XXIV CONVEGNO ED ASSEMBLEA
COLLEGIO DEI DOCENTI DI ISTOLOGIA ED EMBRIOLOGIA UMANA

ABSTRACT BOOK

PLENARY SESSION

The cell as integrated mechanosensor

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Cellular mechanotransduction is a fundamental informational system by which cells read the structural features of their environment to control their own form and function. The YAP/TAZ transcriptional regulators are universal transducers of physical signals into gene-expression programs. Yet, how mechanotransduction is orchestrated at the whole cell level remains largely unknown. Through live imaging and AI-assisted 3D reconstructions of cells subjected to diverse mechanical stimulations we show the workings of a continuum of interconnected subcellular systems, ultimately converging on the nuclear envelope hub, that allows the cell to dynamically restructure itself in response to mechanical cues and to work as a mechanical rheostat controlling YAP/TAZ mechanosensing. Our findings also provide a unifying model that mechanistically merges mechanosignaling with the Hippo cascade. The current model by which YAP/TAZ are regulated by Hippo kinases is through direct YAP/TAZ phosphorylation. Our data in fact provide a more integrated perspective on this model, showing that, at least in the context of mechanotransduction, Hippo signaling inhibits YAP/TAZ indirectly.

New insights in studying bone mechanopathology

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Mechanobiology, an emerging multidisciplinary field of science at the interface of biology, engineering, chemistry and physics, focuses on the study of mechanisms by which cells sense and respond to mechanical signals. In the bone micro-environment, the mechanical stimuli represent an anabolic factor regulating bone homeostasis and function. In this context, the nuclear lamins and their interaction partners are arising growing interest in the field of mechanopathologies, given their pivotal roles in regulating cell response to mechanical stimuli, among which changes in the extracellular matrix composition and stiffness. Here, we demonstrated altered mechano-transduction and mechano-sensitivity in cell lines of osteosarcoma, the most common type of primary bone cancer. Indeed, nuclear lamins are differentially expressed in osteosarcoma cells and inversely correlate with cell line aggressiveness. Moreover, osteosarcoma cells with different aggressiveness respond differently to mechanical stimuli as shear stress, vertical vibration and uniaxial substrate deformation, further confirming a substantial deregulation of the mechano-sensitivity, likely due to altered lamins expression. In conclusion, our results clearly demonstrated the crucial role exerted by the mechano-transduction machinery in the onset and progression of osteosarcoma, thereby suggesting a change of point of view in studying pathological conditions by unravelling the mechanical forces driving disease mechanisms.

ER-phagy regulates ER dynamics during myogenesis

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Endoplasmic reticulum (ER) plasticity and autophagy are tightly interconnected cellular processes. The selective elimination of the ER (ER-phagy) contributes to preserve cell and tissue homeostasis as well as to reshape the ER membranes throughout cellular differentiation. Here we report that, during myogenesis, ER proteome and membranes morphology are deeply modified by ER-phagy activation. ER-phagy flux progressively increases during myoblasts fusion in parallel with the correct reassembly of the ER membranes network into the new sarcoplasmic reticulum structure. The ER-phagy receptor FAM134B is responsible for the ER-phagy induction and ER dynamics during myotubes formation. The classical and dynamic isoform of FAM134B (FAM134B1) is progressively degraded during myogenesis; however, its loss is compensated by the transcription of FAM134B2, a shorter isoform with reduced dynamicity and high avidity for the ER proteins and autophagy modifiers. The ratio between FAM134B1 and FAM134B2 decreases during myotubes maturation, implying a critical role for FAM134B2 in ER reshaping during cell differentiation. FAM134B knockout myoblasts differentiate at a lower rate showing an abnormal and disorganized ER membranes network in myotubes. A time dependent re-expression of FAM134B2 is necessary and sufficient to restore normal myogenesis with a correct ER membrane morphology. Our results show that ER-phagy is spontaneously activated during myoblasts fusion and regulates the dynamics of the ER during muscle cells differentiation. In mature myotubes ER-phagy flux returns to a physiological level leaving the ER membranes re-shaped in a well-organized network after the substitution of the dynamic FAM134B1 with its more rigid FAM134B2 isoform.

Dissecting the Molecular Interactions in endoplasmic reticulum-plasma membrane junctions of muscle and nerve.

