LC-(ESI<sup>-</sup>)-MS/MS). All samples contained between 7 and 21 PFAS. Nearly half of the samples contained between 13 and 15 PFAS. Out of the PFAS included in the TWI of EFSA, PFHxS and PFOS were present in all samples. PFNA and PFOA were detected in 95% and 46% of the samples respectively. Among the sulfonates, those with 6-8 carbons (PFHxS, PFHpS, PFOS) were the most detected (>96%), followed by the long chains with 9-12 carbons (44-75%). Among the carboxylates, the long-chains with 9-14 carbons were detected in almost all samples (> 95%) followed by PFOA (8 carbons). In contrast, emerging PFAS such as ether sulfonic acid F53B (major and minor components), DONA, HFPO-DA and short-chains carboxylates (carbon < 8) and sulfonates (carbon < 6) were poorly detected (<5%). Hence, the exposure of the Belgian angler population consuming their caught fish was evaluated to better understand the potential risks they may face. The mean exposure was 7.1 ng/kg bw/week, with a minimum of 1.8 ng/kg bw/week and maximum of 23 ng/kg bw/week. Therefore, the mean and maximum scenarios exceed the TWI. This study provides important insights into the current state of PFAS contamination and distribution.

#### FROM LAB TO TRACK: THE TAOPATCH® BIOMARKER AND PERFORMANCE DETECTION TECHNOLOGIES

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The focus on enhancing athletic performance is increasingly becoming a central aspect in the sports field, driving researchers and practitioners to explore new technological frontiers. In this context, we've decided to test out new different approaches with the use of innovative nanotechnologies, such as Taopatch®, a medical nanotechnology device that convert sunlight or heat into infrared and ultraviolet light, to investigate their potential effects on athletes' performance and recovery, thus expanding the horizons of sports training. The aim of this pilot study was to assess the effectiveness of Taopatch®, to improve proprioception, balance, and postural re-equilibrium also optimize metabolic and energy processes. For this study, we recruited 10 cross-training athletes. The subjects were randomly assigned into two groups: one that used the device with nanotechnology and a placebo group. Athletes were evalu-

ated at T0 and T1 over an 8-weeks of intervention consisted of tri-weekly training sessions. Hematochemical and strength test assessment were performed; we analyzed biomarkers such as creatine kinase (CK), lactate dehydrogenase (LDH), interleukin-6 (IL-6), azotemia, and creatinine, and Optojump and handgrip test. The results demonstrated greater improvement in lower extremity strength in athletes who used Taopatch® as well as the reduction of IL-6 and CK levels ( $p < 0.05$ ). In conclusion, the study highlighted how the use of Taopatch® can represent a valid support for athletes, offering a new approach to improving performance and well-being, thanks to the acceleration of recovery processes and the optimization of sports training. The results open interesting perspectives on the use of nanotechnologies in the sports field, suggesting further research to explore their long-term benefits.

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#### LINKING NANOPARTICLES TO EMBRYONIC DEFORMITIES: EXPLORING THE TERATOGENICITY OF ZINC OXIDE NANOPARTICLES

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The exponential growth of nanotechnology has led to significant advancements in engineered nanoparticles (ENPs) between 1 and 100 nm in size, with zinc oxide nanoparticles (ZnO-NPs) playing a prominent role across various industries and applications. Particularly in biomedicine, ZnO-NPs have emerged as versatile tools, serving as antibacterial agents, drug and gene delivery platforms for cancer treatment, cellular imaging enhancers, and high-performance biosensors. For these reasons the aim of this study is to explore their potential toxicity on zebrafish early life stage using a combined *in vivo* and *in-silico* approach. In the first phase by SEM-EDS analysis, the ZnO-NPs purity was confirmed. After the Fish Embryo Acute Toxicity Tests according to OECD test guideline No. 236 (OECD, 2013) were performed. The embryos were exposed to five con-

centrations of ZnO-NPs: 50, 100, 150, 200 and 250 mg/L. At 96 hours, LC<sub>20</sub> of about 58.201 mg/L and NOED of <50 mg/L, were determined. The most common sub-lethal alterations were pericardial and yolk edema, blood stasis, reduced blood circulation, reduced heartbeat, skeletal alterations and delayed hatching. Later, to further assess the toxicity of ZnO nanoparticles, oxidative stress was evaluated by quantifying lipid peroxidation using the thio-barbituric Acid Reactive Substances assay. The results showed as the treatment with ZnO-NPs led to a significant increase in lipid peroxidation in zebrafish larvae, as evidenced by the elevated levels of MDA induced by the treatment, indicating a low detoxification capacity of reactive oxygen species. To further confirm these findings, the gene expression of key antioxidant enzymes such as catalase (cat), superoxide dismutase (sod), and glutathione S-transferase (gstm) was also evaluated via RT-PCR. The results demonstrated a decrease in the expression of all the enzymes, suggesting that nanoparticles may interfere with the redox state of zebrafish larvae. Furthermore, since the oxidative stress is often associated with inflammation also key genes related to inflammation *tnfalpha* and *il1beta* were assayed. Our results showed a modulation of inflammation's genes, particularly treatment induced a low downregulation of *tnfalpha* and an opposite regulation of *il1beta* which expression increased at very high levels. Finally, molecular docking and dynamics approach were applied to further explore any potential molecular interactions between ZnO-NPs and critical embryonic proteins, such as hatching enzyme *ZHE1*, and superoxide dismutase, *SOD 1*, enzyme. Results shown that ZnO-NPs interfered with both enzymes inhibiting those activities, and causing a delayed hatching of zebrafish embryos most probably through a multi-modal mechanism. The integration of *in silico* and *in vivo* assessments provides a more comprehensive evaluation of the potential risks associated with exposure to nanomaterials, contributing to the fields of nanotoxicology and developmental biology. However, we are discussing preliminary results that require further testing and examination to fully understand the molecular mechanisms and causes of ZnO-NP toxicity.

#### IN VITRO AND IN VIVO ASSESSMENT OF GLYPHOSATE IN NORMOXIA AND HYPOXIA CONDITIONS

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Glyphosate (N-(phosphonomethyl) glycine) is a systemic and non-selective post-emergence foliar herbicide. It is today known as the most widely used herbicide worldwide. Because of the extensive use of glyphosate in agriculture, traces of this herbicide are nowadays found in soil, water, and air, as well as in food, becoming a growing concern for human health. A distinctive feature of some of these water environments, particularly those highly polluted, is the low water oxygen concentration. This leads to the development

of hypoxic environments which represents a stress factor for the residing organisms. In zebrafish the adverse outcome pathway of the developmental toxicity of GLY and its underlying mechanisms remain still unclear. To date, little is still known about the combined effect of glyphosate and hypoxic conditions on the embryonal and larval forms found in water ecosystems and on *in vitro* model. Therefore, this study aims to assess the biochemical, histological and molecular changes of GLY-induced developmental toxicity in zebrafish embryos also to evaluate these behaviors associated with a hypoxic condition, chemically induced by cobalt chloride (CoCl<sub>2</sub>). The histological observation performed for each glyphosate concentration (25, 50, 75, 100 mg/mL) both in hypoxic and non-hypoxic conditions. The larvae showed major lesions in the liver and the intestine. To gain deeper insights into the role of hypoxia in the damage determined by Glyphosate, the presence of the toxic damage and inflammation markers have been evaluated in 100 mg/mL glyphosate-treated larvae both in normal and hypoxic conditions. To deeply investigate the effects of Hypoxia on glyphosate toxicity we decided to investigate low dose (50mg/L) of glyphosate in hypoxia condition. First we analyzed the mRNA and protein levels of Hif1a in all conditions.

Regarding the expression levels of the major enzymes involved in the oxidative stress response, such as *sods*, *catalase*, *gst*. We observed a different modulation depending on the treatment. This first set of data allowed us to understand that all treatments were responding to oxidative stress, thus we decided to investigate also the enzymatic activity. As expected, the picture of enzymatic activity is also modulated by the treatments, indicating a response to oxidative stress. To better understand the effects of this enzymatic activity we performed the TBARs assay to evaluate the levels of lipid peroxidation products, which is one of the effects of ROS production. The only condition that was not able to counteract the increase of oxidative stress is the condition of glyphosate in hypoxia, suggesting a worsening effect of hypoxia on glyphosate toxicity. Finally to better dissect the effects of glyphosate in hypoxia conditions also *in vitro* models for the gut and liver have been used.

*Acknowledgment: this research was funded by the European Union-Next Generation EU.*

*Project Code: ECS00000041; Project CUP: C43C22000380007; Project Title: Innovation, Digitalization and Sustainability for the Diffused Economy in Central Italy - VITALITY.*

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## AQUATIC ENVIRONMENTS

### SHAPE ANALYSIS AND MORPHOMETRY OF SAGITTAL OTOLITH IN FIVE CRYPTIC MACROURIDAE SPECIES FROM CENTRAL MEDITERRANEAN SEA

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The increase in deep environment exploitation and depletion related to fisheries activities has enhanced the need to improve the knowledge base about deep-sea demersal species. The species belonging to the Macrouridae family, also called grenadiers or rattails, are among the most abundant for biomass and species numbers (405 valid species, 8 in the Mediterranean Sea). These benthopelagic globally distributed species inhabit a wide range of environments, from the continental shelves and slopes between 200 and 2000 m, to the abyssal plains between 2000 and 6000 m. Macrourids are an ecologically essential component of the meso- and bathypelagic community. Otoliths are calcareous structures contained in the teleost's inner ears. Both organs (one for each side), fundamental in balance and hearing, are composed of three semicircular canals, three end organs (*ampullae*) and three otoliths' organs (*sacculus*, *utricle* and *lagena*). These last contain otoliths, respectively *sagitta*, *lapillus* and *asteriscus*. *Sagittae*, or sagittal otoliths, are the largest among them in non-ostariophysian fishes, and they are widely used in many research fields: in paleontology and palaeoecology, to assess past marine teleost biodiversity and populations; in fisheries science, to identify stocks, species, and populations through otoliths shape analysis. The present paper aims to investigate the sagittal morphology, morphometry, and shape in five Mediterranean Macrouridae species, investigating their intra- and inter-specific variations, and comparing data with literature from other geographical areas. A total of 144 individuals (35 *C. guentheri*, 20 *C. caelorhincus*, 24 *H. italicus*, 24 *N. aequalis*, 40 *N. sclerorhynchus*), collected from professional fishing, were analyzed. From each measured, sexed and weighed specimen left and right sagittal otolith was extracted and photographed; images were then elaborated using ImageJ to obtain otolith measurements and to convert images into binary format for contour extraction. Elaboration with Shape R allows us to obtain the otolith shape analysis. Results showed the absence of directional bilateral asymmetry in all the studied species, with clear differences in morphometry and shape at the interspecific level. They detected statistically significant similarity patterns between *Coelorinchus caelorhincus* and *Coryphaenoides guentheri* specimens (*Coelorhynchus Coryphaenoides* group), and even between *Nezumia aequalis* and *Nezumia sclerorhynchus* specimens (*Nezumia* sp group). *Hymenocephalus italicus* showed the most marked differences in otolith features compared to the other investigated species. Findings from the present paper reported and overall morphology of the investigated *sagittae* was not in line with data from the literature for the studied species, with differences detected between morphometrical

parameters and morphological features. While, concerning the shape analysis, the results confirmed its reliability for the discrimination of the main otolith contour in the studied species. Results confirmed the similarity in shape and morphometry of *sagittae* belonging to phylogenetically close species, sharing several aspects of their life habits too. The present paper represents the first intra- and inter-specific comparison among sagittal otoliths morphology, morphometry, and shape of five species belonging to the Macrouridae family from the Southern Tyrrhenian Sea. Further analysis of genetics, growth dynamics, feeding habits and environmental conditions experienced by specimens are required to confirm the environmental influence on *sagittae*, also comparing data from different Macruridae populations to enhance the taxonomical value of these morphological tools.

### DEFENSE SYSTEM IN THE PRIMARY REPRODUCTIVE STAGES OF THE OVARY IN *Scorpaena porcus* (LINNAEUS, 1758)

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Sexual development is a crucial biological parameter for determining the reproductive season of a species and monitoring long-term changes in the reproductive cycle, as well as for other research needs related to fish biology. Although significant progress has been made in this field, fundamental biological information is still lacking for many fish species in the Mediterranean Sea. Research related to reproduction and its interactions with the defense system is still lacking, particularly in fish<sup>1</sup>. In these vertebrates, the immune response plays a role in modulating reproduction. Additionally, in gonad tissues, it is specifically regulated to prevent infertility. Host defense peptides are key components of the fish innate immune system, including antimicrobial peptides (AMPs) such as piscidins. Piscidins are small  $\alpha$ -helical, amphipathic peptides that play a crucial role in the innate defence mechanisms of teleosts. They have a broad spectrum of action against viruses, bacteria, fungi, and protozoa<sup>2</sup>. *Scorpaena porcus* (Linnaeus, 1758) is a significant member of the rocky and reef community in the Mediterranean Sea. The black scorpionfish is a benthic species that typically inhabits shallow rocky areas and the bottom of seagrass beds. It can be found at depths of 5-15 m in the Adriatic Sea, 10-30 m in the Mediterranean Sea, and 10-30 m in the Black Sea. As a carnivorous species, it feeds on small fish such as gobies and gudgeons, as well as crustaceans and other invertebrates. Reproductive strategies in Scorpaenidae have evolved from basic oviparity to matrotrophic viviparity, although oviparity is more common in the genus *Scorpaena*. Their fertilization is external and development is oviparous<sup>3</sup>. This study aims to investigate the primary sexual developmental stages in the female gonads of black scorpionfish, evaluating the evolution of the internal defense system and its interaction with oogenesis via Piscidin1. The findings provide additional knowledge of the reproductive biology of this teleost. Histologically, a morphostructural change is evident from the immature stage to

the early developmental stage. Specifically, there is a reduction in stromal connective tissue from the immature stage to the primary developmental stage, and the oocytes change as they enter the vitellogenesis stage. Immunohistochemistry revealed an increase in the infiltration of immunoreactive defense cells to Piscidin1 in the stage following immaturity, scattered in the stromal connective, among the follicular cells. Interestingly, some oocytes at different stages of maturation exhibited immunopositivity for Piscidin1, indicating that this antimicrobial peptide may play a crucial role in defending this delicate stage, which is vital for the continuation and maintenance of the species. Furthermore, these findings could aid in enhancing comprehension of inter-species immunity to offspring and contribute to fundamental knowledge of the reproductive cycle of this teleost.

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#### EFFECTS OF *Ericaria brachycarpa* EXTRACT ON THE DEVELOPMENT OF *Arbacia lixula* SEA URCHIN EMBRYOS

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Being macroalgae sessile organisms, they need to constantly adapt to either the abiotic and biotic components of the marine ecosystem and have developed complex adaptations to survive, including the production of bioactive molecules. The aim of this work was to evaluate the effects of an extract of the brown macroalga *Ericaria brachycarpa* on the development of *Arbacia lixula* sea urchin embryos, to evaluate the embryotoxic activity from fertilization (0 h) to the pluteus stage (72 h). The range of concentrations tested was chosen to cover a full 0-100 % abnormality curve, with doses ranging from 0 to 40 µg/mL. The extract was added at three developmental endpoints: zygote (0 hpf), gastrula (24 hpf) and pluteus (48 hpf), founding that gastrulae were the most sensitive to the extract with the lowest EC50 (5.366 µg/mL). In all treatments there was a dose-dependent effect. At low concentrations we found a significant variation in two morphometric parameters at the pluteus stage, compared to controls. The highest concentration tested (40 µg/ml) caused 100% mortality of the embryos at all embryo stages. Western Blot experiments showed the modulation of different molecular markers (HSP60, LC3, p62, CHOP and cleaved caspase-7), showing enhanced autophagy at low concentrations and apoptosis at high concentrations. The TUNEL assay confirmed high levels of fragmented DNA in 48 h exposed embryos. Further studies by means of HPLC/MS/QToF are in progress to identify which compound(s) of the *E. brachycarpa* extract are responsible for the embryotoxic activity.

#### PHYSIOLOGICAL AND CELLULAR RESPONSES OF A SYNTHETIC PRESERVATIVE IN PERSONAL CARE FORMULATIONS ON AQUATIC ORGANISMS

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Quaternium-15 (QT-15), widely utilized as a preservative in various personal care products, including shampoos, soaps, shaving items, and cosmetics, has raised environmental concerns as its presence in aquatic ecosystems stems from routine consumer use. With the COVID-19 pandemic, the presence of quaternary ammonium compounds (QACs), in sanitizing and disinfecting products has increased exponentially. Additionally, quaternium-15 has been found to be resistant to degradation, resulting in its persistence in the environment for several years following its release. This persistence raises concerns about the long-term effects on aquatic ecosystems. To detect the potential impact of QT-15 on aquatic communities, bivalves such as *Mytilus galloprovincialis* are particularly suitable bioindicators. For this reason, this study aimed to explore the physiological and cellular responses of the digestive gland and gill cells of *M. galloprovincialis* following exposure to QT-15. The specimens were divided into three experimental groups (CTR: control; E1: 0.5 mg L<sup>-1</sup>; E2: 1 mg L<sup>-1</sup>). Each group comprised 49 animals housed in duplicate aquariums, totaling 294 mussels. The concentrations of QT-15 were chosen based on previous studies<sup>1,2</sup> and on the concentrations of QACs<sup>3</sup>. The animals were maintained at constant temperature, salinity, and pH. Fourteen molluscs, randomly sampled from each experimental condition at 7 and 14 days post-exposure to QT-15, were utilized to assess cellular vitality, the regulatory capacity of digestive gland cells in adjusting their volume, and the expression of genes associated with oxidative stress. Results unveiled a notable decline in both the vitality and volume-regulating ability of digestive gland cells when subjected to a hypotonic solution, suggesting a potential link between exposure and compromised cellular function. The altered expression of genes related to oxidative stress, including *SOD*, *Cat*, *Hsp70*, and *CYP4Y1*, provided molecular insights into cellular responses to the impact of QT-15. In the gills, similar changes in gene expression were observed. These data provide valuable information for understanding the possible environmental impact of QT-15 exposure and highlight the urgency in identifying green and more sustainable compounds that can serve as alternatives in personal care formulations.

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## A PROMISING NOVEL AQUATIC MODEL FOR ASSESSING CELLULAR AND PHYSIOLOGICAL RESPONSE TO PERSONAL CARE PRODUCT ADDITIVES

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Originating from the Pacific Ocean, the pearl oyster *Pinctada imbricata* (Röding, 1798) stands as one of the first “Lessepsian migrants” to reach the Mediterranean Sea via Suez. Since the 2000s, the species has established a successful population in the transitional waterways of the “Capo Peloro Lagoon natural reserve”, exhibiting exceptional abundance owing to its remarkable adaptability across various hydrological, climatic, environmental, and pollution conditions. With reference to recent studies<sup>1,2</sup> and considering its biological traits, *P. imbricata* appears to embody the qualities of an effective bioindicator and model organism for ecotoxicological investigations. The relevance of the availability of suitable bioindicators and model organisms stems from the growing awareness of identifying and addressing stress conditions that affect aquatic ecosystems, primarily due to human-induced pressures. Effectively, aquatic ecosystems are among the main target environments for biomonitoring strategies, as they represent one of the main receptors of pollutants. For instance, the COVID-19 pandemic has increased the use of personal hygiene products, containing compounds, e.g. preservatives, that may threaten aquatic ecosystems. Preservatives include various categories including quaternary ammonium compounds (QACs), recognized for their ability to withstand metabolic degradation and persistence in wastewater due to their lipophilic nature. Among QACs is quaternium-15, which is widely incorporated in various personal care products (PCPs). Therefore, this study aims to assess the physiological and cellular responses of *P. imbricata*'s haemocytes to different concentrations of quaternium-15 (0.1 and 1 mg L<sup>-1</sup>) *in vitro*, to determine the suitability of the species as an experimental model in ecotoxicological studies and the toxicity of the substance. The investigation included cell viability and phagocytosis assays, along with modulation of  $\gamma$ -actin ( $\gamma$ Act) and oxidative stress-related gene expression (Cat, MnSod, Zn/CuSod, GPx). Findings revealed a significant reduction in haemocyte viability and phagocytosis activity after exposure to both concentrations. The decreasing phagocytosis activity was also supported by the gene expression modulation of  $\gamma$ Act, which is involved in cytoskeleton rearrangement. The qPCR data revealed significant alterations in the antioxidant response, resulting in the upregulation of Cat and GPx and the downregulation of MnSod and Zn/CuSod. These data indicate that exposure to quaternium-15 caused harmful effects on the immune-mediated response of haemocytes, which act as the primary defence mechanism for bivalve molluscs against xenobiotics. Additionally, there is evidence of a time and dose-dependent response, highlighting the importance of exploring sustainable alternatives to replace harmful additives in PCPs and promoting *P. imbricata* as a valuable model for future ecotoxicological studies.

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## WATER-SOLUBLE POLYMERS (POLYVINYL ALCOHOL AND POLYETHYLENE GLYCOL): A NEW THREAT TO THE AQUATIC ENVIRONMENT? EVALUATION OF EMBRYOTOXICOLOGICAL EFFECT ON ZEBRAFISH AND AFRICAN CLAWED FROG

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The impact of emerging contaminants (ECs) on the environment, particularly the aquatic environment, has received increasing attention in recent years. Classified as pseudo-persistent pollutants, these chemicals can affect non-target species at many stages of development. ECs are widely used in the formulation of personal care products (cosmetics, creams, detergents etc.), food packaging and pharmaceuticals, which are constantly released into the aquatic environment, making them a potential threat to the aquatic communities. This group includes two water-soluble polymers, also known as liquid plastics, which have increased their presence, particularly in the formulation of personal care products and pharmaceuticals: polyvinyl alcohol (PVA) and polyethylene glycol (PEG), but despite this proliferation, less is known about their environmental accumulation effects on aquatic species. Using embryotoxicity tests, this study aimed to assess the potential hazards of exposure to PVA and PEG for non-target species, using zebrafish (*Danio rerio*) and African clawed frog (*Xenopus laevis*) embryos as models. Fertilised eggs were placed in microwell plates and exposed to the chemicals at different concentrations, for a total of 96 hours. Throughout the experiment, the temperature was maintained at 26 °C for the zebrafish and 23 °C for the frogs, and the water containing the chemicals was changed daily. Embryotoxicity endpoints (mortality, hatching rate, malformations) were observed daily, and heart rate and length were recorded after 48 hours and 96 hours of exposure, respectively. Significant results were obtained for the percentage of mortality for frogs, for the hatching delay for zebrafish, for the occurrence of malformations at almost all concentrations selected for both animals (observation of oedema, body malformations, changes in the pigmentation and spinal and tail deformities) and for the change in the heart rate (decrease or increase in rate) for both zebrafish and African clawed frog. The data and the level of significance obtained during the experiments suggest that PVA and PEG may pose a potential risk to non-target species, but at the same time, further analysis and investigation are needed to understand how these compounds interact with the aquatic environment fully.

## BIOLOGY OF REPRODUCTION AND INFERTILITY

### A PCOS MOUSE MODEL SHOWS EVIDENCE OF GLYCATIVE AND OXIDATIVE DAMAGE AT THE BRAIN LEVEL

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Polycystic ovary syndrome (PCOS) is an endocrine disorder that affects women during their reproductive years, often leading to fertility issues and metabolic challenges. Recently, there has been growing interest in understanding the impact of PCOS on brain function, driven by evidence suggesting a neuroendocrine basis for the syndrome and observed cognitive and functional imaging differences in individuals with PCOS. Moreover, research in recent years has emphasized the connection between PCOS and glycative stress, particularly focusing on advanced glycation end products (AGEs) found in the serum, ovaries, and uterus of women with PCOS. To address the study question, a mouse model of PCOS based on the administration of dehydroepiandrosterone (DHEA) to adult female mice, was generated. Twenty 4-week-old, young CD-1 female mice were randomly assigned to two groups mice daily injected with DHEA (6 mg/100 g body weight), for 20 consecutive days (DHEA mice). The vehicle control group was injected with 0.09 mL sesame oil and 0.01 mL 95% ethanol daily for 20 consecutive days (control mice). The expression of sirtuins (SIRT1, SIRT3), antioxidant enzymes (SOD1, GPX1, CAT, acetyl-NF- $\kappa$ B and acetyl-SOD2), lipid peroxidation marker (4-HNE) and antiglycative enzyme glyoxalase 1 (GLO1) involved in the detoxification of methylglyoxal (MG) was investigated by real time PCR and Western blotting analyses. Besides, whole brains were processed for histology examination of MG-AGE and SIRT1 by hematoxylin-eosin (H&E) and Azan Mallory staining. Treatment with DHEA in CD-1 mice resulted in disrupted estrous cyclicity, an increased number of atretic follicles, and heightened collagen deposition and lipid droplets, all of which are characteristic features of PCOS. Interestingly, no differences were observed in the expression of BDNF and its receptor TrkB at the brain level, suggesting that the biochemical changes observed may not have significant effects on neuronal survival or essential functions. However, this study did reveal elevated levels of lipid peroxidation markers, such as 4-HNE protein, and increased levels of SIRT1, SIRT3, SOD1, acetyl-NF- $\kappa$ B, and acetyl-SOD2 in the brains of PCOS mice, indicating the presence of oxidative stress. This effect was further supported by increased staining of MG-AGE, indicating glycative damage. Additionally, GLO1, the enzyme responsible for detoxifying MG, showed higher expression at the protein level, suggesting an adaptive response to changes in MG levels. Given this evidence, it appears that PCOS can induce changes at the brain level marked by shifts in the redox state, affirming the presence of oxidative stress as a prominent characteristic of PCOS. These findings could enhance our understanding of the molecular alterations associated

with cognitive dysfunction in individuals with PCOS and help establish the multifactorial and systemic aspects of this condition.

### SEASONAL EFFECTS ON SARDA SHEEP OOCYTES QUALITY: ULTRASTRUCTURAL EVIDENCES

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Seasonality of reproduction, a common feature in sheep of temperate latitudes, has been recognized for a very long time. Sheep are domesticated animals, which in temperate latitudes still remain seasonal breeders [1]. In general, the annual breeding season in Sarda sheep is controlled by photoperiod: long days inhibit and short days stimulate sexual activity [2]. Then the season of high sexual activity in Sarda sheep is represented by autumn. The reproductive seasonality of domestic animals is often manipulated to have extended reproductive periods for commercial purposes related to the production of milk and meat. In Sardinia, an extension of the sheep breeding season is finalized to distribute lambing along the year to ensure milk production for several additional months as well as to satisfy the meat market demand. This is achieved using the out-of-breeding mating strategy, which consists of interrupting sexual promiscuity by the use of the male effect and exogenous hormones for oestrus cycle control. In this way the lambing calendar is modified in order to obtain 30–40-day-old lambs (weighing 8–10 kg) during the Easter and Christmas celebrations. However, and despite their wide and frequent use, these strategies may result in decreased fertility [3]. Although the reproductive performance of grazing cattle is lower in summer compared to winter, the effect of season on oocyte developmental competence has not been thoroughly examined [4]. In the present study we evaluated the effects of season, in terms of climate conditions (temperature and humidity), on oocyte quality. Oocytes were collected during winter (January- March, group 1) and summer (May-June and July, group 2) and their quality was investigated through morphological and ultrastructural evaluation by light and transmission electron microscopy (LM and TEM), to evidence possible alterations. By LM and TEM, oocytes of both groups evidenced a round shape with a thin perivitelline space surrounded by a continuous zona pellucida; microvilli covered the oolemmal surface. The ooplasm presented numerous mitochondria clusters, clear vacuoles and high electron-dense lipid droplets that in the group 1 appeared more abundant and mostly located in the cortical ooplasm. The cortical granules beneath the oolemma seemed to be more numerous and more uniformly distributed in group 1 compared with group 2. In conclusion, our data showed very few differences between the two groups examined and these preliminary findings could be useful to better investigate how ovine oocyte quality could be influenced by environmental conditions and by season.

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## EFFECTS OF ENDOCRINE DISRUPTORS ON THE ULTRASTRUCTURE OF MAMMALIAN OOCYTES

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Endocrine disruptors (EDs) (such as pesticides, fungicides, organochlorine compounds, etc.) can interfere with the endocrine system by mimicking or completely blocking the activities of endogenous estrogens by interacting with their receptors. Exposure to EDs is involved in various environmental health hazards, including female infertility [1]. However, the exact mechanism of action of EDs, their direct impact on reproductive functions, and their damage to cell structures in the female reproductive system are still unclear. Mancozeb is a widely used fungicide and is considered an ED. *In vivo* and *in vitro* studies evidenced its reproductive toxicity on mouse oocytes by altering spindle morphology, impairing oocyte maturation, fertilization, and embryo implantation [2,3]. Previously, we demonstrated dose-dependent toxicity exerted by Mancozeb on the ultrastructure of mouse granulosa cells *in vitro* including chromatin condensation, membrane blebbing, and vacuolization [4]. In this study, we aimed to evaluate the effect of this fungicide on the ultrastructure of mouse oocytes, recovered by cumulus-oocyte-complexes (COCs) cultured *in vitro* with or without (control) increasing concentrations of Mancozeb (0.001-1 µg/ml). Transmission electron microscopy was used to investigate the effects of this ED. Results showed at the lowest doses oocytes ultrastructure preserved, comparable to controls, with evident clusters of round-to-ovoid mitochondria, cytoplasmic lattice, smooth endoplasmic reticulum, visible electron-dense round cortical granules, and thin microvilli. Lipid droplets, multivesicular aggregates and lamellar bodies were present in the ooplasm. Mancozeb concentration of 1 µg/ml affected organelle density, with a decrease in the numerical density of mitochondria, which appear moderately vacuolated, cortical granules, and flattened microvilli. *In vitro* cultured systems of COCs provide an easy and useful experimental model of reproductive toxicity to study the harmful effects of pesticides. Preliminary data on mouse oocytes exposed to increasing concentrations of the pesticide Lindane (1-100 µM) showed some ultrastructural changes predominantly at the highest doses, compared with controls, similar to what was found in oocytes treated with Mancozeb. These results contribute to understanding the adverse effects of EDs on the mammalian reproductive system and to identifying their potential impact on fertility.

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## EFFECTS OF L-CARNITINES ON MORPHO-FUNCTIONALITY OF THE FEMALE REPRODUCTIVE SYSTEM IN A POLYCYSTIC OVARY SYNDROME MOUSE MODEL

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Polycystic ovary syndrome (PCOS) is one of the most common reproductive endocrine disorders in young women. It leads to a polygenic, polyfactorial, systemic, inflammatory and dysregulated steroid state (Patel *et al.*, 2018). Multifactoriality is a distinctive feature of the disease, responsible for oxidative, glycativ, and lipoxidative stresses. These traits seem also to be connected to female infertility. Here we investigated morphological alterations on the ovary, uterine horns and oviducts (Di Emidio *et al.*, 2020 (a); Di Emidio *et al.* 2020 (b); Palmerini *et al.*, 2023) by using a Dehydroepiandrosterone (DHEA)-induced PCOS mouse model. Moreover, the effect of antioxidants in the diet was assessed after L-carnitines supplementation. To these aims PCOS was induced in CD-1 mice by subcutaneous administration of DHEA (6 mg/100 g body weight) for 20 days; two groups of mice concomitantly received orally 1) L-carnitine (LC) and acetyl-L-carnitine (ALC) or 2) LC, ALC and propionyl-L-carnitine (PLC). Control animals were untreated. At the end of the treatments, organs were collected and subjected to standard protocols for histology and immunohistochemistry to evaluate morphology, collagen deposition, steroidogenesis, oxidative, glycativ and lipoxidative stress. Respect to controls, PCOS ovaries, uteri and oviducts showed hyperplasia, hypertrophy and hyperfibrosis. MG-AGE accumulation was indicative for glycativ damage. Oxidative, mitochondrial and lipoxidative stresses were evidenced by increased HNE and decreased Tomm-20 immunostaining. PCOS altered steroidogenesis also in the reproductive organs, as indicated by the increased expression of the androgenic enzyme 17 β-HSD4. Diet supplementation with L-carnitines reduced DHEA-induced alterations, even with slight organ-specific differences. The morphological approach on a DHEA-induced mouse model of PCOS provided new insights on the effects exerted on different reproductive organs and highlighted the protective effects of L-carnitines as diet supplement.

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## BENEFICIAL EFFECTS OF D CHIRO INOSITOL ADMINISTRATION IN A MOUSE MODEL OF ENDOMETRIOSIS

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Endometriosis, a disease affecting 5-10% of women of reproductive age, is characterized by the spread of endometrial-like tissue outside the uterine cavity that produces ectopic endometriotic lesions causing pain and infertility. Surgical intervention and hormonal treatments have no long-term effects. In this study, we investigated whether D-chiro inositol (DCI), a natural molecule known to affect ovarian steroidogenesis, may counteract the development of endometriotic lesions and improve therapeutic potential of conventional progestin drugs (*i.e.* Dienogest, DG). To address the study question a mouse model of endometriosis was generated by intraperitoneal inoculation of endometrial tissue fragments in recipient CD1 mice, which were then treated for 28 days with: 0.4 mg DCI; 0.2 mg DCI+0.33 ng DG; or 0.67 ng DG, in drinking water; none (CTRL). Mice were then sacrificed and the intraperitoneal endometriotic lesions were excised. The lesions were measured by number and size and examined for the presence of blood vessels vascularization under stereomicroscope. Endometriotic lesions developed in recipient mice met all criteria for endometriosis, including the presence of endometrial epithelial and stromal cells, and encapsulation in neighboring tissues or organs. Numbers, extensions and vascularization degrees of endometriotic foci were reduced in all the treatment groups when compared to CTRL, in the DCI group the vascularized lesions were not observed. The histological analysis revealed a marked reduction of endometriotic foci in all groups. To understand mechanisms underlying DCI effects, the expression of SIRT1, marker of epithelial-mesenchymal transition involved in endometriotic lesion development was evaluated. The analysis of SIRT1 transcripts highlight a decrease in the DCI+DG group compared to the CTRL; SIRT1 expression was not detected in the DCI group. The lesions were then subjected to immunohistochemical analysis aimed at evaluating the proliferative and vascularization status using PCNA and CD34, respectively. PCNA-positive epithelial and stromal cells were observed in the lesions, the DCI group showed a reduce expression of this marker. CD34 labeling showed similar levels in CTRL compared to DCI+DG and DG treated groups; the DCI group

presents a reduction in the expression of this marker. Then, possible effects of DCI on expression of ovarian CYP19A1 aromatase of modeled mice were investigated. CYP19A1 transcripts in the ovaries decreased in all groups, especially in the DCI group. The follicle count revealed that the ovaries of the DCI group present a significantly higher number of primordial and antral follicles compared to the other groups. This work has provided an easily reproducible, minimally invasive model of endometriosis, which simulates the natural process that leads to the onset of the pathology and has highlighted the beneficial effects of DCI in the treatment of this disorder. In conclusion, these observations make DCI a promising molecule for nutraceutical therapy of endometriosis and open the doors to further investigations aimed at introducing this substance into clinical practice.

## HYPOXIA AND INFLAMMATION INDUCE TESTICULAR DAMAGE INFERTILITY IN FABRY'S DISEASE

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Fabry's disease (FD) is a genetic X-linked systemic and progressive rare disease characterized by the accumulation of globotriaosylceramide (GB3) into the lysosomes of many tissues. (1) FD is due to loss-of-function mutations of  $\alpha$ -galactosidase, a key-enzyme for lysosomal catabolism of glycosphingolipids, which accumulate as glycolipid bodies (GB). In homozygous males the progressive deposition of GB3 into the cells leads to clinical symptoms in CNS, skin, kidney, etc. In testis GB accumulation causes late infertility and alterations of spermatogenesis.(2) However, the precise damaging mechanism is still unknown. Our working hypothesis is that GB accumulation reduces blood vessel lumen and increases the distance of vessels from both stromal cells and seminiferous parenchyma; this, in turn, impairs oxygen and nutrients diffusion leading to subcellular degradation of seminiferous epithelium and, finally, sterility. To test this hypothesis, we have studied a 42-year-old patient presenting a severe FD and infertility, with reduced number of spermatozoa and preserved sexual activity. Before the clinical evidence of Fabry's disease and associated sterility, he fathered three children and had normal blood levels of FSH, LH and androgens. Testicular biopsies have been analysed by optical (OM) and transmission electron microscopy (TEM). Activation and cellular localization of HIF-1 $\alpha$  and NF $\kappa$ B was analysed by immunofluorescence (IF) and RT-PCR on homogeneous tissue fractions after laser capture microdissection (LCMD). OM and TEM showed that GB were abundant in vessel wall cells and in interstitial cells. By contrast, GB were absent in seminiferous epithelium, Sertoli and Leydig cells. However, seminiferous tubular epithelium and Sertoli's cells showed reduced diameter, thickening of basement membrane and tunica propria, and swollen or degenerated spermatogonia. IF showed an accumulation of HIF-1 $\alpha$  in stromal cells but not in seminiferous tubules. On the contrary, NF $\kappa$ B fluorescence was evident in tubules, but very low in interstitial cells. Finally, RT-PCR analysis on LCMD fractions showed the expression of genes of HIF-1 $\alpha$ /NF $\kappa$ B

inflammatory-like pathway in the absence of CD45+ cells. In conclusion, our study demonstrates that the late infertility in FD may be caused by reduced oxygen and nutrients due to GB accumulation in blood vessels cells. Reduced oxygen and nutrients alter HIF-1a/NFkB expression and localization while activating HIF-1a/NFkB driven-inflammation-like response damaging seminiferous tubular epithelium and Sertoli's cells. The clinical history (the patient fathered three children and had normal hormonal level before diagnosis of

Fabry's disease) strongly suggests that the development of testicular insufficiency and OM/TEM alterations leading to late sterility, is a slow process in relation to the progressive accumulation of GB and, therefore, it is unlikely related to Y microdeletions.

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## REGENERATIVE MEDICINE

### OSTEOGENIC DIFFERENTIATION OF hADMSCs ON METHACRYLATED GELLAN GUM SCAFFOLDS FOR TISSUE REGENERATION

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The physiological process of bone repair appears highly compromised when large bone defects occur due to trauma, degenerative diseases (osteoarthritis), inflammatory diseases (rheumatoid arthritis), or tumor resection, representing a serious public health emergency. Currently, the most used treatments in clinical practices for the repair of large bone defects consist of surgical transplants. Nonetheless, there are notable disadvantages of adopting graft, including donor site morbidity, infection risk, immune rejection, and persistent post-operative pain<sup>1</sup>. Therefore, the clinical needs associated with bone repair have stimulated the development of innovative strategies based on bone tissue engineering (BTE) approaches, whose goal is to develop biomaterials with the following features: biocompatible, biodegradable, immunogenicity-free, osteoconductive, and osteoinductive<sup>2</sup>. In last decades, hydrogels based on naturally derived polymers have emerged as a novel class of biomaterials for BTE applications, including Gellan gum (GG), an exopolysaccharide produced by *Sphingomonas*, due to its inherent physical and chemical properties and biocompatibility, although its potential use is limited by the reduced stability under physiological conditions<sup>3</sup>. The stability and mechanical properties of GG can be improved through the functionalization with methacrylate moieties to produce methacrylated GG (GG-MA)<sup>4</sup>. Furthermore, inorganic micro/nanoparticles, such as hydroxyapatite (HAp), are often incorporated within the hydrogel matrix, simultaneously acting as mechanical reinforcement and bioactive signals to guide bone formation<sup>5</sup>. Recently, particular attention has been also devoted to eumelanin as bioactive agent which can specifically support bone repair<sup>6</sup>. Herein, the aim of this study was to evaluate the biological response of hADMSCs on different types of GG-MA scaffolds, in terms of biocompatibility, osteoconductivity and osteoinductivity, from 1 to 21 days of *in vitro* culture. Specifically, the following scaffolds, GG-MA 4% (w/v) (GG-MA4), GG-MA4 functionalized with a naturally derived eumelanin extracted from the black soldier fly (GG-MA4/BSF-Eumel), and GG-MA4 functionalized with 30% of HAp nanoparticles (wHAp/wGG-MA) (GG-MA4/HAp), have been tested by *in vitro* studies. Our results highlighted that BSF-Eumel and HAp triggered a different, time-dependent, physiological response in the osteoblast cells cultured on them. Specifically, while the functionalization with BSF-Eumel was able to improve cell proliferation and osteoconductivity compared to GG-MA4, the addition of HAp into the GG-MA scaffold improved its differentiation and osteoinductive properties. These findings suggest the possible use of BSF-Eumel and HAp to improve the biological properties of 3D-printed GG-MA-based scaffolds for BTE applications, as a promising strategy in the field of orthopaedic surgery for the treatment of large bone defects.