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Endoplasmic reticulum-plasma membrane junctions (ER-PM junctions) are subcellular sites where the plasma membrane comes in close apposition with the endoplasmic reticulum, allowing the crosstalk between proteins in these two compartments and governing local or whole-cell Ca^{2+} signaling events. In skeletal muscle, ER-PM junctions—also known as triads—are formed and maintained by the proteins Junctophilin 1 and 2. These junctions are crucial sites where the electrical excitation of the myofiber membrane is translated into intracellular calcium release, leading to muscle contraction, a process termed excitation-contraction coupling (ECC). Striated muscle ER-PM junctions have been extensively studied from both structural and molecular perspectives. However, questions remained about which of the numerous proteins recruited to these junctions constitute the minimal essential set required for ECC. In contrast, much less is known about neuronal ER-PM junctions and the proteins involved, despite their acknowledged role in neuronal excitability and Ca^{2+} signaling. In recent years, we have sought to investigate the key structural and functional interactions of proteins at ER-PM junctions in muscles and neurons using a *de-novo* reconstitutional approach. This approach aims at recapitulating specific cellular processes or structures in undifferentiated, immortalized cell lines by heterologous expression of essential proteins that are known to participate in these processes or structures. The application of this strategy to muscle and neuronal junctional proteins led us to conclusively define the minimum set of proteins required for skeletal muscle ECC and to provide novel insights into junctophilins' structural and functional role in organizing neuronal ER-PM junction microdomains.

Exploiting advanced human induced pluripotent stem cell (iPSC)-based 3D models to study Globoid Cell Leukodystrophy

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Globoid cell leukodystrophy (GLD) is a lysosomal storage disorder caused by a deficiency in the enzyme ß-galactosylceramidase (GALC), essential for sphingolipid metabolism. This enzyme deficiency leads to the buildup of toxic substrates, triggering demyelination, neurodegeneration, and neuroinflammation in the central and peripheral nervous systems. While the exact mechanisms of GLD pathogenesis remain unclear, glial dysfunction has traditionally been considered the primary driver. However, recent findings suggest that neurons might play a more significant role in white matter damage than previously thought.
In our previous studies, we leveraged patient-specific human induced pluripotent stem cells (hiPSCs) to model GLD and investigate potential gene therapy approaches. Using hiPSC-derived neural stem/progenitor cells (NPCs) and mixed neuronal/glial 2D cultures, we uncovered mutation-dependent defects in neuronal and oligodendroglial differentiation, revealing early pathogenic signs that had gone unrecognized. However, these 2D culture conditions were insufficient to support full maturation of neurons and oligodendrocytes (OLs) (1, 2).
To overcome this limitation, we developed 3D spheroids from hiPSCs derived from both healthy donors (HD) and GLD patients, along with GALC knock-out (KO) and knock-in (KI) isogenic lines generated using CRISPR-Cas9 gene editing. Through a detailed time-course analysis (50, 100, and 150 days), we demonstrated that (i) 3D spheroids were efficiently and reproducibly generated from all hiPSC lines; (ii) both HD and GLD organoids contained immature (OLIG2+) and mature (MBP+) OLs, MAP2+ neurons, and GFAP+ astrocytes; (iii) GLD spheroids displayed pathological hallmarks that were not detected in 2D cultures. We applied single-cell RNA sequencing to analyze neuronal and glial subpopulations in spheroids at 100 and 150 days. This approach allowed us to identify distinct cell subtypes, revealing disease-specific alterations in gene expression and uncovering changes in cellular composition associated with the pathology. Integrating single-cell transcriptomics with phenotypic analysis gave us valuable insights into the molecular alterations driving disease progression.
Our study presents a novel 3D experimental platform that captures key aspects of GLD pathology, revealing molecular signatures, temporal dynamics of gene expression, and critical disease pathways. These findings provide a deeper understanding of GLD and offer a potential framework for studying other neurodegenerative leukodystrophies.

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Modeling cholangiocarcinoma development using biliary organoids

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Abstract: Cholangiocarcinoma (CCA) is a highly aggressive cancer originating from the biliary epithelium, characterized by a complex tumor microenvironment (TME) often marked by prominent desmoplasia. The tumor-associated desmoplastic reaction leads to distorted mechanical properties within the TME, such as increased stiffness and altered viscoelasticity, which significantly influence tumor progression and therapeutic responses. Here, we designed genetically engineered murine biliary organoids as an in vitro model to facilitate an in-depth investigation of CCA development. This advanced organoid system allows for exploration of critical, yet challenging-to-study, aspects of CCA progression, including the dynamic interplay between tumor cells and the mechanical components of the TME. Furthermore, this platform enables high-throughput screening, advancing drug discovery efforts aimed at targeting CCA more effectively. Overall, this platform not only enhances our understanding of CCA pathogenesis but also holds promise for identifying and optimizing therapeutic strategies that target the unique microenvironmental dependencies of this challenging liver cancer.