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### NEW REGENERATIVE AND ANTI-AGING MEDICINE APPROACH BASED ON SINGLE STRAND ALPHA-1 COLLAGEN

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Over the past decade, regenerative medicine, particularly in skin treatment and rejuvenation, has seen significant advancements. This study explores the effectiveness of a new medical device, in treating various skin conditions, including aging, acne scars, and smoking-related effects. The device combines non-crosslinked high-molecular-weight hyaluronic acid (HMW-HA), human recombinant polypeptide of collagen-1 alpha chain, and carboxymethyl cellulose (CMC), aiming not only to hydrate but also to regenerate skin by promoting collagen production and improving skin elasticity and texture. The study involved 100 subjects divided into three groups based on their skin conditions. The treatment protocol included two injections of the medical device one month apart, with evaluations conducted at baseline, 30, and 60 days post-treatment using FACE-Q questionnaires and Antera 3D skin scanner measurements. The results showed a significant improvement in skin quality, reduction in wrinkle depth, and an increase in skin elasticity and texture across all groups. Additionally, the study highlighted the safety and low risk of adverse reactions associated with the use of this new collagen formulation, making it a promising tool in regenerative medicine for skin treatment. The innovative combination of ingredients in the medical device not only provides hydration but also stimulates both *in vitro* and *in vivo*, the skin's natural regenerative processes, leading to significant improvements in skin quality. The study's findings contribute to the field of regenerative medicine by demonstrating the effectiveness and safety of this new treatment approach for various skin conditions, offering a significant advancement in skin rejuvenation and treatment strategies.

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### A NEW STRATEGY FOR TISSUE REGENERATION WITH SPHEROIDS OF ADIPOSE STEM CELLS

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Adipose stem cells represent a reliable source of stem cells for their widely demonstrated potential in regenerative medicine and tissue engineering applications. New recent insights show that 3D models may properly mimic the native tissue properties. The success of the tissue engineering approaches is based on the availability of cells with high regenerative potential and on the properties of the scaffold, which must be able to support all cellular functions. Spheroids of adipose stem cells (SASCs) represent a great promise for tissue regeneration, they displayed enhanced regenerative abilities if compared to 2D cell models (1,2). We studied SASCs-cell quality (2) and tested their viability and differentiation abilities in new hydrogels (3). We also explored the ability of the new hydrogels to evolve in time and remodel their network to support of SASCs proliferation and differentiation (4). Our study also explores the suitability of k-C/PVA systems for the 3D printing (5). These formulations are very promising for the repair of both cartilage and bone defects, that are still a challenge for modern medicine. This study provides a versatile approach to investigate the interactions between cells in controlled settings, opening up novel 3D *in vitro* approaches to mimic the tissues complexity.

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### ANTI-INFLAMMATORY AND REGENERATIVE POTENTIAL OF COMPLEX MAGNETIC FIELDS IN A CO-CULTURE OF DENTAL PULP STEM CELLS AND INFLAMED MACROPHAGES

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Novel technologies such as complex magnetic fields (CMF) represent interesting tools in counteracting chronic inflammation and oxidative stress underlying several diseases. In the oral cavity, pulp inflammation results from the interaction of different cell types, including pulp cells and macrophages. In this light, a co-culture model of dental pulp stem cells (DPSCs) and LPS-inflamed macrophages was set up to evaluate the effects of the anti-inflammatory and regenerative potential of the CMF treatment up to 48 h. Firstly, lactate dehydrogenase (LDH) levels were measured to assess CMF biocompatibility. Next, cell morphology and proliferation rate were monitored through phase-contrast light microscopy upon crystal violet staining. Moreover, cluster of differentiation (CD) markers, such as CD80-14-163 for macrophages and CD105-73-29 for mesenchymal cells, were detected by means of flow cytometry. Finally, the wound healing assay was performed to evaluate the cell migration rate. Taken together, our data show a reduction in the LDH leakage and an increase in DPSC proliferation mainly after 24 h of treatment. Furthermore, CD14 expression was decreased after a cycle of CMF treatment in inflamed macrophages, while markers related to DPSC osteogenic commitment were highly expressed after 24 and 48 hours in the same experimental conditions. Based on the results achieved, the CMF treatment demonstrates its effectiveness in promoting DPSC proliferation and in counteracting LPS-induced inflammation in macrophages, thus revealing complex magnetic fields as a valuable tool in the management of oral cavity pathologies.

### REGENERATIVE PROPERTIES OF *Helichrysum italicum* ON WOUND HEALING IN VITRO

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Wound healing is a dynamic process of regeneration in the human body in which different cell types are involved. The interactions and communication between fibroblasts and stem cells are essential for wound closure and repair. Proper stimulation could help and promote wound healing processes. The use of natural products, especially those derived from plants, is increasing in regenerative medicine for this purpose. *Helichrysum italicum* (*H. italicum*) is a medical plant well described for its pharmacological, antimicrobial and anti-inflammatory activities. Hydrolate of *H. italicum* does not appear to be toxic to either stem cells or fibroblasts. For this reason, the purpose of the present work was to evaluate the effect of *H. italicum*-derived hydrolate on skin-isolat-

ed stem cells and fibroblasts exposed to a wound healing process. Our results provide evidence that hydrolate of *H. italicum* is a valuable candidate in the treatment of wounds to promote tissue healing. Our data open the way for new studies that can translate the *in vitro* results to future cosmetic or therapeutic treatments.

### ODONTOBLASTS DERIVED FROM DOG ENDOMETRIAL STEM CELLS ENCAPSULATED IN FIBRIN GEL ASSOCIATED WITH BMP-2 FOR DENTIN REGENERATION

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Dental hard tissues are known to be extremely important to be safeguarded, since their regeneration does not occur: in particular, enamel is a tissue without cells, and dentin regeneration often appears disorganized. The most advanced tissue engineering techniques, applied to regenerative treatment for dental tissue, include stem cell cultures that can be differentiated into target tissues on a three-dimensional network to provide a scaffold, which plays a crucial role in tissue engineering. Endometrial stem cells (EnSCs) isolated from canine endometrium have been identified as a source of mesenchymal stem cells (MSCs) characterized by a self-renewal capability. Furthermore, growth factors play an essential role in cell proliferation and differentiation: bone morphologic protein-2 (BMP-2), which belongs to the transforming growth factor  $\beta$  (TGF- $\beta$ ) family, is fundamental in the growth and regeneration of skeletal tissues and has shown odontogenic and osteogenic properties, both *in vitro* and *in vivo*. Besides, BMP-2 stimulates the differentiation of dental pulp stem cells (DPSCs) into odontoblasts. This study aimed to solve dental pathologies utilizing tissue engineering methodologies. The study employed an *in vitro* model to analyze the proliferation and odontogenic differentiation of canine endometrial stem cells (C-EnSCs) isolated from the biopsy of the uterine endometrium. The dentin regeneration potential of odontoblast-like cells (OD) derived from C-EnSCs was evaluated in rodent models. C-EnSCs were isolated using alizarin red staining in order to detect the differentiation into odontoblasts and the expression of two odontoblastic markers, the dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP1) genes by qRT-PCR. Then, C-EnSCs were characterized via flow cytometry, using the conjugated antibodies to CD90, CD105, CD34, and CD45. These cells were encapsulated within fibrin gel supplemented with signaling molecules to establish proper conditions for cellular proliferation and differentiation. OD cells were combined with BMP-2 to stimulate dentin formation *in vivo*. The experimental model, employed for assessing the regenerative efficacy of cells and biomaterials, involved the preparation of the left maxillary first molar in twenty male Wistar rats, for direct pulp capping. The animals were divided into four cohorts: group 1 served as the control without treatment, group 2 received fibrin alone, group 3 received fibrin with ODs (fibrin/ODs), and group 4 received fibrin with ODs and BMP-2 (fibrin/ODs/BMP-2). SEM analysis

assessed the organization of C-EnSCs attached and spread in fibrin hydrogel, appeared as a 3D open porous and interconnected porosity. Morphological examinations revealed the differentiation of C-EnSCs into ODs. Additionally, histomorphometric and histomorphological analysis of treated teeth, using hematoxylin-eosin staining, demonstrated that fibrin gel combined with BMP-2 at a concentration of 100 ng/mL provided an optimal microenvironment for dentin tissue regeneration in rodents. In conclusion, these results showed the potential utility of OD cells derived from C-EnSCs encapsulated in fibrin gel supplemented with BMP-2 for direct pulp capping and dentin regeneration.

*Acknowledgement: the present study has been published on current issue in molecular biology. <https://doi.org/10.3390/cimb45040196>*

### NEURAL DIFFERENTIATION OF THE SH-SY5Y HUMAN NEUROBLASTOMA CELL LINE ON P3HT POLYMER THIN FILM

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In the promising field of bioelectronics, the combination of electronics and biology has paved the way for new applications in many medical fields, including implantable devices<sup>1</sup>. Developing techniques, capable to monitor and control the biological systems efficiently and instantaneously, is crucial for many biomedical applications, including drug delivery, electrophysiological recording, and regulation of intracellular activities<sup>2,3</sup>. In this context, conductive polymers-based systems (CPs), provide a useful scaffold to develop multifunctional nano systems able to mimic the properties of biological tissues offering a platform for electrical stimulation, particularly important when targeting differentiation of cells into neurons and glial cells, and application for novel regenerative therapies<sup>4</sup>. Based on the above reported evidences, in this study we investigated the properties of a semiconductive polymer P3HT based-substrate on the neuroblastoma SH-SY5Y cells, in terms of cell adhesion, proliferation, biocompatibility and neural differentiation. For this end, we performed cytotoxicity tests, immunohistochemical analyses with specific neuronal markers, such as  $\beta$ -III Tubulin, MAP2, NF-H, and DAPI staining. Our preliminary results highlighted that the new P3HT based substrate show a good biocompatibility, and high capability to induce neuroblastoma cells adhesion, proliferation and differentiation, from 1 to 15 days even without addition of retinoic acid. These data taken together suggest that the P3HT based substrate represents a step toward creating biocompatible and functional interfaces that hold promise for future biomedical applications, in particular for the development of medical devices for neural tissue engineering.

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### DOPAMINE MODIFIED ALGINATE HYDROGEL FOR CARTILAGE REGENERATION

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Cartilage is a connective tissue with structural function in many body parts including joints as articular cartilage. Its function is related to the composition of the extracellular matrix, primarily constituted by proteoglycans, with the predominant one being aggrecan, alongside collagen. Cartilage does not contain blood vessels and nerves, and this makes it difficult to self-repair. One of the common degenerative diseases that affect over a million people in the world is osteoarthritis which causes pain and joint stiffness; its incidence and severity increase with age. Nowadays, the only possible therapy is to replace the damaged cartilage with implants. For this purpose, cell-based therapies and tissue engineering as regenerative medicine are promising alternative treatments for this pathology. Based on a previous work [1] we investigated the effect of dopamine conjugated alginate/collagen hydrogels as a three-dimensional scaffolds for the differentiation of Mesenchymal Stem Cells (MSCs) without the use of growth factors. After 7 days of incubation, MSCs embedded within the hydrogels showed a spherical shape, forming cell aggregates characteristic of cartilage. Also, an upregulation of genes associated with chondrogenesis (such as aggrecan, collagen 2, and SOX 9) was observed and immunostaining confirmed the production of cartilage extracellular matrix proteins. To sum up, these findings indicate that dopamine-modified alginate is a promising material for use in regenerative medicine field applications, particularly in cartilage regeneration.

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### EVALUATION OF CELL PHYSIOLOGICAL RESPONSES IN 3D OSTEOBLAST SPHEROIDS

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Organoids provide a more accurate picture of human health than traditional 2D cell culture and animal models. This makes them an important tool for biomedical research [1]. In recent years, there has been significant progresses in the development of spheroids that are three-dimensional (3D) *in vitro* cells as models for *in vivo* tissue surrogates. Besides these systems exploit the self-organizing properties of stem cells, however it is often difficult to control both the spheroid organization and cell-cell/cell-matrix interactions [2]. Biomimetic molecules stimulating microenvironment for *in vitro* cell proliferation and differentiation have been recognized as a key tool to overcome the above described issues. In this context, peptides, short protein fragments, can emulate the functions of their full-length native counterparts. In particular, peptides mimicking growth factors, signalling molecules and receptors have a crucial function in modulating cell survival and differentiation in tissue regeneration. Recently, peptides derived from phage display technology are attracting the attention of many researchers, since they show a high ability to penetrate tissues and interact with native components of the extracellular matrix [3]. In this work, we evaluated cell physiological responses in terms of proliferation, migration, differentiation and formation of 3D osteoblast spheroid using a cocktail of peptides derived from phage display selection. Specifically, these phage peptides were mimotopic of different biomolecules such as osteogenic growth factors, extracellular matrix components and several bone tissue formation proteins. Our results, show that in the peptides-derived mimic microenvironment, human osteoblast cells maintain viability and are promoted in aggregation, cell-cell interaction and osteogenic differentiation. The formation 3D osteoblast spheroids using peptides-derived mimic microenvironment could represent a new promising strategy for both physiological and therapeutic studies. The proposed approach can be extended to several types of tissues and it is suitable to be implemented in organ on chip system for the development of an effective preclinical surrogate.

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## MICRO- AND NANOVESICLES

### ELABORATION OF A SYSTEMATIC APPROACH FOR THE BIOENGINEERING OF TUMOR-DERIVED EXTRACELLULAR VESICLES: INCORPORATING PRELIMINARY *IN VITRO* RESULTS FOR TARGETED CHEMOTHERAPEUTIC DELIVERY IN MALIGNANT PLEURAL MESOTHELIOMA

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Conventional interventions for malignant pleural mesothelioma (MPM), which primarily include surgical resection and chemotherapeutic regimens, typically culminate in recurrence, indicating an urgent need for innovative therapeutic strategies. Despite exploration of diverse approaches, leveraging both the tumor's microenvironmental dynamics, such as anti-angiogenic modalities, and exploiting tumor-specific antigens, for instance, the PDGF receptor or Mesothelin, clinical efficacy remains suboptimal. MPM's unique biomolecular profile presents limited targets for precision medicine, necessitating novel delivery systems for treatment. In this study, we explore the concept of utilizing the tumor's own paracrine signaling pathways by reengineering extracellular vesicles (EVs) that the tumor naturally secretes. These EVs potentially serve as a 'Trojan horse,' infiltrating the tumor's communication network to introduce chemotherapeutic agents directly to neoplastic cells. Building upon this hypothesis, we have refined a protocol for the isolation and modification of EVs sourced from MPM, enhancing their potential as nanocarriers for site-specific therapeutic delivery. This research presents the initial phase, wherein we have successfully isolated and delineated the characteristics of tumor-derived vesicles. We have further innovated the vesicles, incorporating chemotherapeutic agents, and evaluated the efficacy of these drug-loaded vesicles *in vitro* using MTT assays and immunofluorescence to measure their cytotoxic impact and targeting precision. The findings from these preliminary investigations are promising and indicate a progressive step towards the development of a novel, targeted delivery system for MPM treatment, potentially overcoming the limitations of existing therapeutic modalities.

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### DISSECTING THE ROLE OF EXTRACELLULAR VESICLES IN OVARIAN CANCER MICROENVIRONMENT

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Ovarian cancer (OC) is still the most lethal gynecologic tumor, due to the rapid and silent development of omental metastasis. Thus, a deeper understanding of the mechanisms regulating OC progression may have crucial impact on the outcomes of this deadly disease. There is consistent evidence of an association between obesity and increased OC aggressiveness. As omentum is rich in adipocytes, a key pro-tumor role for visceral adipose tissue has been postulated. Indeed, a cross-talk between OC and omental adipose cells has been demonstrated; however, the study of this dialog has been limited to metabolites and adipokines, although recent findings point to a key role of extracellular vesicles (EVs) in the control of tumor evolution. In the present study, we found that OC EVs could induce the production of multiple adipokines, namely interleukin 6, interleukin 1 $\beta$ , MCP-1 and TNF $\alpha$ , in adipocytes. In particular, these changes were accompanied by ERK1/2, p38 and JNK activation, which was demonstrated to modulate the release of all the above cytokines. Interestingly, conditioned media from EV-treated adipocytes stimulated both macrophage and neutrophil recruitment, also favoring their polarization toward the pro-tumor M2 and N2 state, respectively. More importantly, this media promoted OC cell migration and invasion as well as anoikis resistance; in particular, an increase in tumor spheroid formation ability was observed. Overall, these data indicate that an EV-mediated bidirectional crosstalk exists between OC and adipocytes, endowing the latter with pro-tumor properties.

*Acknowledgements: F.F. was supported by Fondazione Umberto Veronesi.*

### UNLEASH THE POTENTIAL OF FOURIER-TRANSFORM INFRARED SPECTROSCOPY TO STUDY EXTRACELLULAR VESICLES IN BREAST CANCER

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Breast cancer remains a primary health concern among women worldwide, and insight into its initiation, progression, and metastasis are of the utmost importance. The role of Extracellular Vesicles (EVs), cell-derived membrane-surrounded vesicles, in cell-to-cell communication has long been recognized as a crucial component in these processes. A wide range of studies based on EVs are carried out on *in vitro* models, based on bi-dimensional (2D) cultures; these studies led to a greater understanding not only of the different mechanisms by which EVs contribute to tumor progression but also of their potential clinical use in diagnostics as biomarkers. Thus, investigating the molecular composition of EVs is of considerable importance, since it reflects the molecular composition of the parental cells [1]. Among all the techniques already set up, Fourier Transform Infrared spectroscopy (FTIR) has recently been introduced to the study of EVs. This innovative approach, commonly used in the material sciences field, enables fast label-free profiling, obtaining a distinctive molecular 'fingerprint' of EVs from minimal sample quantities, allowing the discovery of potential differences in molecular composition [2]. For this reason, for our study, EVs were isolated from two breast cancer cell lines, with different aggressiveness levels, cultured in 2D; EVs were isolated by ultracentrifugation technique and, once resuspended in a saline solution, they were analyzed for their FTIR spectra, to evaluate whether there could be differences that could reflect the different tumor grades. The results showed that there are some differences in several spectral regions of the two EVs samples, suggesting that the FTIR could allow the recognition of EVs, based on cell origin. However, the 2D models aren't able to truthfully mimic the true complexity of the *in vivo* tumor microenvironment and the interactions mediated by EVs in it. Thus, more recent studies concerning EVs were focused on *in vitro* three-dimensional (3D) cell cultures, using tumor spheroids models [3]. To evaluate if there could be a difference in the molecular fingerprint of EVs derived from cells cultured in 2D or 3D, we isolated the EVs from breast tumor cells cultured by these two models and FTIR was performed on them. The results showed that the EVs isolated from 3D culture have different content in some molecular components compared to the EVs isolated from the respective cell lines cultured on 2D, suggesting that the composition of EVs is profoundly influenced by the cell culture methods and leading us to reflect on a careful evaluation of the most suitable cellular models in the *in vitro* studies. In conclusion, our preliminary data suggest that the FTIR offers new directions for EVs molecular fingerprints, making this tool potentially useful for biomarker discovery in liquid biopsy based on EVs.

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#### UNRAVELING THE POTENTIAL ROLE OF A SMALL EXTRACELLULAR VESICLE LYOPHILIZED ENRICHED IN IMMUNE AND INFLAMMATORY REGULATOR FACTORS AS IMMUNORESPONSE MODULATOR IN PATIENTS SUFFERING FROM AUTOIMMUNE THYROID DISEASES

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In Autoimmune Thyroid Diseases (AITDs), a loss of tolerance to thyroid antigens and lymphocyte infiltration into the thyroid gland occurs. An imbalance in T helper type (Th)-17 and regulatory T lymphocytes (Tregs) contributes to perpetuate the inflammatory state leading to thyroid dysfunction and disruption. Although the replacement of hormone production represents the main aim in therapeutic treatment, a deeper understanding of the mechanism regulating the immune response improving inflammation could represent an important outcome in AITDs. In our previous study, we described an *in vitro* coculture model elucidating the ability of Th-1 cytokines licensed fibroblast-like Limbal mesenchymal Stem Cells (f-LSCs) to regulate T-cell activity in patients suffering from AITDs by an altered cytokine profile via downregulation of human heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) isoforms, which were found overexpressed in peripheral blood mononuclear cells (PBMCs) collected from patient suffering from AITDs<sup>1-2</sup>. Unfortunately, the clinical application of human mesenchymal stem cells (MSCs) is hampered by legal and ethical issues. Noteworthy, MSC immunomodulator capability depends also by soluble products, which could be conveyed by Small Extra Vesicles (sEVs), nanoscale cell-derived structure of 200 nm less in size. Data here reported demonstrate for the first time that a reconstituted lyophilized sEVs (sEV<sub>Ly0</sub>) derived TH-1 cytokines licensed f-LSCs retains the anti-inflammatory and immunomodulation capabilities in activated PBMCs from AITD patients, similar to f-LSCs. As the lack of comparative studies assessing the efficacy of different EV isolation techniques hinders clinical use of sEVs, we firstly compared two different sEV isolation methods tangential flow filtration (TFF) and precipitation. Once obtained, sEVs were lyophilized and characterized for size distribution, protein content, and purity by detection of CD63, CD9, CD81, specific sEVs surface markers. To explore the sEV anti-inflammatory and immune modulation properties, activated PBMCs from AITD patients were exposed to different concentrations of sEV<sub>Ly0</sub> and several functional biological assays were performed. We demonstrate that sEVs improve the autoreactive response in PBMC from AITD patients, inhibiting CD8<sup>+</sup>Tcell proliferation, CD69<sup>+</sup> and CD25<sup>+</sup> expansion within CD4<sup>+</sup>Tcells. Among several anti-inflammatory and immunosuppressive markers modulated, *i.e.* IDO, PDL-1, MCP-1 and IL-4, hnRNP A2/B1 were found downregulated in activated PBMC under sEV<sub>Ly0</sub> exposure. Finally, we propose that TH-1 licensing enriched sEV in specific beneficial factors, including PD-L1, COX-2, TXN-1. Indeed, we confidently promote the lyophilization as a valid storage method to satisfyingly preserve functional sEVs, encourage their emerging role as an alternative approach to stem cell therapy highlighting the possibility to modulate their protein content, once again suggesting hnRNP A2/B1 as potential target in AITDs.