Patient-derived organoids from advanced colorectal cancer patients for precision oncology

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Chromosomal instability (CIN) consists in the progressive accumulation of aneuploidies due to chromosome segregation errors, that cause genome instability. About 70% of colorectal cancers (CRCs) display a CIN phenotype, associated with poor prognosis. We previously showed that oxaliplatin non-responder PDOs present enrichment of the t-RNA aminoacylation process and a shift towards oxidative phosphorylation dependence, while an exceptional response to palbociclib was detected in a PDO with MYC activation and an enrichment of chaperonin T-complex protein Ring Complex (TRiC) expression, involved in proteome integrity.

In the present study we aimed at characterizing the CIN profile of our organoids cohort in terms of genome, transcriptome and proteome, and to evaluate their capacity to reproduce the original CIN phenotype of tissues. We applied Weighted Genome Instability Index (wGII) to identify chromosome instability in our PDOs cohort and showed that not only there is a good correlation between organoids and tissues as regards wGII, but also that their genome expression profile recapitulates that of the TCGA cohort. Moreover, thanks to a proteotranscriptomic approach we unraveled differential processes in CIN+ and CIN- PDOs and uncovered significant relation between metabolic rewiring and epithelial-mesenchymal transition in CIN+ CRC PDOs.
In conclusion, our organoids are good models of CIN+ advanced CRC recapitulating both genomic and transcriptomic CIN features. Therefore they allow functional studies that could shade lights onto this molecular subtype with possible therapeutic implications.

Decoding the Role of TCF7L2/TCF4 in Shaping Colorectal Cancer Phenotypes and Aggressiveness

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The essential partnership between TCF72, a transcription factor belonging to the TCF/LEF family of DNA binding proteins, and the transcriptional co-activator β-catenin, has long been recognized as the central hub of the Wnt/β-catenin signalling pathway, a key regulator of homeostatic renewal and proliferation of intestinal stem cells. In 90% of colorectal cancers (CRCs), mutations in APC or β-catenin lead to unrestrained activity of the β-catenin/TCF7L2 complex, which recruits transcriptional and epigenetic cofactors to instigate pro-tumorigenic signals. The exclusive nature of TCF7L2/β-catenin interplay aligns with the notion that TCF7L2 acts as an oncogene in CRC; however, conflicting reports in humans and mice suggest a less orthodox interpretation. Of note, TCF7L2 is commonly mutated in microsatellite-stable, APC-mutant CRC (12-15% of cases). Mutations, evenly distributed across the gene, include heterozygous truncating, frameshift, and missense variants, supporting a loss-of-function, tumor-suppressive role, at odds with TCF7L2 alleged oncogenic function. The exact explanation for this apparent discrepancy is still ambiguous, and our understanding about the functional relevance of TCF7L2 mutations is frustrated by the lack of adequate experimental scrutiny in clinically relevant models. Against this framework, we assessed the biological, transcriptional and proteomic consequences of TCF7L2 mutations by capitalizing on the extensive resources available through XENTURION (XEnografts and TUmouroids for Research in Oncology), a collection of 128 CRC tumoroids and matched PDXs, extensively annotated with molecular and biological response data. In particular, we found that: i) intact TCF7L2 function is necessary for the survival of wild-type tumoroids (‘TCF7L2 dependent), whereas TCF7L2-mutant models become independent of TCF7L2 function, still maintaining reliance on β-catenin (‘TCF7L2-independent’); ii) the transcriptional consequences of TCF7L2 depletion are less pronounced in TCF7L2 mutant tumoroids as compared to wild-type models, in the face of pervasive and comparable effects produced by β-catenin silencing; iii) the expression of LEF1, a TCF7L2 paralogue belonging to the TCF/LEF family, is significantly higher in TCF7L2 mutant tumoroids, possibly sustaining ancillary transcriptional activity in a context of hypomorphic TCF7L2 function; iv) the TCF7L2/β-catenin interactome, including several epigenetic and transcriptional co-factors, is differentially represented in TCF7L2-mutant versus wild-type tumoroids, and denotes idiosyncratic biological functions; v) TCF7L2 mutations significantly associate with sensitivity to the clinically approved anti-EGFR antibody, cetuximab. All in all, these results indicate that TCF7L2 mutations yield a hypomorphic phenotype that enforces alternative β-catenin partnerships and encourages a distinct trajectory of gene expression programs (possibly including increased reliance on EGFR), with potential clinical applicability.

PARALLEL SESSIONS 1:
CELL SIGNALLING AND CANCER

Cell motility and nuclear architecture regulation through MET/YAP-driven modulation of perinuclear actin cap alignment

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The actin cytoskeleton surrounding the nuclear envelope on the apical side and regulating nuclear shape and translocation is organized in a dome-like structure named "perinuclear actin cap". Although MET receptor pathway has been widely associated with actin remodeling, no studies are available on receptor-mediated selective regulation of the perinuclear actin cap fibers. In a cancer cell model with hyperactivation of MET receptor, we registered an altered arrangement of the actin cap filaments, crashing into perinuclear patches associated with spherical nuclei, meandering cell migration and inactivation of the mechanotransducer YAP1. CRISPR/Cas9 knock-out of MET receptor in the same model induced YAP reactivation and proper assembly of the cap, resulting in flat cell and nuclear phenotype and polarized cell motility. In line, by experimentally inducing the constitutive activation of MET pathway in normal epithelial cells we induced actin cap misalignment and nuclear vertical expansion. Moreover, the expression of the constitutively active YAP mutant YAP5SA was found to be sufficient to overcome the effects of oncogenic MET.

Finally, the enlarged nuclear phenotype prompted by MET hyperactivation and consequent YAP inhibition resulted in a global decrease of nuclear stiffness, pointing to a pro-tumorigenic effect that could foster metastatic dissemination by facilitating cell confined migration through the increase of nuclear deformability. Overall, our work identifies for the first time a signaling pathway involving MET-mediated YAP1 inhibition and actin cap misalignment, with potentially crucial implications for nuclear integrity and cell migration in MET-dependent cancer cells.

Understanding the determinants of the invisible phase of metastasis

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The clinical manifestation of metastases in some cancers, such as breast and prostate, often takes years. Several intrinsic and extrinsic factors have been shown to influence the quiescence and reawakening of disseminated dormant cancer cells (DDCCs). However, the signals and processes that maintain the survival of DDCCs in a foreign environment remain poorly understood. An in vivo loss-of-function screen identified several proteins involved in the crosstalk between lung epithelial cells and breast DDCCs. Our recent studies employing an organotypic lung system demonstrated that the lysosomal compartment is activated in DDCCs and is modulated by the pro-survival ephrin receptor EphB6. Furthermore, lysosomal components are enriched in DDCCs in vivo and correlate with relapse in ER+ breast cancer patients. Direct coculture of DDCCs with alveolar type I-like lung epithelial cells and dissemination into the lung drive lysosomal accumulation and EphB6 induction. Furthermore, EphB6 signalling promotes proliferation of surrounding lung parenchymal cells in vivo. In addition to microenvironmental-driven mechanisms, our preliminary results with a functional CRISPRs-based lineage tracing tool also support the existence of pre-encoded traits in some recurrent metastatic clones.

Time-lapse observations identify distinctive types of emperipolesis between neutrophils and megakaryocytes from the bone marrow of Gata1^{low} mice with myelofibrosis

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In the bone marrow of patients with myelofibrosis, the presence of naked nuclei of megakaryocytes (MK) presumably originating from para-apoptosis induced by pathological emperipolesis between these cells and malignant neutrophils (Neu) has been observed[1-2]. The discovery that MKs from these patients express high levels of the chemoattractant interleukin-8 (CXCL1 in mice)[3], led us to discover that treatment of the Gata1^{low} mouse model with a non-competitive allosteric inhibitor of CXCR1/CXCR2 (Reparixin), prevents both fibrosis and pathological MK-Neu emperipolesis[4]. However, the mechanistic detail of this interaction remains elusive. Huang et al[5], combining transmission electron microscopy and time-lapse observations by confocal microscopy, demonstrated that emperipolesis between normal murine Neu/MK occurs in two fashions: fast (<10 min), with Neu assuming an amoeboid morphology as it rapidly traverses the MK, and slow (>60 min), during which Neu is retained within the MK cytoplasm near the nucleus. In both processes, the interacting cells remain alive. The functions of these interactions are still unknown.