*Acknowledgments: European Union (NextGeneration EU), through the MUR-PNRR project SAMOTHRACE (ECS00000022).*

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**MULTIOMIC PROFILING AND NEUROPROTECTIVE BIOACTIVITY OF *Salvia* HAIRY ROOT-DERIVED EXTRACELLULAR VESICLES IN A PARKINSON'S DISEASE MODEL**

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Extracellular vesicles (EVs) have become highly promising instruments in the realm of nanomedicine. The isolation of mammalian-derived EVs entails intricate procedures, and their therapeutic utilization introduces numerous safety and regulatory considerations. Recently, plants have emerged as unconventional reservoirs of therapeutically significant EVs. In this context, we have identified hairy roots (HRs) from medicinal plants as innovative biotechnological platforms for manufacturing EVs with the goal of improving human health. In this study, we present a comprehensive exploration of the purification, omics profiling, and bioactivity of EVs derived from HRs of medicinal plants, specifically *Salvia sclarea* and *S. dominica*. A detailed biophysical analysis of *S. sclarea* HR EVs has been conducted, along with the characterization of their proteome, revealing a distinctive molecular signature. Metabolomic analyses of HR EVs from both *S. sclarea* and *S. dominica* unveiled a conserved cargo of secondary metabolites, predominantly triterpenoids renowned for their antioxidant properties. Utilizing an *in vitro* model of Parkinson's disease involving SH-SY5Y cells treated with 6-hydroxydopamine (6-OHDA), we demonstrated the safety, cellular entry, and potent anti-apoptotic effects of HR EVs. Cell metabolomics showcased that EVs maintain metabolic homeostasis and alleviate cellular oxidative stress when co-administered with 6-OHDA. Mechanistically, HR EVs impede 6-OHDA autoxidation and significantly reduce the accumulation of its oxidative byproducts, mitigating the toxicity induced by 6-OHDA. In summary, our results offer compelling evidence that EVs derived from the hairy roots of *Salvia* species represent promising non-mammalian alternatives for innovative therapies in neurological disorders.

## NEUROSCIENCE

### INTRANASAL DELIVERY OF NGF RESCUES HEARING IMPAIRMENT IN AGED SAMP8 MICE

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Background: hearing loss impacts the quality of life and influences communication resulting in social isolation and reduced well-being. Although its impact on society and economy, no therapies for age-related hearing loss are available so far. Loss of mechanosensory hair cells of the cochlea is a common event of hearing loss in humans. Neurotrophins are growth factors involved in neuronal survival, development, differentiation, and plasticity. Nerve Growth Factor (NGF) has been implicated in the interaction between auditory receptors and efferent innervation in the cochlea during development. It has been reported that NGF is involved in the differentiation of auditory ganglion and hair cells. The main obstacle to the development of hearing impairment therapy is that effective methods of delivery for selected drugs to the cochlea are missing. Aim: thus, it has been suggested that NGF administration can reduce neuronal damage and prevent hearing loss. Herein, in this study NGF was administered by the intranasal route. Methods: the first part of the study was focused on a biodistribution study, which showed the effective delivery in the cochlea; while the second part was focused on investigating the potential therapeutic effect of NGF in senescence-accelerated prone strain 8 mice (SAMP8). Results: remarkably, intranasal administration of NGF resulted protective in counteracting hearing impairment in SAMP8 mice, ameliorating hearing performances (analyzed by auditory brainstem responses and distortion product otoacoustic emission) and hair cells morphology (analyzed by microscopy analysis). Conclusions: the results obtained were promising suggesting that the neurotrophin NGF was efficiently delivered to the inner ear and that it was effective in counteracting hearing loss.

Funding: this work has been supported by the European Union PON R&I 2014–2020 FSE-REACT EU- CUP D.M. 1062/2021 E15F21004290001 and by the European Union - NextGenerationEU under the Italian University and Research (MUR) National Innovation Ecosystem grant ECS00000041 - VITALITY - CUP E13C22001060006 and by Ministry of Enterprises and Made in Italy (MISE) - - D.M. 31 December 2021 and D.D. March 18, 2022- Project n. F/310074/02/X56 - CUP: B19J23000180005.

### 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<sup>1</sup>. DR initially was considered as a

microvascular complication of diabetes and classified as either nonproliferative or proliferative based on the presence of neovascularization<sup>2,3</sup>. 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<sup>4</sup>. 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<sup>5</sup>. Above all, *in vitro* and *in vivo* studies showed that NGF exerts a protective action also on retinal photoreceptors, Müller cell, and vascular pericyte<sup>6</sup>. 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.

Funding: this work has been supported by the European Union PON R&I 2014–2020 FSE-REACT EU- CUP D.M. 1062/2021 E15F21004290001 and by the European Union - NextGenerationEU under the Italian University and Research (MUR) National Innovation Ecosystem grant ECS00000041 - VITALITY - CUP E13C22001060006 and by Ministry of Enterprises and Made in Italy (MISE) - - D.M. 31 December 2021 and D.D. March 18, 2022- Project n. F/310074/02/X56 - CUP: B19J23000180005.

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### UNRAVELING THE “METABOLIC SYNDROME AND COGNITIVE IMPAIRMENT” DETRIMENTAL LOOP: WHAT ROLE FOR MEDITERRANEAN PHYTONUTRIENTS?

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Metabolic Syndrome (MetS) and obesity correlate with cognitive impairment, heightening the susceptibility to cognitive decline and dementia. Noteworthy, cognitive impairment exacerbates weight management challenges in MetS, giving rise to a "cognitive decline-MetS" loop. Indeed, various cognitive and affective functions, including memory and executive functions, have been found to be compromised. Our research delves into the nuanced realm of cognitive alterations in MetS by examining reliable biomarkers and the protective influence of specific Mediterranean Phytonutrients

(MediPhy) in a high-fat diet (HFD) rat model. We explored multi-faceted parameters unraveling behavioral, molecular, cognitive, and dysmetabolic alterations. The interventions carried out in our study, administering MediPhy from sources like "golden" tomato juice and *Opuntia ficus indica*, exhibit promise in modulating neural function, potentially due to concurrent amelioration of systemic dysmetabolism and redox homeostasis. Nutritional supplementation with MediPhy for one month demonstrates notable enhancements in behavioral reactivity, anxiety, anhedonia, and declarative memory in rats with cognitive alterations associated with MetS. Specifically, the behavioural reactivity was indeed improved by MediPhy in HFD groups as emerged in an open field maze and by burrowing test. Besides, rats consuming MediPhy had lower feeding time in a novel environment, they consumed more highly palatable saccharine and spent more time in the light zone of a light-dark box, all considered a measure of reduced anxiety and anhedonia. Furthermore, MediPhy consumption rescued the memory impairment determined by HFD in the recognition of novel objects. At the molecular level, MediPhy supplementation could counteract HFD-induced neuroinflammatory signaling pathways such as mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated kinase (Erk) and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) in regions like prefrontal cortex, hippocampus, and hypothalamus. Collectively, these findings emphasize the crucial role of functional food supplementation in ameliorating neuroinflammation and cognitive profile associated with MetS, tracing paths for developing effective prevention approaches.

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### EXPLORING THE ROLE OF OXIDATIVE STRESS IN PARKINSON'S DISEASE PATHOGENESIS: INSIGHTS FROM MICROGRAVITY STUDIES

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Parkinson's disease (PD) is a progressive neurodegenerative disorder primarily affecting individuals over 60 years old, with its prevalence expected to rise due to an aging population. The identification of alpha-synuclein (a-syn) aggregates, known as Lewy Bodies, in post-mortem brain tissue remains the definitive diagnosis for PD, highlighting a-syn as a promising biomarker and therapeutic target [1]. Oxidative stress is a significant contributor to the pathogenesis of PD, and can promote the aggregation of a-syn through multiple mechanisms including oxidative modification of the protein and impaired protein degradation systems. Furthermore, a-syn aggregation itself can exacerbate oxidative stress creating a vicious cycle where oxidative stress promotes a-syn aggregation, which in turn increases ROS production, further driving disease progression in PD [2,3]. Microgravity research offers valuable insights into various pathophysiological processes, and in this study, we utilized microgravity condi-

tions to accelerate the aging process and enhance the pathophysiological pattern of PD *in vitro* [4,5]. Particularly, we investigated the impact of microgravity conditions on oxidative stress levels in SH-SY5Y and PD mutated 3K-SNCA neuroblastoma cell models [6,7]. Our findings demonstrate that microgravity induces elevated levels of reactive oxygen species (ROS) and oxidative stress markers, including malondialdehyde (MDA), indicative of enhanced lipid peroxidation. Additionally, we observed fluctuating levels of antioxidant markers, suggesting a dynamic protective response to oxidative stress. These results highlight the role of oxidative stress in PD pathology and underscore the potential of microgravity conditions to accelerate and emulate PD-related oxidative stress mechanisms in cellular models.

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### ENDOCANNABINOID AND KISSPEPTIN SYSTEMS STIMULATION REDUCE ADULT NEUROGENESIS BY MODULATING ERK1/2 SIGNALLING PATHWAY

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The Endocannabinoid System (ECS) and the Kisspeptin System (KpS) are two important signalling systems involved in the central and peripheral control of reproduction along the hypothalamic-pituitary-gonadal axis [1]. Outside the hypothalamus, these systems are also expressed, and ECS has been characterized in hippocampus, one of the few brain regions where adult neurogenesis occurs [2]. However, the involvement of the ECS in neurogenesis and the role of the KpS, comprising kiss proteins (Kiss1) and their receptor (KissR), in neurogenesis remains largely unknown. To address this knowledge gap, we investigated the specific roles of these two systems *in vivo*. To this aim, male rats (n=4/group, PND38) were injected intraperitoneally (i.p.) with a single dose of BrdU. After one week, Kp-10 peptide, AEA

(anandamide), and the CB1 selective antagonist SR141716 were administered for an additional three weeks until rats' sacrifice. The brains were then removed and processed to obtain serial coronal sections from bregma -2.04 to -5.04 mm, following the Paxinos and Watson rat brain atlas [3]. Sections were subsequently processed for immunofluorescence (IF) and western blot (WB) analyses of the hippocampus and BrdU/NeuN positive cells were counted. Results showed that both Kp-10 and AEA treatments decreased adult neurogenesis and the AEA-induced effect was CB1 dependent since SR141716 was able to antagonize AEA action. Moreover, distribution and expression of the CB1 and Transient Receptor Potential Vanilloid 1 (TRPV1) - ECS receptors as well as kiss1 and kissR components of the KpS in the Cornus Ammonis 3 (CA3) and dentate gyrus (DG) of the hippocampus revealed a lack of significant variations in the expression of CB1 and KissR while indicating an increase of the TRPV1 expression. To gain deeper insights into these findings, we evaluated the expression of proteins essential for proliferation, differentiation and involved in synaptic plasticity such as ERK1/2, both Brain Derived Neurotrophic Factor (BDNF) and sirtuin 1 (SIRT1). The treatment of rats with Kp-10 and AEA caused a significant reduction of ERK1/2 phosphorylation together with an increase in BDNF and SIRT1 expression levels in rats treated with Kp-10, suggesting a possible role of the KpS in regulating neuronal differentiation and synaptic plasticity. Interestingly, WB analysis also showed that Kp-10 induced glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and estrogen receptor alpha (ER $\alpha$ ) proteins that behind their known role, are involved in neurogenesis, differentiation, and plasticity of hippocampal neurons. On the contrary, ECS stimulation did not show significant expression variations of the same proteins except for ERK1/2 activation. Collectively, the above-mentioned results suggest that ECS and KpS systems can reduce neurogenesis by inhibiting the ERK signalling pathway even though only the KpS may act as an inducer of neuronal differentiation. However, the two systems may cooperate both inducing TRPV1 receptor expression. Altogether these results provide some evidence for a new role of the KpS in hippocampal neurogenesis possibly involving an interplay with the ECS.

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#### SERUM NEUROFILAMENTS LIGHT CHAIN AS PROMISING BIOMARKER FOR DIAGNOSIS AND MONITORING OF ATTR DISEASE

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Neurofilament light chain (NfL) is a protein biomarker in body fluids that is unique to neurons, with elevated levels when neurons are damaged. NfL has emerged as a leading bio-

marker candidate relevant to therapy development for a host a broad range of neurological disorders due to its non-specific nature as a marker of axonal degeneration. Transthyretin variant amyloidosis (A-ATTRv) is a debilitating inherited disease caused by pathogenic variants in the transthyretin (TTR) gene that can be fatal without effective treatment. It is, which is inherited in an autosomal dominant pattern, and the most common TTR variant V30M (p.V50M) is associated with ATTR amyloidosis-related polyneuropathy (A-ATTRv-PN). In South Italy, according to our experience, only three mutations are found with an endemic distribution (Glu89Gln, Phe64Leu, Thr49Ala), while Val30Met mutation is absent. Generally, diagnosis of the disease is primarily based on clinical data, neurophysiological test, and genetic analysis to confirm the presence of pathogenic TTR variants. Valid biochemical biomarkers that reflect the pathophysiology of the disease and highly specific could be useful for early diagnosis, monitoring of disease progression and response to treatment. Aim of the present study was primarily focus on the use of serum biomarkers of peripheral neuropathy for early diagnosis and clinical management in patients with polyneuropathy associated with different TTR variants. We carried out a single centre study on 28 ATTRv patients and 7 carriers in the predicted age of disease onset (PADO). Serum samples were collected at time 0 after six month and after one year from both healthy control and presymptomatic TTR mutation carriers and serum levels of total tau and NfLs were analysed. Disease severity was assessed with polyneuropathy disability score, renal and cardiological function were also monitored. All patients were treated with patisiran, inotersen or tafamidis. Results obtained evidenced that the most frequent variants in our cohort were p.Phe84Leu and p.Glu109Gln. We used the SiMoA technique to measure serum content of total tau protein and neurofilament. Our data evidenced that NfLs was elevated in ATTR patients and correlated with disease severity, moreover NfLs discriminated asymptomatic mutation carriers from early symptomatic mutations. Taken together, our results strongly suggest that serum NFLs are reliable biomarkers for the diagnosis and monitoring of ATTR and to establish ATTR disease conversion and progression.

#### IRISIN ATTENUATES NEUROINFLAMMATION TARGETING NLRP3 INFLAMMASOME

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In neurodegenerative diseases, neuroinflammation plays a dominant role. Activated microglial cells are the major source of pro-inflammatory factors and cytokines, which are closely associated with disease progression. Microglia can be activated by lots of invading pathogens and endogenous danger molecules. The NLRP3 inflammasome is highly expressed in

activated microglia. The NLRP3 inflammasome consists of the Nod-like receptor protein NLRP3, the adaptor protein ASC and pro-caspase-1. Once activated, it leads to the autocatalytic cleavage of caspase-1. This ultimately promotes the maturation and release of IL-1 $\beta$ . Activation of the NLRP3 inflammasome also plays a key role in microglia-mediated neuroinflammation. Irisin is a small myokine derived from the proteolytic processing of the transmembrane precursor fibronectin type III domain-containing protein 5 (FNDC5). Importantly, the expression of irisin and its precursor protein FNDC5 are increased in muscle in response to many forms of exercise. In addition, irisin is expressed skeletal muscle and in selected brain regions, including the hippocampus and frontal cortex, is able to cross. Irisin crosses the blood-brain barrier and exerts a neuroprotective effect. In this study, we investigated the effects of irisin on the NLRP3 inflammasome pathway using LPS-treated BV2 cells as a cellular model of neuroinflammation. We found that irisin alleviated the inflammatory responses and improved neuroprotection. In addition, the anti-inflammatory M2 phenotype appeared to dominate over the pro-inflammatory M1 phenotype and microglial reactivity was reduced in response to irisin treatment.

#### DISSECTING THE NEURONAL FEATURES OF PARKINSON'S DISEASE

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Parkinson's disease (PD) stands as the second most prevalent neurodegenerative disorder, characterized by the progressive loss of motor function attributed to a depletion of dopaminergic neurons in the substantia nigra pars compacta. The disease's trajectory is marked by the accumulation of alpha-synuclein insoluble aggregates, lysosomal impairment, and oxidative stress. Despite the absence of a definitive mechanism for PD pathogenesis, aging remains the primary risk factor. Notably, mutations in the lipid hydrolyase gene GBA1 emerge as a primary genetic risk factor, conferring a 15% increased risk compared to the general population. Such mutations result in enzyme loss of function, culminating in the accumulation of lipid substrates. However, GBA1 depletion is discernible even in PD patients lacking genetic mutations, strengthening the association between lipid accumulation and PD development. This study extensively characterizes the functional, morphological, and lipidomic features in dopaminergic neurons derived from PD patients' induced pluripotent stem cells (iPSCs), comparing those with and without GBA1 mutations. The findings unveil a distinctive lipidome signature in dopaminergic neurons with GBA1 mutations, concomitant with a noteworthy accumulation of alpha-synuclein aggregates and phosphorylated forms of synuclein. Moreover, the conducted functional analysis exposes that lipid and alpha-synuclein accumulation precipitates functional defects, evidenced by impaired calcium signaling and neuronal network dysfunction. These revelations mark a pioneering report on the effects of GBA1 mutations at omic and functional levels, paving the way for the identification of a multi-targeted pharmacological approach to PD. The comprehensive insights provided by this study offer a significant basis toward understanding the intricate interplay of genetic and molecular factors contributing to Parkinson's disease.

## NUTRITION

**HOW GOLDEN TOMATO JUICE SUPPLEMENTATION AFFECTS LEPTIN LEVELS AND OXI-INFLAMMATION IN RATS WITH METABOLIC SYNDROME**

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Chronic inflammation is an adaptive response triggered when other homeostatic mechanisms fail. Leptin is a pathophysiological regulator of inflammation that also plays a key role in energy homeostasis. Excessive production of reactive oxygen species has been shown to be responsible for the initiation, progression, and resolution phases of inflammatory processes. Based on previous evidence on the association between metabolic syndrome (MS) and biomarkers of redox homeostasis, and on the ameliorative effects of golden tomato on metabolic and anti-inflammatory status in MS-induced high-fat diet (HFD) rats. (Di Majo *et al.* 2023; Gambino *et al.* 2023). The aim of this work was to evaluate whether a daily intake of 2 mL of golden tomato juice can counteract oxidative stress and improve inflammatory status in an HFD rat model of metabolic syndrome. The Golden Tomato is made from a mixture of tomatoes that would normally be rejected at harvest because they are not fully ripe. The juice obtained from this mixture is rich in phytonutrients, which have significant antioxidant properties. The study showed that eight weeks of HFD in rats produced a metabolic syndrome condition characterised by low levels of inflammation, similar to that seen in humans. Supplementation with Golden Tomato Juice for a further five weeks improved metabolic status, reducing plasma triglyceride and cholesterol levels while improving HDL levels and glucose tolerance. In terms of energy homeostasis, it is also able to reduce body weight and levels of leptin, a pro-inflammatory adipokine. In this context, a regulatory effect on systemic oxidative stress has been observed, explained by the reduction of reactive oxygen and nitrogen species, hydroperoxyl and lipoperoxide radicals. This effect is also reflected in the reduction of oxidation processes at the expense of thiol groups and the improvement of the plasma antioxidant barrier as assessed by the anti-ROM assay. This work highlights the antioxidant and anti-inflammatory role of daily consumption of golden tomato juice, opening up new perspectives on its use for preventive and integrative purposes in the treatment of inflammatory disorders.