Using the paper by Huang et al as a foundation, we investigate here the features of the pathological MK/Neu emperipolesis in myelofibrosis by performing time-lapse observations of co-cultures between Neu and MK (1:10 ratio) purified from the bone marrow of old CD1 and Gata1^{low} mice in the presence of thrombopoietin. MK/Neu from Gata1^{low} mice were cultured with or without Reparixin (10 μM). Furthermore, RNAseq profiling of CD41+ cells (>90% pure by reanalysis) from the bone marrow of these mice was compared. Time-lapse experiments were performed with 30 sec time intervals during 48 h of co-culture. The total number of MK analyzed was 45 CD1, 94 Gata1^{low}, and 51 Gata1^{low} with Reparixin for a total of 147 h of observations. The observation time window was divided into four groups: 0-12; 13-14, 25-36 and 37-48 hours. We observed four types of cellular interactions that were identified by the numerical code: 0=none; 1=low, touch and go (<90 sec); 2=long (>90 sec) with possible emperipolesis; 3=long leading to MK disarrangement and death. Changes in MK morphology leading to death occurred within 15 min. Type 0 interactions were mainly observed in the 0-12 time window and the frequency was 25% for CD1 and only 3% for Gata1^{low} MK, with or without Reparixin. Type 1 and 2 interactions were mainly observed in the 13-14, 25-36 and 37-48 hour time windows and their frequency was not statistically different between groups. Of note, type 2 interactions were not observed in the Gata1^{low} co-culture in the 37-48 hour time window. In this time window, Gata1^{low} co-cultures mainly showed type 3 interactions which accounted for 33% of all interactions observed between Gata1^{low} MK and Neu. In contrast, type 3 interactions were rarely observed in the co-culture with CD1 cells and Gata1^{low} cells with Reparixin (Chi-Square $p<0.0001$ vs. Gata1^{low} without Reparixin). The fact that in both the CD1 and Gata1^{low} groups, type 2/3 interactions were observed in large numbers after a few hours of co-culture suggests that they are induced by chemoattractants released by the MK. The observation that Reparixin reduced type 3 interactions without affecting type 2 interactions indicates that these are not emperipolesis and that they are dependent on CXCL1. Furthermore, RNAseq analyses identified that Gata1^{low} MKs have an activated CXCR1/CXCR2 signature compared to controls and are enriched for genes implicated in phagocytosis: upregulation of 16 genes that promote phagocytosis (C3, Camk1d, Cfp, Clec7a, Cyba, Fcgr1, Fpr2, Il1b, Lbp, Ptprj, Rab27a, Rab31, Sirpa, Sirpb1b, Sirpb1c, Slc11a1) and of only 8 genes that inhibit phagocytosis (Atg3, Atg7, Cnn2, Prtn3, Pten, Siglece, Sirpa, Tlr2). Of note, purified Gata1^{low} MKs had a clear Neu signature that is likely a consequence of the Neu embedded in the MK which contaminated the cell preparations.

In conclusion, unlike Huang et al[5], MK-Neu interactions observed in old CD1 and Gata1^{low} co-cultures are more rapid and occur after a temporal delay of cell inactivity, suggesting that they are dependent on the release of chemoattractants. The Gata1^{low} co-culture display a novel type of MK-Neu interaction that occurs after at least 24 hours, leads to cell death, and is dependent on CXCL1.

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Epigenetic/transcriptional targeting of Flotillin-1 gene promoter by nuclear miR-223: unveiling a new mechanism underlying myelopoiesis