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**FROM THE ANALYSIS OF THE HAIR BULB TO THE RESTORATION OF THE IDEAL CONDITIONS OF WELL-BEING OF THE ORGANISM**

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In this work we want to explore the close correlation between the analysis of the follicular matrix of the hair bulb with the measurement of the deficits of the main micronutrients (or excess of heavy metals) whose restoration allows the recovery of the ideal conditions of well-being of the organism. The reference lies in the specificity of the microvesicles of the bulb as biomarkers of modified cell functionality. The Bio Molecular Test (BMT) system has undergone a series of evolutions that allow for an in-depth analysis of the presence-absence of micronutrients and heavy metals. Numerous measurements of the cells composing the cap of the hair bulb were carried out using spectromicroscopy; in this way it was possible to precisely define the presence marker for a series of micronutrients. Similarly was done for heavy metals considered harmful to human health. Thanks to the RAMAN systems it was also possible to weigh the quantity of each micronutrient and heavy metal observed. The BMT system has been enriched with a series of algorithms specifically developed to obtain an expected value of micronutrients and heavy metals. The expected values were placed, using a multivariate analysis method, in contrast with the values measured by spectromicroscopic investigations. The results of the comparisons were translated into deficiencies or excesses and, consequently, the components and dosages of the supplements to be offered to the customer were identified. This algorithm represents a real paradigm shift capable of quantitatively measuring the distance between the expected and the real value of each individual micronutrient. For the first time we can really talk about a personalized food supplement integration solution "tailored" to the each single subject.

**IN VITRO EVALUATION OF THE POTENTIAL SYNERGISTIC EFFECTS OF COLLAGEN AND HYALURONIC ACID (TendoGenIAL™) ON HUMAN TENDON-DERIVED CELLS AND MACROPHAGES**

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*In vitro* preclinical studies showed how high and low molecular weight hyaluronic acids (HMWHA and LMWHA, respectively) contribute to tendon healing thanks to their mechanical support and anti-inflammatory properties. Considering the role of collagen in tendon and extracellular matrix composition, this study examines whether a potential synergistic effect of collagen and hyaluronic acids might be exploited on human tendon-derived cells to disclose a usefulness in the treatment of tendinopathies. The *in vitro*

ratio collagen/HAs was developed starting from the one contained on a commercial food supplement named TendoGenIAL™. The anti-inflammatory activity was evaluated on both human LPS-stimulated macrophages and human tenocytes conditioned with the LPS-stimulated macrophages-derived medium whereas the effects on cell proliferation were monitored on tenocytes under basal conditions. Levels of expression of proteins involved in redox homeostasis and inflammation, such as Nrf2, NF- $\kappa$ B and COX-2, were monitored in parallel with ROS and RNS generation. The modulation of markers related to the extracellular matrix (ECM) remodeling such as collagen type I was investigated to elicit the regenerative pathway. Moreover, the morphology of tenocytes and their organelles was observed through transmission electron microscopy (TEM), disclosing an increased vesiculation. Our data confirm the anti-inflammatory and antioxidant activity of both hyaluronic acids and collagen, highlighting a synergistic effect. Similarly, the association promotes tenocytes' proliferation and thus ECM remodeling.

#### CHEMICAL COMPOSITION AND BIOCHEMICAL CHARACTERIZATION OF COFFEE SILVER SKIN EXTRACTS OBTAINED BY GREEN TECHNOLOGY

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The silver skin of coffee (CSS) is one of the by-products of the industrial processing of coffee. It is a highly available waste product and more stable due to its low water content. It is known that the bioactive compounds in silver skin have potential protective action on the skin, counteracting skin aging and inflammation. Skin inflammation can result from the deleterious effects of extrinsic factors, such as overexposure to UV solar radiation (mainly UVB), which over time lead to pathological conditions such as erythema, edema, hyperpigmentation, premature skin aging, and cancer. UV radiation induces an overproduction of ROS, resulting in oxidative stress; for this reason, the use of compounds with antioxidant effects could be a promising approach to inhibit skin damage induced by their irradiation. Therefore, this study aimed to characterize qualitatively and quantitatively an extract of CSS and its purified fractions, obtained through green technologies, and to evaluate their potential dermo-protective effects against a UV-induced skin cellular model, represented by a human keratinocyte cell line, HACAT. Overall, our results indicate that CSS, particularly its melanoidin-rich fraction, exerts protective and antioxidant effects, suggesting a new possible way to prevent or, at least, attenuate skin damage induced by UV.

*Funder: Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 - Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU.*

*Award Number: Project code CN\_00000033, Concession*

*Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP: D43C22001260001, Project title "National Biodiversity Future Center - NBFC".*

#### NUTRITIONAL INTERVENTION WITH LETTUCE FUNCTIONALIZED BY SEAWEED EXTRACT TO PREVENTING MINERALS DEFICIENCY IN HEALTHY POPULATION

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Minerals are primarily obtained through diet and have essential metabolic biological functions for human health. Globally, about 2 billion people suffer from mineral deficiency that severely affects physiological homeostasis, triggering alterations at the immune level, impairing the function of the nervous, endocrine and bone systems. Nowadays, large quantities of fertilizers and pesticides are administered to vegetables, like lettuce, to meet market demands. Fertilizers and pesticides are extremely toxic, even in small traces, to human health and the ecology of agricultural systems. In recent decades, *Ecklonia* species have received enormous attention for their wide range of therapeutic properties and multiple health benefits, such as great nutritional value and richness in vitamins and minerals. Biostimulants using algae extracts (SwE), by increasing the concentration of minerals in plants, are new tools used in agronomic practice to stimulate plant growth in a natural and environmentally friendly way. Because of these numerous health benefits, they have been a focal point for researching nutraceutical potential. The present study evaluated, in healthy volunteers, whether supplementation for 4 weeks with SwE lettuce affects endogenous mineral levels and the potential metabolic pathways involved. A cohort of 48 volunteer subjects was recruited and double-blind divided into experimental groups that consumed 100 grams a day of control or SwE lettuce or one tablet of iron for 4 weeks. It was examined and compared among the groups blood tests at baseline (t0) and after 4 weeks (t2) for differences in serum mineral concentrations (sodium, calcium, potassium iron, magnesium, phosphorus) and iron, glucose and bone homeostasis. The results showed that consumption of SwE lettuce after 4 weeks of treatment did not affect blood sodium, calcium, potassium, magnesium and phosphorus concentration but significantly enhanced iron serum levels. The nutritional intervention with SwE lettuce improved iron homeostasis by increasing transferrin saturation but did not affect proteins of iron metabolism (ferritin, transferrin). The consumption of SwE lettuce did not affect glucose (fasting glucose, insulin, insulin resistance,  $\beta$ -cell function, and insulin sensitivity) and bone (osteocalcin, CTX, PTH, calcitonin) homeostasis. Supplements of iron in tablets, for four weeks, did not affect

glucose and bone metabolism but increased serum iron and transferrin saturation similar to SwE lettuce group. Side effects like diarrhea or constipation were reported by the iron tablet group. The study suggests that the intake of SwE lettuce is safe and could be a suitable tool to counteract mineral deficiency. It shows another approach to devel-

op functional food varieties in an environmentally sustainable and cost-effective way.

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## ONCOLOGY

**A PROSPECTIVE STUDY ON GLIOBLASTOMA MULTIFORME: FOCUS ON THE ROLE OF THE CCT COMPLEX SUBUNIT 5**

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The human chaperonin CCT or TRiC consists of eight non-identical subunits, and its protein-folding activity is critical for cellular health (1). The CCT chaperonin is emerging as a key molecule in cell division thanks to its essential role in the folding of several proteins involved in the cell cycle suggesting it could have a crucial role in uncontrolled proliferation (2). Several studies correlate the role of the complex and its subunits in the pathogenesis of glioblastoma multiforme (GBM) (3). GBM is the most diffused and malignant type of primary astrocytoma and represents more than 60% of all brain tumors in adults. Tumor heterogeneity, influenced by epigenetics and metabolism, is its distinctive hallmark. In this work, we focused on the role of subunit number 5 of the CCT complex (CCT5), as it has been shown upregulated in different types of cancers, including breast cancer, colon cancer, lung cancer, hepatocellular carcinoma and ovarian cancer (4). Furthermore, the CCT subunits, including the CCT5, have been found in GBM-derived extracellular vesicles (3). It has also been demonstrated that CCT subunits can translocate into the nucleus in a leukemia cell line and other interaction proteins of CCT, involved in processes such as protein folding, cellular metabolism, RNA processing and apoptosis have been identified in the nucleus (5). First, an *in silico* analysis was conducted for the CCT5 subunit using the bioinformatic portal cBioportal, which uses the cohort of patients diagnosed with GBM from The Cancer Genome Atlas (TCGA-GBM) (6). The CCT5 mutations identified in this cohort of patients were analyzed, as well as their significance and the proteins most expressed in the two groups, with and without CCT5 mutations; genes with increased co-expression with CCT5 and survival curves were also analyzed. It was seen that the most closely related proteins were involved in the cell cycle and proliferation. An *in vitro* analysis was also conducted in the T98G and G166 glioblastoma cell lines, whose second possesses stem-like characteristics. The subcellular localization of the CCT5 subunit in these two lines was analyzed using immunofluorescence techniques. Western blotting techniques were performed after compartmentalized nuclei-cytoplasm protein extraction. The HDF (human dermal fibroblasts) cell line was used as a non-tumor control. This analysis highlighted the nuclear localization of the CCT5 subunit. Considering these results, we hypothesize that subunit 5 translocating into the nucleus may act as a transcriptional regulator for genes involved in cell proliferation. Consequently, it may have a significant role in tumorigenesis and could therefore be considered a possible therapeutic target.

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**THE LNCRNA H19 MODULATES ALTERNATIVE SPLICING OF GTPASE-RAC1 IN COLORECTAL CANCER CELLS**

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Alternative splicing (AS) is a fundamental process by which the same gene can generate multiple distinct mRNA transcripts to increase protein diversity. Aberrant alternative splicing (AS) events are considered one of the major factors influencing the occurrence and development of various diseases. Moreover, AS plays a critical role in several aspects of cancer biology, such as tumor invasion, metastasis, epithelial-mesenchymal transition (EMT), and drug resistance. Recent studies have shown that AS is a key feature for transcriptomic variations in colorectal cancer (CRC) which ranks third among malignant tumors worldwide in both incidence and mortality. Long non-coding RNAs (lncRNAs) can modulate AS by acting as trans-regulatory agents, recruiting splicing factors, or driving them to specific targeted genes. lncH19 is an ncRNA dis-regulated in a variety of tumors and listed among the oncogenes. In CRC, it plays a critical role in tumor onset, progression, and metastasis. It is known that lncH19 can affect cancer development by working as a sponge for miRNAs, as miRNAs precursor or epigenetic modulator. Our data represent the first evidence of new mechanisms of action by which lncH19 prompts CRC through the modulation of AS. Analysis of RNA sequencing from lncH19 antisense precipitation has revealed that in CRC cells, the long non-coding RNA is linked to immature RNAs. Furthermore, Western Blot analyses have indicated that H19 binds with the splicing factors hnrRNPM and RBFOX2. The latter has been widely documented as the mediator of a pro-tumoral alternative splicing signature. Through bioinformatic analysis, we identified 53 transcripts associated with lncH19 and containing binding sites for both SFs, hnrRNPM, and RBFOX2. Among these transcripts, we identified the mRNA of the GTPase-RAC1, whose alternatively spliced isoform RAC1B has been ascribed several roles in the malignant transformation. Firstly, by RNA Immuno Precipitation (RIP) we confirmed the binding of the splicing factors to both the transcripts RAC1 and lncH19. Analyses from patients with colorectal cancer (CRC) showed higher levels of both lncH19 and RAC1B in tumoral biopsies compared to healthy controls. These findings support the hypothesis that lncH19 may enhance RAC1B expression. This hypothesis was confirmed by *in vitro* studies of gain and loss expression of the lncH19 in two CRC cell lines (SW620 and HCT-116). Our data showed that lncH19 over-expression can induce c-Myc and Cyclin-D expression by upregulating RAC1B while lncH19 silencing impedes the expres-

sion of RAC1B without altering RAC1. Finally, we demonstrated that IncH19 drives the SFs, RBFOX2, and HNRNPM, to RAC1 to allow AS and RAC1B expression. These data revealed a new mechanism of action by which the IncH19 carries out its functions as an oncogene.

### MIMICKING GLIOBLASTOMA MULTIFORME MICROANATOMICAL ARCHITECTURE VIA PATIENT-DERIVED 3D SPHEROIDS

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Glioblastoma multiforme (GBM) represents one of the most malignant forms of central nervous system tumors [1]. Despite advances in treatment modalities, prognosis remains poor, due to the remarkable resistance to therapies attributed to molecular intertumoral and intratumoral heterogeneity within GBM [2]. Consequently, to foster the development of more effective antitumor therapies, it is necessary to develop novel experimental models able to mimic the *in vivo* spatial and molecular organization of GBM, to better understand its biological complexity. Among *in vitro* models, 3D spheroids mimic the morphological and molecular complexity and heterogeneity of the tumor, as they recapitulate the cell-cell and cell-extracellular matrix interactions present *in vivo*. Patient-derived 3D spheroids are increasingly considered more physiologically relevant [3]. In this study, our focus lies on regional intratumoral differences and on the potential use of patient-derived 3D spheroids as model able to recapitulate the GBM niches. In GBM patient specimens, we assessed the architecture of vascular niche characterized by pronounced angiogenesis, accompanied by increased VEGF (vascular endothelial growth factor) expression level. In addition, we assessed the morphology of the hypoxic niche that contributes to tumor growth and resistance, wherein HIF (hypoxia-inducible factor) contributes to the upregulation of VEGF and supports cell proliferation in hyperproliferative and invasive niche, overexpressing PCNA (proliferating cell nuclear antigen) and c-KIT (tyrosine-protein kinase KIT). Subsequently, primary cell lines were isolated from GBM tumor tissues and characterized for morphological, phenotypical and growth features. To investigate the spatial organization and cellular heterogeneity of GBM patient-derived 3D spheroids were generated, particularly focusing on the c-KIT pathway. C-kit, also known as stem cell factor receptor (SCF), is a proto-oncogene implicated in both normal growth and development of neoplastic processes, frequently overexpressed and amplified in gliomas. Uncontrolled activity of the SCF/c-KIT pathway by glioma cells can activate brain microvascular endothelial cells, supporting proliferation, angiogenesis, stemness, and metastasis [4]. In summary, we have developed a 3D culture model

that faithfully mimics the *in vivo* architecture of GBM, providing a valuable tool for future mechanistic studies aimed at elucidating the effects of anti-angiogenic therapies targeting the c-KIT pathway.

*Acknowledgments: finanziato dall'Unione europea – Next Generation EU” componente M4C2, investimento 1.1, “Fondo per il Programma Nazionale di Ricerca e Progetti di Rilevante Interesse Nazionale (PRIN)” – CUP: B53D23032960001, Implementation of a patient-derived 3D model of glioblastoma multiforme for the developing of extracellular vesicles-based drug delivery system (EXANDROID-EXtracellular vesicles pAtieNt-DeRived 3d gbm mOdel drug dellvery Developing).*

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### APTAMER-BASED THERAPEUTIC TARGETING FOR GLIOBLASTOMA

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The rate of primary malignant brain tumors stands at around 7 cases per 100,000 people. Glioblastoma (GBM) represents the most prevalent and lethal form of malignant brain tumor, with a 5-years survival rate of only 7 percent. The heterogeneity of tumor cells and tumor microenvironment (TME) are well described and remain the main obstacle in finding an efficient cure. Another challenge is the difficulty of drug-delivery across the blood-brain barrier. Consequently, it is essential to identify new therapeutic targets and develop more efficient therapeutic tools to improve the probability of successful therapy. Aptamers are short oligonucleotide sequences, are selective and specific to their biological target upon folding into unique 3D structures. Their easily modifiable nature makes aptamers a good choice for the conjugation of pharmacologically active molecules, acting as a transport vehicles in the brain to target the TME of GBM. Literature shows that nucleolin expressed on the surface of GBM cells is a potential tool for drug targeting therapy. The aptamer AS1411 forms G-quadruplex, binds specifically and with high affinity to this protein and is internalized [1]; at a concentration of 5  $\mu$ M, it has been shown to induce apoptosis and cell cycle arrest of glioma cells by up-regulation of p53 and down-regulation of Bcl-2 and Akt1. Another suitable target is EGFRvIII: Unlike EGFR, its mutated form, EGFRvIII, is constantly active and can signal independently of ligand binding, leading to persistent activation of downstream signalling pathways, cell growth, survival, and proliferation. This mutation is found in approximately 30% of GBM cases. U2 is an aptamer targeting the mutated epidermal growth factor EGFRvIII and has been seen to inhibit proliferation, migration, invasion and downstream signalling of cells, demonstrating that it is an excellent candidate for GBM therapy [2]. Two aptamers can be combined, generating bifunctional molecules, to overcome renal expulsion and prove the potential for specific binding to multiple targets. In this work, aptamers AS1411 and U2 were conjugated, using Poly-A and Poly T as linkers. Linear linkers were chosen so that

they could not interfere with the G-quadruplex formation of AS1411. We generated four chimeras: Hybrid3 (10A), Hybrid4 (18A), Hybrid5 (10T), Hybrid6 (18T). Cytotoxicity studies were performed to compare the cytotoxic effect of chimeras *versus* individual aptamers. Different cell lines were used: a stabilised human glioblastoma cell line, U87 MG (ATCC HTB-14), expressing nucleolin on the surface; a rat glioma cell line transfected with EGFRvIII expressed on the cell surface, F98 np EGFRvIII (ATCC CRL-2949); F98 wild type (ATCC CRL-2397) as control; U87 np EGFRvIII, expressing both nucleolin and EGFRvIII. All cell lines were exposed to different concentrations of AS1411, U2, Hybrid3, Hybrid4, Hybrid5, Hybrid6, at different times, from 48 to 96 hours. It was found that the most effective and specific chimera is hybrid5, which had an estimated IC<sub>50</sub> of 10  $\mu$ M. Cell confluence experiments were performed on all cell lines and cell shrinkage and rounding were shown. Once these preliminary studies are completed, DM4, Maytansinoid Ravtansine, a potent chemotherapeutic agent [3], will be conjugated to the most effective chimera to further increase its cytotoxicity.

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#### MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF BREAST CANCER ORGANIDS AFTER TREATMENT WITH MITOCHONDRIAL SIRTUINS MODULATORS

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Breast cancer has different subtypes. One subtype is triple-negative breast cancer, which is difficult to treat with targeted therapies and often requires chemotherapy and radiotherapy. A cellular model called MDA-MB-231 is generally used to study this subtype of breast cancer. Sirtuins are enzymes that have a role in cell stress, cellular metabolism, inflammation, and cancer biology. In this study, we investigate how mitochondrial sirtuins affect the growth, metabolism, and structure of MDA-MB-231 spheroids. In order to determine the most effective treatment with sirtuins modulators, viability and clonogenicity assays were conducted on 2D MDA-MB-231 cell cultures. Based on the results obtained, we have chosen to employ a sirtuin 5 activator (MC3238), a sirtuin 3 inhibitor (3-TYP), and the combination of the two for the experiments on MDA-MB-231 spheroids. Molecular and morphological features of untreated and treated spheroids were analyzed by immunofluorescence and OM/TEM (Transmission Electron Microscopy). MDA-MB-231 spheroids were grown for 7 days and then treated for 48h. After 48h, treated spheroids were significantly smaller than controls. This time point was considered as the baseline to carry out the ultrastructural analysis of MDA-MB-231 spheroids in the presence of the following treatments: DMSO for the control, MC3138, 3-TYP and MC3138 plus 3-TYP. Control MDA-

MB-231 spheroids exhibited a compact mass of cells, with varying spherical to oval shapes, approximately 10  $\mu$ m in cell diameter. Ultrastructural examination revealed variable nuclear shapes, one or more nucleoli, well-defined cellular membranes, and tight junctions indicative of cell adhesion. Mitochondria and endoplasmic reticulum appeared normal. Spheroids treated with the SIRT5 activator MC3138 displayed morphology similar to controls. The notable difference was the presence of osmiophilic lipid droplets both inside, outside the cells and during secretion. Recently, a role has been associated with mitochondrial sirtuins in the control of autophagy and mitophagy. Therefore, we performed immunofluorescence analyses on MDA-MB-231 spheroids treated with DMSO and MC3138 for 48 hours, which showed reduced autophagy and mitophagy in MC3138-treated spheroids compared to the control group. Spheroids treated with SIRT3 inhibitor 3-TYP exhibit a structure similar to the control. We also observed abundant osmiophilic droplets inside and outside the cell. More evident compared to the control and MC3138 treated group is the difference between the outermost areas of the spheroid, with a well-organized structure, as compared to the internal areas, where there are various necrotic areas. The combination of SIRT5 activator MC3138 with SIRT3 inhibitor 3-TYP induced heightened cellular stress, with mitochondrial alterations, endoplasmic reticulum stress, cell membrane rupture, and necrosis. Osmiophilic lipid droplets were also evident both inside and outside the cells. Our results suggest that mitochondrial sirtuins play an important role in the structure and differentiation of triple negative breast cancer cells, increasing autophagy and mitophagy. However, more experiments will be performed to better understand the nature of lipid droplets and the role of SIRT3 and SIRT5 on malignant progression and differentiation.