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Developmental cell programs, including the hematopoietic cell lineages specification and fate, are intricately regulated by epigenetic signals and microRNAs (miRNAs). MiRNAs primarily mediates post-transcriptional silencing of target mRNAs. However, they also exert significant roles within the nucleus. Here, they can bind to complementary DNA sequences at specific chromatin sites, providing the driving force for the DNA targeting activity of chromatin modifiers affecting gene transcription. The nuclear function of miRNAs appears crucial for somatic stem cell proliferation, lineage specification, and differentiation.
To address this issue, we set-up a new technology based on the transfection of Cy5-labeled double-stranded oligonucleotides, mimicking the activity of miR-223, or a scramble Cy5-miRNA (negative control), in myeloid cells undergoing granulo-monocytic differentiation. MiRNA-bound chromatins were immunoprecipitated using anti-Cy5 antibodies for the identification of DNA sequences bound by Cy5-labelled miR-223. In addition, chromatins were immunoprecipitated with antibodies specifically recognizing trimethylation of lysine 4 or 27 on histone H3 (H3K4me3, H3K27me3), to detect the chromatin status at these sites. Massive parallel sequencing (ChIP-Seq) of immunoprecipitated chromatins and computational-bioinformatic analysis allowed the detection, at the whole genome level, of the genomic sequences complementarily bound by Cy5-labeled miR-223 and the epigenetic changes occurring at these sites during myeloid differentiation.
Among the DNA sequences bound by nuclear miR-223, we selected an evolutionarily conserved region in the promoter of Flotillin-1 (FLOT1) gene, encoding a lipid-rafts associated protein whose role in hematopoiesis is still largely unknown. We found that during myeloid differentiation, this FLOT1 promoter region is bound by a complex comprising miR-223, RISC component AGO1, and the trithorax (TrxG) protein RBBP5 and is enriched in H3K4me3 marks. Accordingly, FLOT1 mRNA and protein levels are increased in primary human CD34+ hematopoietic progenitors and myeloid cell lines undergoing granulo-monocytic differentiation.
In these cells, the silencing of miR-223 significantly impairs the upregulation of FLOT1 expression and granulocytopoiesis, while FLOT1 overexpression enhances the expression of myeloid differentiation markers CD11b and CD14. Notably, FLOT1 overexpression drives the receptor for Colony Stimulating Factor 1 (CSF1R), a cytokine which controls myelopoiesis, to Rab4 endocytic vesicles, thus increasing receptor recycling to cell membrane after CSF1 stimulation.
The analysis of publicly available datasets (TCGA and GSE13159) and bone marrow (BM) samples from 25 *de novo* acute myeloid leukemia (AML), from our clinical centers used for validation, showed that FLOT1 mRNA expression is significantly downregulated in AML blasts when compared to healthy BM cells; the lowest FLOT1 expression levels were measured in acute promyelocytic leukemias (APLs) blasts.
Collectively, these results enhance our understanding on the molecular mechanisms underlying myeloid differentiation and highlights the physiological roles of nuclear miRNAs and FLOT1 in hematopoiesis. Dysregulation of their functions might cause the blockage of progenitors' differentiation program resulting in leukemic transformation.

Intracellular cholesterol reduction mediated by NFATc1 deficiency affects cell signaling and mitochondria metabolism

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NFAT is a family of five different transcription factors (NFATc1-5), four of which are activated by the Ca^{2+}/calmodulin-dependent phosphatase calcineurin. Notably, the calcineurin/NFAT signaling pathway is an effector of the LCK kinase activity, fundamental to maintaining normal T-cell physiology, and has been found to be deregulated both in B/T-cell lymphomas and leukemias, as well as in solid tumors (i.e prostate, breast and pancreatic cancer). Consistent with our previous evidence of LCK kinase hyperactivation in glucocorticoid (GC) resistant T- cell acute lymphoblastic leukemia (T-ALL) cells, in this study we identified NFATc1 and NFATc2 family members as mediators of GC resistance. Next, we aimed to unveil the molecular mechanisms through which either NFATc1 or NFATc2 support GC resistance. To achieve this goal, we applied omics approaches (Gene Expression Profile, Nuclear Magnetic Resonance and metabolomic analysis) on an experimental model represented by NFATc1 and NFATc2 knockdown cells. In particular, we observed a dramatic decrease of the intracellular cholesterol abundance together with a shift toward a more glycolytic phenotype in NFATc1 knock down cells. In good agreement with this result, by Chromatin Immune Precipitation we revealed that NFATc1 directly controls the transcription of key enzymes of cholesterol biosynthesis process such as HMGCS1, EBP and DHCR7. Since cholesterol is a key component of the plasma membrane lipid raft (LR) elements, by immunofluorescence we demonstrated that its downregulation decreases the number of LRs, as well as the anchoring and activation of key proteins and co-receptors such as the lymphocyte-specific protein tyrosine kinase (LCK) as well as CD4 and CD8, thus impairing the TCR signaling cascade. Intriguingly, by coupling Transmission Electron Microscopy observations and metabolic assays we unveiled how NFATc1 knock down switches cells toward a more glycolytic phenotype despite functional mitochondria. Finally, by High-Throughput drug synergism Screening (HTS) and Highest Single Agent (HSA) approach we observed that the inhibition of cholesterol biosynthesis by simvastatin, both in cell lines and primary cells, sensitizes cells to GC action by restoring glucocorticoid receptor transcriptional activity. All together these results reveal for the first time that NFATc1 regulates intracellular cholesterol abundance and in turn modulates intracellular cell signaling, T-cell metabolism and response to glucocorticoids, paving the way to novel therapeutic interventions.