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#### SELECTIVE HDAC6 AND PROTEASOME INHIBITION 1 IN COLON CANCER CELLS: THE EFFECTS ON LIPID METABOLISM

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Metabolic reprogramming is a hallmark of cancer and tumor cells highly consume fatty acids to sustain uncontrolled proliferation. To reverse this situation, lipid accumulation and consequent lipotoxicity may be promoted using specific compounds. Histone deacetylase 6, (HDAC6) has been found overexpressed in colon cancer (1) and related with reduced lipogenesis and increased lipid catabolism (2). Therefore, selective HDAC6 inhibition may provide a rationale to target lipid metabolic reprogramming and favour lipid-dependent cell death. Here, the selective HDAC6 inhibitor ITF3756 was used and combined with the proteasome inhibitor Bortezomib (BTZ) in HCT116 colon cancer cells. BTZ, inhibiting the 26S proteasome, has been also shown to promote lipogenesis (3). The results showed that subtoxic con-

centrations of ITF3756 and BTZ markedly reduced HCT116 cell viability when used in combination. The two compounds together also promoted lipid accumulation as evidenced by red-oil staining. These effects were associated with a marked decrease in the levels of the full length SREBP protein and increase in PPAR $\gamma$ , both events correlated with lipogenesis. Ongoing studies aim to clarify the molecular mechanism of cell death induced by the combination ITF3756 and BTZ and the extent of lipid involvement in these events. Overall, these preliminary results suggest that inhibiting HDAC6 and the proteasome may represent a key strategy to target colon cancer unbalancing lipid metabolism.

*Acknowledgements: we thank ITALFARMACO S.P.A for providing ITF3756.*

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#### CYTOTOXIC EFFECT OF A NOVEL METAL SCHIFF BASE COMPLEX ON HEPG2 HUMAN TUMOR CELLS

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Metal-based compounds show numerous applications in the field of medicine and among these their anticancer effects stand out. Most of the latter are likely due to their binding with DNA, even in the form of non-canonical folding, such as guanine-quadruplexes<sup>1,2</sup>. In this scenario, three Ni(II), Cu(II), Zn(II) complexes of a salphen-like N4-donor ligand were synthesized and their potential cytotoxic effects on HepG2 human hepatocarcinoma cells investigated. First, interesting data emerged from cell viability studies which showed that among the compounds, only the Cu(II) complex exerted a markedly dose-dependent cytotoxic effect at 24 h vs. control. Moreover, no significant viability-restraining effect was exerted by either the sole scaffold at all the concentrations tested, or Cu(II) acetate except for the maximum concentration assayed (100  $\mu$ M). The IC<sub>50</sub> value at 24 h of the Cu(II) complex was determined and the IC<sub>50</sub>-treated cells were submitted to a panel of analyses aimed to the evaluation of the biological aspects of cytotoxicity, as reported<sup>3,4</sup>. First, staining with propidium iodide for cell cycle analysis showed the accumulation of treated HepG2 cells in the sub-G<sub>0</sub>G<sub>1</sub> fraction without perturbations of the morphological appearance and cell cycle profile, thereby suggesting the occurrence of a higher level of DNA fragmentation, which is associated with cell death. Staining with annexin V-FITC and PI indicated that after exposure the percentage of the viable annexin V-/PI- cells decreased from about 88% of the controls to about 59% and, on the other hand, the percentage of the apoptotic annexin V+/PI+ cells increased from about 6% of the controls to about 35%. The activity of the caspase proteases possibly involved in the cytotoxic effect was tested and the results obtained showed the activation of caspase-3, executor of classical apoptosis, and -5, an inflammatory caspase known to interact with caspase-3

and induce pyroptotic cell death<sup>5</sup>. Interestingly, the increase of the proteolytic cleavage of GSDME protein, a key event in pyroptosis activation, was detected through Western blot. Further, we investigated whether cell exposure could impair the mitochondrial function and cell redox status, demonstrating that the Cu(II) complex induced the dissipation of the mitochondrial membrane potential and the up-regulation of ROS. The preliminary data obtained represent a valuable starting point for the study of the biological mechanisms underlying the potential beneficial effect of the Cu(II) complex on liver tumor cells, so far tested only *in vitro*, and further studies will be necessary to better define the intracellular pathways targeted by this cytotoxic molecule.

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#### NOVEL ULTRASTRUCTURAL INSIGHTS INTO CLEAR CELL CARCINOMA OF THE PANCREAS: A CASE REPORT

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Clear cell primary adenocarcinoma of the pancreas (CCCP) is a rare histological subtype of pancreatic ductal adenocarcinoma (PDAC), still poorly characterized (1, 2). We reported a case of CCCP in a 65-year-old male with the aim to dissect the complexity of this morphological variant by means of molecular and ultrastructural investigations. Molecular genetic testing did not detect any mutational variants for BRCA1 and BRCA2. Immunohistochemical staining with Mucicarmin and Alcian-Pas demonstrated that the clear-cell foamy appearance was not due to a hyperproduction of mucins. Ultrastructural characterization revealed the massive presence of mitochondria in the clear cell cytoplasm. Mitochondria showed disrupted cristae and various degrees of impairment of structural integrity. To evaluate the functional state of mitochondria, immunohistochemistry staining for NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 4-like 2 (NDUFA4L2), a subunit of Complex I of the mitochondrial respiratory chain which transfers electrons from NADH to ubiquinone, was carried out. Interestingly, while the clear cells were NDUFA4L2 negative, ductal adenocarcinoma areas proved positive to the stain, suggesting a different functional state of mitochondria. Thus, our ultrastructural

and molecular data indicate that the clear cell “foamy” appearance of CCCP is specifically due to the accumulation of disrupted mitochondria.

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### INHIBITION OF PDE4D PREVENTS MIGRATION OF HCC CELLS THROUGH MODULATION OF IGF2/H19 CLUSTER

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Cyclic nucleotide phosphodiesterases (PDEs) play major roles in several different signalling pathways and thus control key cellular events, including cell proliferation, differentiation, and survival. Among PDEs, PDE4 is abundantly expressed in liver and appears deregulated in many tumours. The PDE4 family is encoded by four genes, namely PDE4A, PDE4B, PDE4C and PDE4D. Our previous studies showed that PDE4D is a major regulator of cAMP expression in hepatocarcinoma (HCC) cells and tissues. Its silencing or inhibition reduced cell proliferation and increased apoptosis of HCC cell lines by interfering with the expression of key cell cycle effectors. In addition, PDE4D silencing, or inhibition, affected the expression of several cancer-related genes, with a significant down regulation of the pro-oncogenic insulin growth factor 2 (IGF2), an imprinted gene whose transcription is regulated in a cluster with the region encoding for the H19 long non-coding RNA deeply involved in HCC proliferation, migration and invasion. Specific objectives of this research were to investigate the possible correlation between PDE4D overexpression, epithelial-mesenchymal transition (EMT) and uncontrolled cell migration, as well as to verify whether pharmacological inhibition of PDE4D can reverse EMT by acting on the IGF2/H19 cluster. The correlation between PDE4D and IGF2 expression patterns were confirmed by the CancerLivER database. Low tumorigenic (HepG2) and highly tumorigenic (Hep3B and Huh7) cell lines were used as HCC *in vitro* models. PDE4D activity was selectively inhibited using Gebr-7b. Western blotting experiments were performed to analyze the expression of proteins involved in epithelial-mesenchymal transition and cell migration, while qRT-PCR was employed to analyse the expression of IGF2 and H19 mRNA. Finally, Real-Time cell migration was evaluated using the IncuCyte video microscopy system in cell treated and non-treated with Gebr 7b. Selective pharmacological inhibition of PDE4D, using Gebr-7b, induced an upregulation of the epithelial marker E-cadherin and a down-regulation of the mesenchymal markers Twist and Snail. Gebr-7b treatment also significantly reduced HCC cell migration and induced upregulation of H19 gene expression, thus reducing the expression of IGF2 protein. We hypothesize that an aberrant up-regulation of PDE4D might impact the transcription of the IGF2/H19 cluster by disrupting its epigenetic regulation, potentially involving various already identified H19inc regulators such as cAMP, PKA, and paxillin. Therefore, targeting the PDE4D/IGF2/H19 cluster with pharmacological selective inhibitors of PDE4D (e.g. Gebr-7b) may prevent metastatic

dissemination and increase the efficacy of current HCC treatments while reducing toxicity. However, since the upstream signals governing the regulation of the IGF2/H19 cluster are only partially understood during hepatocarcinogenesis, further exploration is warranted to corroborate our hypothesis.

### EXTRACELLULAR MATRIX ORGANIZATION IN 3D PRIMARY TUMOR SPHEROID AND THE ROLE IN DOXORUBICIN RESPONSE

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In solid tumors, the extracellular matrix (ECM) modulates cancer cell behavior and response to chemotherapy. We developed an *in vitro* model to mimic the *in vivo* microenvironment and specifically the interaction of tumor mass with the surrounding ECM. Breast carcinoma in rats was generated with a single dose of 7,12-Dimethylbenzotracene (DMBA), intraperitoneally administered in 5 weeks old female Wistar rats. After 24 weeks, a solid tumor mass was explanted, and the tumor tissue was treated with a mixture of ultrapure (>99%) recombinant collagenases (Class I and Class II) and thermolysin. A heterogeneous cell population, such as epithelial-tumor cells and fibroblast, was isolated and cells were seeded in low attachment plate to generate three-dimensional tumor spheroids. After 6 days, the presence of collagen matrix was observed by confocal microscopy, while proteomic analysis identified a complex matrix organization on 3D spheroids compared to 2D cell culture. Therefore, to investigate *in vitro* the ECM involvement in the modulation of Doxorubicin effects, spheroids were treated with recombinant collagenases (in the best formulation) for the digestion of the ECM to reduce the thick and dense collagen matrix around them. Doxorubicin cellular uptake studies highlighted the role of ECM as a barrier, limiting drug penetration, and consequentially, the doxorubicin cellular internalization increases after collagen degradation. Furthermore, treatments of spheroids with collagenases before Doxorubicin treatment, significantly increase drug cytotoxicity effects. Overall, we developed a standardized isolation protocol to obtain primary cancer cells, which preserves important cell properties to obtain reliable sources for *in vitro* 3D spheroids with a characteristic ECM organization. Taken together, these data suggest the ECM role in increasing chemoresistance in spheroids, impairing the efficacy of anticancer drugs.

### ROLE OF EXTRACELLULAR VESICLES IN THE TUMOUR MICROENVIRONMENT AND MULTIDRUG RESISTANCE

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The tumor microenvironment represents a complex network that contributes significantly to cancer progression, invasion, and metastasis, in which tumor cells communicate with each other but also with stromal and immune cells. One of the main mechanisms of therapeutic failure and consequent mortality of cancer patients is the development of resistance to anticancer drugs. Although there are several mechanisms through which tumor cells develop resistance, the over-expression of ABC family transporters, particularly P glycoprotein (P-gp), is one of the main causes of multi-drug resistance (MDR). Furthermore, it is known that tumor-derived extracellular vesicles, (EVs), are highly specialized structures that carry numerous surface markers and signaling molecules, and which, in addition to their important role in communication between cells, can mediate the transfer of drug-resistance to sensitive tumor cells, through the horizontal transfer of specific bioactive factors including the drug efflux pump, survival factors, apoptosis inhibitors and non-coding RNAs (Bucci-Muñoz *et al. Life* 2023, 13, 1633). The aim of our work was, therefore, to isolate the extracellular vesicles released by a model of sensitive cell line and its MDR variant, characterize them and verify that the EVs released from resistant cells are responsible for the transfer of P-gp to sensitive cells, significantly contributing to the spread of resistance in the tumor microenvironment. For this purpose, we used the human leukemia cell line HL60 and its MDR variant HL60R, obtained by exposure to gradually increasing concentrations of doxorubicin, as a model of acquired resistance in a liquid tumor. Our data show that HL-60R cells release about 70% more EVs, compared to the sensitive HL-60 cells; this was further confirmed by scanning electron microscopy (SEM) analysis, in agreement with literature data indicating that resistant cells release a greater quantity of large vesicles than sensitive cells (Lopes-Rodrigues V. *et al. Biochimica et Biophysica Acta (BBA) - General Subjects*, 2016). By Western Blotting analysis we observed the presence of P-gp only in the HL-60R cell extracts and in the respective vesicles; the protein is absent in sensitive HL-60 cells and its related vesicles. To verify the ability of HL-60R to carry out the horizontal transfer of P-gp through the release of EVs, immunofluorescence analysis was performed on sensitive HL-60 cells, treated for different steps of time with EVs released from HL-60R and stained with polyclonal P-gp antibody. Our data show P-gp expression only in the positive control HL-60R and in HL-60 treated with EVs released from resistant cells. In contrast, if HL-60 cells were incubated with EVs released from the same sensitive cells, they do not express P-gp on their membrane; this confirms the western blotting data that indicated the absence of P-gp in HL-60 cells and its related EVs. Since EVs released from HL-60R transfer P-gp, we are now carrying out some functional assays to verify whether, after horizontal transfer of the efflux pump into sensitive HL-60 cells, they acquire the resistant phenotype regarding sensitivity to doxorubicin. In parallel, we are conducting the same type of analysis on another model of acquired drug resistance: the MCF-7 breast cancer line and its MDR variant MCF-7R. Previous data had demonstrated several common characteristics between these two different tumor models, such as the constitutive activation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) and the overexpression of P-gp and different IAPs. (Poma P. *et al. Crit Rev Oncog.* 2021) Since EVs released by tumor cells are implicated in various stages of tumor progression and drug resistance, we believe that the inhibition of their secretion, and therefore of the horizontal transfer of their contents, is a very promising strategy to counteract these phenomena.

## BIOCHEMICAL CHARACTERIZATION OF *Lycium barbarum* EXTRACT MECHANISM: PYROPTOSIS INDUCTION IN BREAST CANCER CELLS WITH RESCUING HEALTHY COMPARTMENT FROM OXIDATIVE STRESS

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Nowadays, great attention has been paid to plant-derived antioxidant compounds with potential onconutraceutical properties. In this regard polyphenols, including phenolic acids and flavonoids (anthocyanins, flavonols, flavones, flavanones, isoflavones, flavanols, and flavonolignan), and carotenoids represent a large family of phytochemicals ubiquitous in nature with a significant antioxidant activity. Their association with the health benefits derived from consuming substantial quantities of fruits and vegetables has been established. Several studies have focused their attention on the dual behavior of dietary polyphenols and carotenoids. These compounds are frequently utilized to shield the body from oxidative stress but, under specific conditions, such as the redox imbalance present exclusively in cancer cells, they may exhibit pro-oxidant properties useful in fighting cancer. *Lycium barbarum*, commonly known as goji berry or wolfberry, is a fruit renowned for its perceived health-promoting properties, including its antitumor properties, particularly in breast cancer. However, the precise mechanisms by which goji berry exerts its effects on breast cancer cells remain not completely understood, and the detailed composition of different studied goji berry extracts is often inadequately documented or mainly associated to polysaccharide component. Our study aimed to investigate the characterization of a polyphenolic and carotenoid-rich fraction of *Lycium barbarum* fruit extract (LBE) using UHPLC-HRMS/MS analytical techniques with a focus on elucidating its impact on new biochemical pathways. LBE demonstrated cytotoxic effects against various breast cancer cell lines (MCF-7, MDA-MB-231, SK-BR-3), exerting a pro-oxidant effect that triggers pyroptosis activation via endoplasmic reticulum (ER) stress and subsequent activation of the P-IRE1 $\alpha$ /XBP1/NLRP3 axis. Conversely, LBE did not exhibit cytotoxicity towards non-tumorigenic breast cells MCF-10A; instead, it displayed antioxidant properties by neutralizing reactive oxygen species (ROS) generated by the anthracycline drug doxorubicin. These results collectively suggest that goji berries hold significant promise as nutraceuticals for chemoprevention. Moreover, considering these findings and that many chemotherapeutics act precisely by activating pyroptosis, as anthracyclines, the co-administration of LBE with these drugs could represent a new synergistic potential therapeutic that aims at enhancing the antitumor action of these drugs but at the same time dampens their side effects in healthy cells thanks to its high antioxidant action.

## LONG NON-CODING RNA H19 FAVOURS APOPTOSIS INDUCED BY THE HISTONE DEACETYLASE INHIBITOR ITF2357 (GIVINOSTAT) IN COLON CANCER CELLS

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Long non-coding RNA H19 (lncH19) is canonically considered an oncogene due to its involvement in tumor development and progression. Recently, the expression of lncH19 has been associated with reduced sensitivity of colorectal cancer (CRC) cells to 5-Fluorouracil (5-FU), which is the conventional chemotherapeutic drug used to treat CRC. Our data highlighted a new role of lncH19, able to promote apoptosis in CRC cells treated with the Histone Deacetylase Inhibitor (HDACi) ITF2357 (Givinostat). In particular, we demonstrated that ITF2357 reduced the viability of HCT-116 CRC cells and significantly induced lncH19 expression. Evaluation of autophagy and apoptosis in CRC H19-silenced

cells revealed that lncH19 knockdown markedly attenuated the effect of the HDACi reducing annexin V positivity, caspase-3 cleavage and Poly ADP-Ribose Polymerase (PARP-1) degradation compared to the respective control CRC cells. On the other hand, non-significant effects in the levels of autophagic markers were observed upon lncH19 silencing. These data suggest that autophagy induced by the HDAC inhibitor represents a pro-survival adaptive response to the effects of the drug. To elucidate the pro-apoptotic role of lncH19, bioinformatic analyses were performed. The results suggested that lncH19 may act as an endogenous competitive sponge (ceRNA) for miRNAs, stabilizing TP53 and its pro-apoptotic targets NOXA and PUMA. Accordingly, ITF2357 upregulated, together with lncH19, all these apoptotic factors. In addition, we provided evidence that ITF2357 reduces the viability of 5-FU-resistant HCT-116 cells that express high levels of H19. Overall, these findings support the hypothesis that lncH19 expression can be exploited to favour HDACi-induced cell death and to overcome 5-FU chemoresistance. ITF2357 can thus be considered a promising epi-drug in the treatment of CRC and lncH19 as a new putative biomarker for the outcome of epigenetic therapy in patients with 5-FU-resistant CRC.

## CELL STRESS

**EFFECT OF AQUEOUS EXTRACTS FROM *Posidonia oceanica* SEAGRASS ON MOUSE MACROPHAGES AND HUMAN BLOOD BRAIN BARRIER**

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Bioactive compounds from aquatic species exert several beneficial effects in human health, including anti-inflammatory and antioxidant. In particular, extracts derived from green leaves (GLE) and rhizomes (RE) of *Posidonia oceanica* have been shown to exert antitumoral activity *in vitro* against liver cancer cells<sup>1</sup>. Since these extracts have a prominent content of polyphenols, the aim of this study was to assess their potential anti-inflammatory effect on LPS-treated mouse RAW 264.7 macrophages and TNF- $\alpha$ -treated human endothelial cells belonging to an *in vitro* model of blood brain barrier (BBB)<sup>2</sup>. No cytotoxic effect and a reduction of nitrite production by inflamed macrophages were found after 24 h-treatment with increasing concentrations of both extracts. In addition, a modulation of mRNA expression of inflammatory markers was shown by Real Time PCR. Subsequently, on the basis of these data referring to the peripheral level, an *in vitro* model of the human blood-brain barrier (BBB) has been used to investigate the potential anti-inflammatory effect at the central level. We used a co-culture model with the presence of human endothelial cells and pericytes and we added the extracts, at the same concentrations used in the previous experiments with RAW cells, to the luminal compartment and induced inflammation with TNF- $\alpha$ <sup>3</sup>. Results obtained by Real Time PCR on inflammatory markers of BBB cells showed that both extracts were ineffective in reducing inflammation. Interestingly, studies performed on BBB permeability have shown that both extracts do not alter its integrity, reduce the TNF- $\alpha$ -induced permeability alteration and counteract the release of nitrites. Of note, only RE induces an increase in mRNA and protein expression of molecular markers of tight and adherens junctions, leading to a recovery of protein delocalisation after exposure to TNF- $\alpha$ , as shown by immunofluorescence. These promising results prompt further investigation to evaluate more in detail the potential immunomodulatory role of GLE and RE and to unveil the molecular cascade underlying the observed recovery of the integrity of the BBB.

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**EFFECT OF MELATONIN ON HEPATIC ALTERATIONS IN A BTBR T + Itpr3tf/J MOUSE MODEL OF AUTISM**

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Autism spectrum disorder (ASD) is defined as a heterogeneous set of neurodevelopmental disorder compromising social communication and social interactions and inducing restricted and repetitive behavior. Although the etiology of autism is not well understood, previous findings suggested that the mechanism underlying ASD involves genetics, environmental and biological factors. Among others, oxidative stress, neuroinflammation and apoptotic mechanisms seem to be associated with the pathogenesis of autism. ASD is also described to be associated with various physiological abnormalities in different organs, such as liver. Melatonin (N-acetyl-5-methoxytryptamine) is considered a strong antioxidant due to its ability to scavenge free oxygen radicals. It also has a potential therapeutic action, based on beneficial effects shown on liver injuries and diseases. The aims of the study were to investigate morphological and functional alterations in liver of an autistic mouse model BTBR T+Itpr3tf/J (BTBR) mice and to identify therapeutic strategies for alleviating hepatic damages using melatonin administration. BTBR mice and C57BL6/J (CTR) as healthy control mice have been divided in 4 groups, then treated and not treated respectively with melatonin. We studied hepatic cytoarchitecture, oxidative stress, inflammation and ferroptosis. The results showed more elevated oxidative stress and inflammation in BTBR mice than CTR mice. We also demonstrated the expression of ferroptosis markers in BTBR mice liver. Moreover, we assessed the beneficial potential of melatonin on hepatic alterations of BTBR mice. The results suggested positive effects on cytoarchitecture and metabolic functions due to melatonin treatment.

**CELLULAR AND MOLECULAR MECHANISMS RELATED TO AAPH EFFECTS IN HUMAN ERYTHROCYTES: BENEFICIAL ROLE OF ANTHOCYANIN EXTRACTED FROM *Callistemon citrinus***

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Oxidative stress decreases antioxidant capacity, irreversibly damages red blood cells (RBCs), causing their eventual damage by hemolysis and their removal through the circulation. Since mature RBCs are cells without a nucleus and other cellular organelles, they do not have the ability to repair damaged components and therefore in their short life they exhibit effective defense mechanisms against multiple stressors, including increased reactive oxygen species (ROS). This investigation aimed to explore the cellular and molecular mechanisms related to oxidative stress underlying anion exchanger 1 activity (band 3, SLC4A1/AE1) in human RBCs. To achieve this aim, the relationship between RBC morphology and functional and metabolic activity has been explored. Moreover, the potential protective effect of an anthocyanin-enriched fraction extracted from *Callistemon citrinus* flowers was studied. Cellular morphology, parameters of oxidative stress, as well as the anion exchange capability of band 3 have been analyzed in RBCs treated for 1 h with 50 mM of the pro-oxidant 2,2'-azobis (2-methylpropionamide) -dihydrochloride (AAPH). Before or after the oxidative insult, subsets of cells were exposed to 0.01 µg/mL of an anthocyanin-enriched fraction for 1 h. Exposure to AAPH caused oxidative stress, exhaustion of reduced glutathione, and over-activation of the endogenous antioxidant machinery, resulting in morphological alterations of RBCs, specifically the formation of acanthocytes, increased lipid peroxidation and oxidation of proteins, as well as abnormal distribution and hyper-phosphorylation of band 3. Expected, oxidative stress was also associated with a decreased band 3 ion transport activity and an increase of oxidized haemoglobin, which led to abnormal clustering of band 3. Exposure of cells to the anthocyanin-enriched fraction prior to, but not after, oxidative stress efficiently counteracted oxidative stress-related alterations. Importantly, protection of band 3 function from oxidative stress could only be achieved in intact cells and not in RBC ghosts. These findings contribute a) to clarify oxidative stress-related physiological and biochemical alterations in human RBCs, b) propose anthocyanins as natural antioxidants to neutralize oxidative stress-related modifications, and 3) suggest that cell integrity, and therefore a cytosolic component, is required to reverse oxidative stress-related pathophysiological derangements in human mature RBCs.

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### EXPOSURE TO NANO-PLASTICS PROVOKES OXIDATIVE STRESS AFTER INTERNALIZATION IN HUMAN ERYTHROCYTES: ROLE OF ESTROGEN RECEPTORS

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Nowadays, plastics remain indispensable materials used in several sectors of our everyday lives. While the effects caused by plastics in the environment, namely in marine ecosystems, is a well-recognized problem, more recently, concern regarding human exposure to nano-plastics (NPs) has increased. Human erythrocytes are the prime target for most toxic xenobiotics after entering the bloodstream. It has been widely demonstrated that, due to their smaller size, NPs may adhere to the human erythrocyte surface and exert adverse effects following aggregation and adhesion to blood vessels. In the present investigation, we explored the effects of NPs on human erythrocytes. In particular, we investigated the mechanisms underlying their cytoplasmic uptake, with a focus on estrogen receptor-mediated internalization, and analyzed erythrocyte surface morphological modifications. Moreover, we studied cellular and molecular mechanisms associated with increased intracellular oxidative stress, as well as the anion exchange capability through band 3 protein (anion exchanger 1; SLC4A1). Cellular morphology, binding/internalization of NPs, oxidative stress parameters, as well as the distribution and anion exchange capability of band 3 have been analyzed in human erythrocytes exposed to 1 µg/mL NPs for 3 and 24 hours, respectively. The data obtained showed significant structural modifications in the cellular shape after exposure to 1 µg/mL NPs for 3 and 24 h. In particular, acanthocytes, echinocytes (cells with surface blebs), and leptocytes (cells with a flattened shape) were detected. In contrast, the percentage of eryptotic cells (<1%) was comparable to a physiological condition. An increased trafficking from the cytosol to the erythrocyte plasma membrane and an abnormal clustering of estrogen receptors were also observed. These phenomena led to estrogen receptor binding and internalization of NPs, as confirmed via co-localization analysis and confocal microscopy. An increased phosphorylation of ERK1/2 and AKT kinases and eNOs enzyme indicated an activation of the estrogen-modulated non-genomic pathway. Interestingly, NPs caused a significant production of reactive oxygen species, resulting in an increase of TBARS levels (a lipid peroxidation biomarker)

and a reduction of total protein sulfhydryl groups. Oxidative stress was also associated with a decreased band 3 ion transport activity and increased oxidized haemoglobin, which led to abnormal clustering of band 3 on the plasma membrane. Taken together, these findings clearly identify mechanisms underlying the internalization of NPs in human erythrocytes and contribute to elucidating their potential oxidative stress-related harmful effects, which may affect erythrocyte homeostasis.

### CHARACTERIZATION OF 2D HaCaT CELL MODEL EXPOSED TO A PSORIATIC PROINFLAMMATORY MICROENVIRONMENT

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To study skin biology and explore a rapid epithelial response to exogenous stimuli, HaCaT cells, a spontaneously immortalized line of human keratinocytes, represent a useful 2D cell experimental model, allowing to analyze *in vitro*, under normal and altered conditions, the cell proliferation and differentiation. Keratinocytes interplay with proinflammatory cytokines during the development and progression of psoriatic lesions, but the early pathogenetic cellular mechanisms are still being elucidated. In our experimental design, 2D cultures of HaCaT cells were induced to differentiate with [1.8 mM] CaCl<sub>2</sub> and exposed to a complete proinflammatory psoriatic microenvironment, containing a cytokine combination (MIX) of interleukin (IL)-17A (10 ng/mL), IL-22 (20 ng/mL), IL-23 (10 ng/mL) and Tumor Necrosis Factor  $\alpha$  (20 ng/mL), for 24h and 48 h. We investigated by immunofluorescence the modulation of claudin 1 (CLDN-1), a transmembrane junctional protein, Zonula Occludens 1 (ZO-1), a cytoplasmatic scaffold between the cytoskeletal filaments and cell membrane proteins, keratin (K)10, and K14, *i.e.* keratin isoforms synthesized, respectively, by suprabasal and basal keratinocytes. Keratinocyte proliferation and transmission electron microscopy (TEM) analysis were carried out. We observed that HaCaT control samples mimicked an initial phase of epidermal differentiation after high calcium concentration, as demonstrated by the presence of desmosomes at TEM observations and the immunopositivity for K10. In parallel, ZO-1 and the proliferative activity grew at 48h. After MIX, CLDN-1 and ZO-1 decreased; immunofluorescence of K10 and cell proliferation were also reduced, suggesting that the psoriatic environment early affects the delicate differentiation process. Instead, K14 distribution was unaffected by cytokine MIX and no ultrastructural morphological changes

were detected. Ongoing research in our laboratories aims to optimize and standardize the proper experimental conditions of this 2D *in vitro* model as a tuneable cell system for mimicking either the basal keratinocyte phenotype in the presence of low calcium concentration or the most differentiated upper epidermal layers in prolonged culture time with high calcium concentration. In conclusion, HaCaT cell culture is a useful 2D experimental model allowing us to analyse i) specific molecular mechanisms triggered by cytokine administration, ii) the susceptibility to processes leading to ferroptosis, *i.e.* a new form of programmed cell death, and iii) the release of microvesicles and their biological effects.

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### BLACK PERSIMMON (*Diospyros digyna* JACQ.) EXTRACTS MITIGATE IL-1 $\beta$ -INDUCED INFLAMMATORY RESPONSE IN INTESTINAL EPITHELIAL CELLS WHILE PRESERVING EPITHELIUM BARRIER FUNCTION

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*Diospyros digyna* Jacq. is a tropical fruit tree that has largely remained unfamiliar in Europe. However, recent successful cultivation trials in the Mediterranean region, particularly in Sicily, have shed light on its potential adaptability. Renowned for its peculiar soft and dark chocolate pulp, the fruit is commonly referred to as black persimmon or chocolate pudding fruit. Our previous investigations have unveiled black persimmon's richness in bioactive compounds, uniquely distributed across its different parts. These compounds exhibit notable radical scavenging and metal-reducing activities, alongside their ability to counteract oxidative damage in cells (Mannino *et al.*, 2022). Expanding our understanding of the functional properties of black persimmon, in the present study, we investigated the potential anti-inflammatory effects of its extracts on IL-1 $\beta$ -induced inflammation in intestinal epithelial cells. For our experiments, we employed differentiated Caco-2 cell monolayers subjected to the proinflammatory stimulus of IL-1 $\beta$ . Proinflammatory cytokine and antioxidant enzyme production were quantified through gene expression analysis using qRT-PCR and protein level assessment via ELISA or Western blotting. The impact of the extracts on epithelial barrier functions was examined by assessing paracellular permeability, MMPs expression, and distribution of adhesion proteins. Additionally, we assessed the extracts' influence on the activation of the transcription factors NF- $\kappa$ B and Nrf2, aiming to elucidate the mechanisms involved in the observed anti-inflammatory effects. Our results demonstrate that black persimmon pulp, seeds, and

peel extracts significantly mitigate the IL-1 $\beta$ -induced inflammatory response, as evidenced by decreased release of proinflammatory cytokines and reduced expression of inflammatory enzymes. Furthermore, treatment with black persimmon extracts preserves epithelial integrity by maintaining barrier function, as evidenced by attenuation of IL-1 $\beta$ -induced increase in paracellular permeability and attenuation of redistribution of tight junction proteins. Notably, non-edible components, particularly seed extracts, demonstrated significant anti-inflammatory effects, suggesting the potential value of typically discarded fruit parts. The anti-inflammatory activity of black persimmon extracts was associated with modulation of the NF- $\kappa$ B signaling pathway, as evidenced by reduced I $\kappa$ B- $\alpha$  phosphorylation and altered p65/p50 translocation. Furthermore, the upregulation of antioxidant enzyme genes (CuZnSOD, MnSOD, and GPx) and increased Nrf2 expression elucidate a clear correlation between the anti-inflammatory and antioxidant actions of the bioactive compounds in the extracts. These findings suggest that black persimmon extracts hold promise as a natural therapeutic agent for alleviating inflammation and preserving epithelial integrity in the context of intestinal inflammatory disorders.

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doi: 10.1016/j.foodchem.2022.132137.

#### NANO-ZnO-ERYTHROCYTE DUET INTERPLAY: UNRAVELING THE MOLECULAR MECHANISMS OF ZINC NANOPARTICLES IN HUMAN ERYTHROCYTE TOXICITY

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Zinc oxide nanoparticles (ZnO-NPs) finds attractive applications in different fields of biomedicine, environment, and cosmetics. They have shown potential interactions with blood cells, particularly erythrocytes, raising concerns about potential adverse effects. This study delves into the intricate interplay between nano-sized zinc oxide (Nano-ZnO) particles and human erythrocytes, aiming to provide a comprehensive understanding of the molecular mechanisms governing red blood cell toxicity response. After incubation of human erythrocytes with different concentrations of ZnO-NPs (12.5, 25, 50 and 100  $\mu$ g/ml) for six hours, hemolytic activity, oxidative stress, and morphological alterations were determined. Furthermore, molecular docking and dynamic simulations were applied to prove any potential intermolecular interactions and binding affinity of ZnONPs for erythrocyte mem-

brane proteins. Our results demonstrate that zinc oxide nanoparticles caused hemolytic effects through formation of ROS, cell membrane distortions leading so toward eryptosis. Combination of *in vitro* exposure assays with *in silico* docking simulations offers valuable insights for better understanding the implications of Nano-ZnO exposure on erythrocyte function.

#### H<sub>2</sub>O<sub>2</sub>-INDUCED OXIDATIVE STRESS IMPAIRS BAND 3 PROTEIN FUNCTION IN HUMAN ERYTHROCYTES: PROTECTIVE ACTIVITY OF QUERCETIN

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During their 120-day life span, erythrocytes are continuously exposed to many stressors. Therefore, they are considered a suitable model to study cellular responses to oxidative stress. The purpose of this study was to evaluate the potential beneficial effects of the natural antioxidant quercetin (Q) on an oxidative stress model represented by H<sub>2</sub>O<sub>2</sub>-treated human erythrocytes. Oxidative stress markers, including % hemolysis, reactive oxygen species (ROS) production, thio-barbituric acid reactive substances (TBARS) levels, protein sulfhydryl groups oxidation, and % methemoglobin (MetHb), as well as CD47 and Band 3 protein (B3p) expression and anion exchange capability assessment *via* B3p were analysed in erythrocytes treated for 1 h with H<sub>2</sub>O<sub>2</sub> (20 mM). The antioxidant effect of Q (10  $\mu$ M) was evaluated as pre-treatment or alternatively as post-treatment to H<sub>2</sub>O<sub>2</sub> exposure. Results show that pre-treatment with Q is more effective than post-treatment to counteract oxidative changes. Specifically, Q pre-exposure prevented acanthocyte formation, decreased oxidation markers and restored abnormal B3p distribution and CD47 expression. As a result of the preventive beneficial effect of Q, restoration of anion exchange capability *via* B3p, previously affected by H<sub>2</sub>O<sub>2</sub> treatment, was observed. In conclusion, these results contribute to elucidate the impact of oxidative events on erythrocytes homeostasis and may propose Q as a natural antioxidant contributing to counteract oxidative stress-related diseases.

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## MISCELLANEOUS

## CLINICAL AND MORPHOLOGICAL ASPECTS OF NON-COMPACTION CARDIOMYOPATHY

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Left ventricular non-compact cardiomyopathy (LVNC) is known under several names, including spongy myocardium, fetal myocardium, non-compact myocardium, hyper-trabeculation syndrome, and left ventricular non-compact, depending on clinical features observed in patients and during its clinical evolution to hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), arrhythmic cardiomyopathy (ACM), terminal heart failure or to a form of reversible outcome with repair. These polymorphisms suggest different pathogenetic mechanisms and possibly different forms of the disease. Non-compact myocardium has been observed already at early ages of life, including during normal cardiac development, in neonatal, paediatric, and young patients, and then during adult or advanced ages (professional athletes, pregnancy, aging). Due to the absence of overt symptoms, non-compact myocardium may be unexpectedly diagnosed after Cardiac Magnetic Resonance, ventriculography, or endomyocardial biopsies (EMB) performed for other purposes. In 40-50% of cases, the pathogenesis of LVNC has been associated with a number of mutations of two classes of genes. One (transcription factors and related genes) is speculated to produce an arrest of the final stage of myocardial morphogenesis. A second set of mutated genes appears to be involved in the correct organization and composition of supramolecular aggregates that stabilize cardiomyocytes into the connective tissue scaffold (cytoskeletal intracellular elements and extracellular cell-to-scaffold junction proteins) [1]. In this study, an Italian family of 7 subjects, 4 aged 10 (II-1), 14 (II-2), 43 (I-4), and 46 years (I-5), presenting abnormal ECG changes, dyspnea and palpitation (II-2, I-4, and I-5), and recurrent cerebral ischemic attack (I-5), underwent 2-dimensional echo, cardiac magnetic resonance, Holter monitoring and, EMB. EMB were subjected to next-generation sequencing gene analysis and morphological evaluation [2]. Two-dimensional echo and cardiac magnetic resonance documented LV myocardial non-compactness in all. Coronary arteries were normal. LV angiography showed transmural crypts progressing to spongy myocardial transformation with LV dilatation and dysfunction in the older subject. At histology and electron microscopy lateral detachment of cardiomyocytes was associated with cell shape alterations, subcellular changes, myofibrillar disarray, and degradation of intercalated discs causing also dysancorage of myofilaments from Z-discs and cell membrane. Next-generation sequencing showed in affected members an unreported p.(Ala21Val) mutation of ACTC1. We hypothesize that the novel p.(Ala21Val) mutation of ACTC1 may cause alterations of myofibrillar function, intercalated disc, and other cytosolic skeleton interaction, leading to familial paediatric spongy myocardium, hypertrophic cardiomyopathy, and LV myocardial non-compactness

with transmural crypts with variable clinical features. However, this hypothesis may also explain why several types of LVNC occur, such as primary myocardial forms, a form associated with arrhythmias, and a form associated with congenital heart disease, including septal defects, right heart obstructive abnormalities and, hypoplastic left heart syndrome. While in late/reversible forms other mutations could be only a susceptibility condition in which triggering factors are still unknown.

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## CONVENTIONAL GLUCOCORTICOID REPLACEMENT THERAPY VS. DUAL-RELEASE HYDROCORTISONE: EFFECTS ON INFLAMMATION AND IMMUNE PROFILE IN PATIENTS WITH PRIMARY ADRENAL INSUFFICIENCY AND IMPLICATIONS OF THERAPY RESPONSE PREDICTORS

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Primary adrenal insufficiency (PAI), whose autoimmune adrenalitis is the main cause, is characterised by an inadequate cortisol secretion, due to a destruction of adrenal gland and a consequent lack of glucocorticoid and mineralocorticoid production. Autoimmune conditions are caused by a loss of tolerance for self-antigens, with regulatory T lymphocytes failing in limiting auto-reactivity, and the increase of pro-inflammatory processes. Generally, corticosteroids exert anti-inflammatory and immunosuppressive actions by regulating the expression of inflammatory and immunomodulatory proteins and by modulating the lymphocyte pattern, including the regulatory T-cells (Tregs) induction. Conventional glucocorticoids - including cortisone acetate and hydrocortisone administered two or three times a day - are the mainstay of the treatment of patients with PAI, but in the most recent years the innovative dual-release hydrocortisone (DR-HC) - administered once daily - has been used as replacement therapy. DR-HC has shown favourable effects on cardiovascular risk factors, glucose metabolism and bone parameters compared to conventional steroids. Moreover, it has shown to improve the immune system cell profile expression, restoring a more physiological circadian glucocorticoid rhythm. According to this evidence, this study investigates whether conventional replacement steroid (CRS) or DR-HC elicit different response in terms of anti-inflammatory or immunomodulatory effects, by measurement of anthropometric, metabolic, serum inflammatory parameters and gene expression levels of IL-6, IL-17A, COX-2, HSP-70,IDO, PD-L1, hnRNPA2/B, iNOS and TXN-1. Additionally, a cytofluorimetric analysis was performed to evaluate a modulation in the activation status of T-cells, including CD4+CD25+Foxp3+Treg population. The study included 15 patients with PAI on conventional glucocorticoid

therapy and 15 patients on DR-HC. The outcomes of the study were evaluated by isolation of peripheral blood mononuclear cells at baseline and after 12 months of treatment. 10 healthy patients (controls) were evaluated at the time of enrolment. In patients treated with CRS, a significant increase in c-reactive protein, erythrocyte sedimentation rate and fibrinogen were observed after 12 months of treatment compared to baseline. At 12 months, significantly lower waist circumference, glucose, c-reactive protein, erythrocyte sedimentation rate and neutrophil-to-lymphocyte ratio was observed in patients treated with DR-HC compared to those treated with conventional treatment. A significant decrease in the transcription of COX-2 and HSP-70 (along with IL-6) was observed together with a significant increase in mRNA expression of IDO and PD-L1 in patients treated with DR-HC after 12 months of observation. Compared to CRS, DR-HC treatment improves the inflammatory and immune patterns in patients with PAI modulating several proteins involved in inflammatory response in a Treg-independent manner. Moreover, we can suppose that the transcription levels of IL-6, COX-2, HSP-70, IDO and PD-L1 could be considered to predict and evaluate the response to steroidal therapies in PAI and their different efficacies, based on our findings on anthropometric, metabolic, and biochemical outcomes. However, further larger and controlled studies are needed to confirm our preliminary results and the relevance of this assumption.