PARALLEL SESSION 1

DEVELOPMENT, HOMEOSTASIS AND REGENERATION

Molecular regulation of placental development: the role of EGFL7

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Placentation is a highly regulated process aimed to create a proper fetal-maternal interface in order to sustain fetal growth throughout pregnancy. A variety of factors and molecular pathways are involved in the regulation of trophoblast differentiation and invasion, fetal vasculogenesis and maternal vascular remodelling. An imbalance in the expression and activation of such molecules and signalling pathways can result in placental pathologies such as preeclampsia (PE), a disease characterized by an insufficient trophoblast invasion of maternal tissues and an inadequate maternal vascular remodelling, together with an imbalance in the release of pro- and anti- angiogenic factors, resulting in maternal gestational hypertension, proteinuria and oedema, the classical clinical manifestations of the disease. Some years ago, our group identified Epidermal Growth Factor-Like domain 7 (EGFL7) as a largely endothelial-restricted secreted factor critical for embryonic vascular development. More recently, we unravelled the role of EGFL7 in the molecular regulation of placental development. Indeed, using gain- and loss- of function studies, we demonstrated that, beyond its classical role in angiogenesis, EGFL7 is involved in promoting migration and invasion of cyto- and syncytio- trophoblast, which both express EGFL7. To confirm its pivotal role in placentation, we found that EGFL7 expression is dramatically reduced in placenta of women affected by PE. Considering that EGFL7 is a secreted factor, we measured its circulating levels during pregnancy and found that they are undetectable in nonpregnant women, increase during pregnancy and decline toward term, being increased in case of PE. In order to explore new potential therapeutic strategies, very recently, using an ex-vivo model of chorionic villous culture, we proposed the increased expression of EGFL7 as a new potential mechanism of action of pravastatin, that has gained interest for the prevention and treatment of PE. Overall, our data demonstrate EGFL7 as a novel secreted factor involved in proper placental development.

IZUMO1 binding to the orphan receptor LILRA5 mediates sperm recognition by the innate immune system

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The interaction between the sperm protein IZUMO1 and its egg binding partner JUNO is essential for mammalian fertilization[1]. While it is well established that the pair is required for adhesion of sperm and egg, their role in cell fusion has not been fully elucidated[2], and the existence of a second receptor for IZUMO1 has been postulated. We used a systematic protein interaction assay to identify new IZUMO1 binding proteins and identified the orphan receptor LILRA5 (leukocyte immunoglobulin-like receptor A5) as a candidate. We showed that IZUMO1-LILRA5 binding is conserved in Humans and Gorillas but not in mice, and it does not interfere with the IZUMO1-JUNO interaction. Predictions of the 3D structure of the IZUMO1-LILRA5 complex, suggest that the N-terminal helical domain of IZUMO binds to residues in the LILRA5 Ig-like domains 1 and 2. We experimentally mutated some of the residues at the predicted interaction interface and found that the mutations disrupted the binding in vitro, supporting the AlphaFold models. The introduction of semen in the female reproductive tract induces a physiological reaction known as the leukocytic reaction[3] and it has been reported that surplus sperm not involved in fertilization are phagocytized. Because LILRA5 is expressed on monocytes, macrophages, and neutrophils and is not detected in human eggs, we hypothesize that its interaction with IZUMO1, mediates the clearance of supernumerary sperm from the female reproductive tract.

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Role of Dipeptidyl peptidase 3 (Dpp3) in disease tolerance and immune resistance against bacterial pulmonary infection.