#### A NOVEL PROMISING STRATEGY FOR MANAGING OBESITY AND OBESITY-RELATED DISORDERS: A MOLECULAR DYNAMICS STUDY

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Fat tissue represents an important source of adipose-derived stem cells (ADSCs), which can differentiate towards several phenotypes under certain stimuli, contributing to tissue regeneration both *in vitro* and *in vivo*. ADSC adipogenic differentiation is controlled by the activation of a specific transcriptional program, including epigenetic factors and key adipogenic genes<sup>1</sup>. Dysregulation in stem cell recruitment and differentiation during adipogenesis is linked to a chronic low-grade inflammation and macrophage infiltration inside the adipose tissue, cardiovascular diseases, and obesity<sup>2</sup>. Several molecules, as vitamin D, are able to commit stem cells towards defined cellular phenotypes, acting on the expression of specific genes, counteracting lipid accumulation and regulating adipogenesis<sup>3</sup>. Understanding the main mechanisms involved in adipogenesis, from a physiological condition to obesity, could help in increasing current therapeutic strategies for the management of obesity and its related metabolic syndrome<sup>4</sup>. In the present study we aimed to evaluating the role of metformin and vitamin D, alone or in combination, on ADSC differentiation. We also investigated the effect of metformin and vitamin D in targeting ADSC differentiation towards an intermediate phenotype, as beige adipocytes and in modulating inflammation and autophagy in ADSCs during adipogenic commitment. Within this context, ADSCs were cultured for 21 days in a specific adipogenic

conditioned medium (DM), in the presence of metformin, alone or in combination with vitamin D. We evaluated the levels of expression of main markers of adipogenesis, aP2, LPL and ACOT2. We also analyzed the gene and protein expression of thermogenic UCP1 protein, and the expression of PARP1 and the beige specific marker TMEM26. We then assessed the role of specific epigenetic modulating genes and miRNAs in controlling stem cell adipogenesis. Furthermore, the lack of knowledge of the mechanism of action of drugs hampers the development of new therapies. Within this context, knowing the binding site selectivity and the mechanism of action of inhibitors and substrates is crucial for drug discovery and optimization processes. Among other objectives of the study, the interaction between metformin and different cellular targets using molecular dynamics (MD) simulations was also investigated. The result of this research could unravel the possible molecular interactions of metformin and vitamin D at the cellular and nuclear level and their role in cell fate determination, identifying new drug targets for the treatment of obesity and its related disorders.

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#### COMPARISON OF HEMOSTATIC TOPICAL FORMULATIONS

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A skin wound, even if superficial, damages the anatomical structures involved and alters the function of the affected area. The skin wound healing is a dynamic and complex process involving a sequence of coordinated cellular events that can be influenced by different factors. Abnormalities in this sequence of events can impaired healing and/or reopening the wound. To date, traditional materials are potentially effective in the cutaneous wound healing process and various types of hemostatic materials can be used in the clinical application and in home-setting; however, the traditional materials have still important limitations and the development of effective, safe, easy-to-use and cheap hemostatic materials is therefore essential. In this study was analyzed the hemostatic effect of the two different Nova Argentia Srl (Gorgonzola, Milan, Italy) formulations: (1) NOVA.emoSTOP powder and (2) NOVA.emoSTOP pad. In this study was evaluated the time of clot forming and the morphological features of the blood clots. We observed that both NOVA.emoSTOP powder and NOVA.emoSTOP pad promote a rapid formation of a medium/small clot. Disproportionate clot formation or abnormalities in the hemostasis phase may result in excessive accumulation of extra-

cellular matrix at the wound site so altering the wound healing process. The morphological and morphometric analyses performed in the present study showed that the NOVA.emoSTOP pad promotes the formation of a blood clot richer in platelets respect to NOVA.emoSTOP powder. Interestingly, the NOVA.emoSTOP pad used in the nose-bleed simulation promotes a very rapid formation of a platelet-rich clot underlining its valuable hemostatic effect together with the capability to absorb blood. In conclusion, the pad formulation may represent a functional, effective, safe, easy-to-use and cheap hemostatic innovative-tool for healing superficial skin wounds such as abrasions, excoriations, and small surgical wounds.

### CUTANEOUS LEISHMANIASIS AFTER TREATMENT WITH GOLIMUMAB FOR PSORIATIC ARTHRITIS

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Cutaneous leishmaniasis is a zoonosis caused by flagellate protozoans of the genus *Leishmania* transmitted by sandflies. Immunosuppression is an important risk factor for developing leishmaniasis in patients undergoing treatment with TNF-alpha inhibitors for autoimmune diseases. We report the case of a patient developing cutaneous leishmaniasis after treatment with golimumab for psoriatic arthritis. A 65 years old Italian man with a 14-year history of psoriatic arthritis with minimal cutaneous manifestations went to our hospital with bilateral ulcerations of the buttocks and an additional smaller lesion of the upper back that had appeared 8-9 months earlier. He had been treated with methotrexate (15 mg per week) for the past 14 years and with golimumab (50 mg per month) in the last 2,5 years. His last travel abroad (China) dated back to 8 years earlier. Punch biopsies from the borders and the inner portions of both gluteal lesions were fixed in 10% buffered formalin and embedded in paraffin to obtain 4 micrometers-thick sections stained with hematoxylin-eosin. Additional slides were cut to be stained with Giemsa, PAS, and Grocott methods, and immunostained with monoclonal antibodies against CD1a (clones 010 and MTB1). Histology showed a dense nodular lympho-histiocytic dermal infiltration with superficial ulcerations. Intracellular amastigotes with peripheral nuclei and kinetoplasts were detected within histiocytes. Amastigotes were strongly immunoreactive for CD1a using MTB1 monoclonal antibody, but not with 010 clone. Mucosal and visceral involvement was excluded based on clinical parameters and ultrasonography. These findings allowed us to yield the diagnosis of cutaneous leishmaniasis. Therapy with golimumab and methotrexate was suspended after histologic diagnosis and the patient was referred to another hospital to undergo an appropriate treatment. To the best of our knowledge, this case represents the fifth report of leishmaniasis in a patient treated with golimumab. The increase in the use of TNF-alpha antagonists has been associated with new cases of leishmaniasis, as TNF-alpha and other cytokines are central in the early control of the infection. Lesions may be due to infection during immunosuppressive therapy or reactivation

of a latent leishmaniasis. Physicians should be aware of this disease in patients on this biotechnological drug, especially when they have previously lived in endemic areas.

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### STRUCTURAL CHARACTERIZATION OF THIOREDOXIN REDUCTASE FROM *Cryptosporidium parvum* AND ITS INTERACTION WITH AURANOFIN

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*Cryptosporidium parvum*, an apicomplexan protozoan parasite, is the causative agent of cryptosporidiosis, one of the most common causes of diarrheal disease worldwide. Cryptosporidiosis is strongly related to early childhood mortality, with prevalence in tropical countries, and is also a potential life-threatening complication in individuals with poor health or weakened immune systems (HIV/AIDS patients, cancer, and transplant patients) (1). Because of its link with malnutrition, poverty and poor hygiene, cryptosporidiosis was included in the WHO Neglected Disease Initiative in 2004 (2). No fully effective treatments or vaccines are currently available as the only FDA-approved drug, nitazoxanide, is effective in immunocompetent patients while showing reduced efficacy in immunocompromised ones (2). Over the last decades, the screening of existing drugs has been used as a convenient strategy for new antiparasitic drugs development. Auranofin (AF, Ridaura<sup>®</sup>), an FDA-orphan drug and a gold-containing compound, has been identified as an antiparasitic drug for the treatment of many human parasitic diseases. Reprofiled auranofin has been shown to be active against several parasites, including *Schistosoma mansoni*, *Brugia malayi*, *Onchocerca volvulus*, *Leishmania* spp., *Trypanosoma brucei*, *Entamoeba histolytica*, and *Giardia lamblia* (3). Moreover Debnath A. et al. (2) found that auranofin was effective *in vitro* against *C. parvum* in the micromolar range, which was comparable to the aforementioned nitazoxanide. It is well known that auranofin can target thioredoxin reductase (TrxR), a crucial parasite enzyme involved in the detoxification of reactive oxygen species, among other functions (3). Using X-ray crystallography, we solved the crystal structure of *C. parvum* TrxR (CpTrxR) in the apo form (1.95 Å) and in complex with AF (3.3 Å). The 3D structure classifies CpTrxR as a type II high molecular weight thioredoxin reductase, sharing a characteristic spacer of four residues between the two redox active Cys residues at the C-terminus. This distinctive redox motif characterizes the

TrxRs from apicomplexan protozoa, including *Plasmodium falciparum*, a malaria parasite (4), and, to the best of our knowledge, it has been observed for the first time in our crystallographic structure. This result will allow a thorough investigation of the catalytic mechanism of these enzymes. The second structure results from co-crystallization of CpTrxR with AF in reducing conditions. This structure shows the gold atom bound to the protein, providing insights into the molecular mechanism of AF against these parasites.

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#### STUDY ON ORGAN-ON-CHIP TO DEVELOP AN ALGORITHM THAT ALLOWS THE PERSONALIZATION OF THE OXYGEN-OZONE MIXTURE IN AUTOEMOTHERAPY

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In recent years, increasing scientific knowledge and modern high-tech advancements in micro- and nanoscale fabrication technologies have had a significant impact on various scientific fields. The microfluidic technology, commonly referred to as microfluidic technology, has rapidly emerged as a potent tool for a multitude of applications, particularly in the realm of bioengineering and biomedical engineering research. Hence, a significant impact has been observed, for instance, in the handling of biological samples, the utilization of analyte sensing cells in assays, tissue engineering, molecular diagnostics, and drug screening. Besides the immense range of functions it possesses, microfluidic technology also affords the possibility to imitate various organs in order to tackle the complexities inherent in animal-based testing methodologies with efficiency. The amalgamation of fluid physics and three-dimensional (3-D) cell compartmentalization has maintained its popularity as an organ-on-a-chip. In this particular context, the development of simple humanoid model systems, which hold significant significance in a wide range of research fields, is dependent on the development of a microfluidic system. The basic goal is to provide an artificial testing subject that resembles the human body in every aspect. For instance, drug testing in the pharma industry is crucial to assure proper function. The application of oxygen-ozone mixture infusions was studied on different tissues, with the aim of verifying their effectiveness and, above all, evaluating the possibility of customizing the ozone component to be infused through the blood. To resolve the difficulty of *in vivo* tests, the aim was to set up the tests on Organ-on-Chip systems. The in OoC from the company Molecular Device were selected and the microfluidic tissue models were taken into consideration. System have been simulated with specific growth-stimulating proteins in order to make them active and operational towards anomalous trophic

problems. Parallel samples were divided into 4 batches in order to have a "reserve" group (preserved in cryogenic Dewar containers with nitrogen at -160°C), a "neutral" group not treated with growth proteins, a "comparison" group treated with growth proteins and, finally, an "O<sub>3</sub>" group, again treated with growth proteins and in turn divided into 16 parts so that these were treated with 16 different doses of Oxygen-Ozone mixture. The studies, already in the initial phases, highlighted a difference in cell development between the "O<sub>3</sub>" samples and the "Control" samples, also showing a substantial difference in the times of cell reproduction and tissue proliferation. Particular attention was paid to the cellular arrangement and it was highlighted that the "O<sub>3</sub>" samples present greater regularity in the dimensions of the intracellular matrices. The continuation of the research involves an in-depth study on the dosages of O<sub>2</sub>-O<sub>3</sub> mixtures specific for each cell type and their adaptations linked to the apoptotic speed correlated to the biological age of each human being.

#### SERUM miRNAs PROFILE IN DIABETIC RETINOPATHY AND CORRELATION ANALYSIS WITH IMAGING MARKERS AND CLINICAL OUTCOMES

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Diabetic retinopathy (DR) is one of the mayor complication of diabetes mellitus resulting from multiple pathogenetic processes leading to retinal microvascular defects and neuroretinal dysfunction and degeneration. MicroRNAs (miRNAs) are a class of highly conserved, 19-25 nucleotide non coding RNAs sequences that regulate the activity of target mRNAs and control gene expression at the post-transcriptional level. Dysregulated miRNA expression has been identified as a risk factor in several diseases and metabolic pathways, and seems to play an important role also in diabetes and its complications. Their use in the clinical setting of diabetic macular edema (DME) is limited by lack of data regarding the correlation with clinical and imaging features. In the present study we evaluated miRNAs profiles obtained by serum samples of newly diagnosed DME patients. The study was designed as prospective study and included a baseline visit (V0), a 4-months visit (V1) and a 12-months visit (V2). At V0 patient underwent optical coherence tomography (OCT) imaging and serum samplings for the detection of miRNAs, at V1 and V2 the patients repeated the OCT evaluations. During the observation time, treatments were set as for clinical convenience. Enriched microRNAs were extracted and real-time PCR was performed. OCT features were used to assess the severity and progression of the disease. We collected data of a cohort of type 1 and type 2 diabetic patients. All the patients presented DME and were naive to intravitreal treatments. The follow-up was of 12 months. Diabetes-related miRNAs were found up- or down-regulated and associated with the course of DME (resolved/recurrent/persistent). Correlations were found with imaging biomarkers and results obtained prompted us to speculate that the some miRNAs exert a protective role and a better response to treatment while other miRNAs are

linked to negative effects. More specifically, the cluster of miRNAs constituted by miR-192-5p, miR199-5p, miR-320d, miR-338-3p and miR-486 is suggested by the results of ROC curves, showing that when they are highly expressed there is a good response to treatments with a resolution of macular edema. On the basis of these findings, serum miRNAs assessment is a feasible and clinically relevant assessment to be performed in the diagnostic work-up of diabetic patients affected by DME to customize treatment strategies and to optimize the management of DME.

### CRYSTAL STRUCTURE OF HUMAN HISTONE DEACETYLASE 8 IN COMPLEX WITH A NOVEL HYDROXAMIC ACID INHIBITOR

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Histone deacetylases (HDACs) are enzymes involved in the control of gene expression. They catalyze the removal of acetyl moieties from lysine  $\epsilon$ -amino group residues in histones and other non-histone protein and play a key role in the regulation of many biological processes, such as cell differentiation, proliferation, senescence, and apoptosis. HDACs are also involved in the occurrence and progression of a number of pathophysiological conditions and diseases, including cancer. These enzymes are validated targets for drug design: treatment with HDAC inhibitors (HDIs) leads to restrict the DNA repairing, cell cycle arrest, alteration in genetic expression and induces cellular apoptosis. Same HDIs have been approved by FDA for the treatment of cutaneous T-cell lymphoma, but they are pan-HDAC inhibitors, that interact with all classes of HDACs and lead to adverse effects.<sup>1</sup> Among the 18 different isotypes of HDACs known, HDAC8, a Zn<sup>2+</sup>-dependent class I HDAC, is an emerging anticancer target for structure-based drug design, because it exhibits several unique characteristics. Structural studies have shown the high flexibility of the L1 loop (Ser30-Lys36) in the proximity of the active site: it can adopt two different conformations, open and closed, upon binding of specific inhibitors and substrates. As a consequence, the size of the binding pocket can change and this aspect can be exploited to develop selective inhibitors, given that other isoforms possess more conformationally static active sites than HDAC8. Aromatic hydroxamic acids show HDAC8 inhibition in lower nanomolar concentration, but they are also active against other isotypes of HDACs.<sup>1</sup> Therefore, designing of selective HDAC8 inhibitors is a promising strategy to treat the diseased conditions involving the HDAC8 activity. Novel hydroxamic acid inhibitors, that are tetrahydroisoquinoline (TIQ)-based HDAC8 inhibitors, have shown an improvement in potency and selectivity for HDAC8.<sup>2</sup> We solved the X-ray crystal structure of human HDAC8 (hHDAC8) in complex with a TIQ-based HDAC8 inhibitor to 3.3 Å resolution and in an unprecedented crystallographic space group. The 3D structure

obtained by co-crystallization of hHDAC8 with this compound shows that the inhibitor occupies a hydrophobic, long, and narrow tunnel at the end of which the catalytic machinery, with the Zn<sup>2+</sup> ion is located. The hydroxamic acid moiety of the inhibitor chelates the active site Zn<sup>2+</sup> ion. An important aspect concerns the L1 loop, that is in the closed conformation. These findings will be significant to understand the binding mode to clarify the improved potency and selectivity of the TIQ-based HDAC8 inhibitor.

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### MORPHOLOGY OF THE PINE PROCESSIONARY (*Thaumetopoea pityocampa*)

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The pine processionary moth *Thaumetopoea pityocampa* (Denis and Schiffermüller, 1755) is a species widely distributed in Mediterranean areas where its larval phases colonise different *Pinus* species often inducing a complete defoliation (Bonamonte *et al.*, 2013). The biological cycle consists of two phases: an aerial one with adults and larvae and a terrestrial one with pupae. The females lay their eggs (70-300) on the ends of the pine branches, building a sticky cocoon and covering it with the scales from the last segments of their abdomen. In 35-40 days, the larvae hatch and weave a web, creating a silky nest positioned on the tops of the trees where they overwinter. The larvae emerge, and after going through a series of 5 phases, they move, and begin their characteristic procession, searching for suitable ground to infiltrate. Pupae rest in a cocoon below the ground until the adult flies at night during summer. The life cycle is characterized by one generation per year, but some pupae frequently enter diapause for one year or more (Trematerra and Colacci, 2019). Pine processionary infestation is considered a major public health since larvae air can induce severe reactions in both humans and pets (Azcárate *et al.*, 2023). According to the most common pattern in Insects, *T. pityocampa* shows a size dimorphism, with males being smaller than females. In the males, the thorax is covered by dark brown hairs, and the abdomen is lighter, ochraceous, and provided with fewer hairs. Antennae are pectinate and ochraceous. In females, the thorax is covered by light hairs, and the abdomen is bigger and ochraceous with dark bands; antennae are filiform and ochraceous (Basso *et al.*, 2017). Despite its great socioeconomic relevance, the morphological characteristics of this species are still not well-documented. Available studies mainly focus on general morphology, biological cycle and ecological traits, thus leaving a knowledge gap on this topic. Here, we provide for the first time a detailed morphological description of *T. pityocampa* adults through an in-depth analysis based on observation under the stereomicroscope and scanning electron microscope. This approach allowed us to depict and analyze the wing scales'

shapes and the antennae's sensory equipment in both males and females. Our comparative morphological investigation evidenced sex-related differences in both wing scale and the number and type of sensilla.

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#### **Artemisia absinthium EXTRACT: A PROMISING CANDIDATE FOR ANTIBACTERIAL MOUTHWASH AGAINST ORAL PATHOGENS"**

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*Artemisia absinthium*, commonly referred to as absinthe, is a perennial plant characterized by broad ovate pointed leaves of a silvery-gray hue, attaining a height of 1.5 meters. Its essential components encompass thiol, thione, flavonoid ascorbic acid, and artemisinin within its essential oils. The antimalarial and antibacterial properties of artemisinin on various pathogens have evoked ongoing debates regarding its mode of action. This study pioneers the examination of *Artemisia absinthium* extract, enriched with artemisinin, against oral pathogens, specifically *Porphyromona gingivalis* and *P. Intermedia*. Initially, artemisinin levels were meticulously quantified using High-Performance Liquid Chromatography (HPLC), revealing a concentration of 25 µg/ml. Subsequently, total phenolics were assessed using the Folin method to elucidate their antimicrobial role, confirming their presence in the extract at 1.68 mM +/- 0.11. The antibacterial efficacy against oral anaerobic bacteria was scrutinized through common bacterial assays, morphological optimization, and measurement of Minimum Inhibitory Concentrations (MIC). The *Artemisia absinthium* extract exhibited 100% toxicity against *P. gingivalis* and *P. Intermedia* at the highest tested concentration, with MIC values of 2.5µg/ml for *P. gingivalis* and 5µg/ml for *P. Intermedia*. To augment our understanding of the extract's potency, a biocompatibility assay was conducted on human periodontal ligament cells, affirming its biocompatible profile. In summation, our preliminary findings suggest that *Artemisia absinthium* extract could serve as a viable herbal addition to mouthwash, offering antibacterial potential against oral pathogens while demonstrating non-toxic effects on other oral cells.

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