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ABSTRACT: *Klebsiella pneumoniae* is a common cause of pneumonia, particularly in elderly and immunocompromised patients, often leading to sepsis. Over the past decades, the emergence of multi-drug resistant strains made the treatment of *K. pneumoniae* a big challenge. Recent studies have demonstrated that host defense plays a critical role in eradicating *K. pneumoniae*.
The dipeptidyl peptidase 3 (DPP3) is a ubiquitous cytosolic metallopeptidase acting in the degradation of various bioactive peptides. In addition, it has been involved in the Nrf2 antioxidant pathway. Its high blood concentrations, related to massive cell death and inflammation, predict high risk organ dysfunction and mortality in patients with septic shock. To address the relevance of DPP3 in *K. pneumoniae* infections, we applied a model of lung infection in the DPP3 knock-out mice. We evaluated the mortality and the pulmonary and systemic bacterial load at different time points post infection. The levels of pro- and anti-inflammatory cytokines were measured, histopathological damage and inflammation were assessed in the lung. Results showed that DPP3 deficiency was associated with markedly decreased lung colonization and systemic bacterial burden, resulting in a significant survival advantage. Upon bacterial infection, DPP3-/- mice exhibited reduced tissue damage and weakened pulmonary and systemic inflammation compared with WT mice. Adoptive transfer experiment indicated that deficiency of DPP3 in immune cells was sufficient to enhance the lung bacterial clearance. Thus, we further used transcriptomics and cellular assays to define the specific role of DPP3 in lung immune cell biology. We found that lack of DPP3 induced transcriptional and metabolic programs characteristic of effector cells, associated with enhanced inflammatory signaling pathways. Accordingly, both innate and adaptive DPP3-deficient immune cells exerted more potent antimicrobial responses, particularly ROS and Th1/Th17 cytokines production. These findings point to DPP3 as novel immune checkpoint that shapes immunity by controlling the threshold for activation and resistance pathways. These results provide a framework for new therapeutic strategy against K. pneumoniae through improving host immunity.

Ultrastructural morphological study as a tool for identifying early heart damage: the protective effects of a high-fiber diet against dysbiosis

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Recent clinical and experimental findings in human microbiota research highlight that gut microbiota dysbiosis plays a role in the development and progression of breast cancer (BC), and they suggest dietary intervention as a potential strategy to synergize with anticancer therapies. Moreover, it's well established the role of dysbiosis also in the onset of cardiovascular diseases (CVDs).

Patients with BC, especially those with the HER2-positive subtype, are particularly vulnerable to cardiovascular events due to the adverse effects of cancer therapies on cardiac tissues. CVDs represent the most common and significant comorbidities in BC patients, with many post-treatment recurrences and cancer-specific deaths linked to cardiovascular events.

We hypothesize that targeting dysbiosis through dietary interventions could benefit BC patients by providing cardioprotection in addition to anti-tumor effects.

In this study, we used an in vivo HER2-positive BC mouse model to investigate the effects of gut microbiota homeostasis alteration, induced by administering the antibiotic vancomycin (VAN) in drinking water, on cardiac muscle tissue. Additionally, we explored whether a high-fiber diet (HFiber) could provide cardioprotection following antibiotic-induced dysbiosis.

Light microscopy analysis of heart tissue structure showed no visible morphological changes in response to VAN treatment. However, digital transmission electron microscopy (TEM) analysis of cardiomyocyte ultrastructure revealed that, although the myofibril arrangement was preserved, mitochondria in the VAN group were significantly larger, more elongated, and often partially or entirely damaged compared to the untreated control group (CT). No significant ultrastructural alterations were observed in the HFiber group, suggesting potential protection against mitochondrial damage. Molecular characterization via real-time PCR indicated that antibiotic treatment upregulated genes associated with mitochondrial fusion (e.g., OPA1, DNM1, MFS1) and oxidative damage (e.g., SOD2), while showing a trend toward reduced expression of biogenesis-related genes (e.g., NRF1, PPRargc1). These findings are consistent with the observed mitochondrial enlargement in dysbiotic mice. Proteomic analysis by FT-Orbitrap highlighted alterations of the mitochondrial respiratory chain in dysbiotic mice and also an increase of interstitial collagen, not observed with the followed high-fiber diet administration. Luminex analysis of circulating inflammatory markers showed changes relevant to CVD pathogenesis.

In conclusion: i) dysbiosis drived by VAN treatment induces early ultrastructural alterations in intermyofibrillar mitochondria of cardiomyocytes; ii) TEM analysis is effective in detecting ultrastructural damage in the absence of overt histological tissue changes, as confirmed by molecular and proteomic data; iii) a high-fiber diet may counteract the adverse cardiac effects triggered by antibiotic-induced dysbiosis, providing potential protective benefits for BC patients at risk for CVDs.

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IPs from autologous human gingival stem cells and extracellular vesicles: a new tool for cardiac regeneration.

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Abstract

The growing number of chronic diseases afflicting the global population underscores the urgency of identifying alternative efficient technologies. Technological innovation and advances in regenerative clinical applications are promising tools in transformative therapeutic impact. The core of regenerative medicine is stem cells. The regenerative field has focused on the study and use of multipotent adult stem cells (MSCs). Furthermore, in recent years, to have a greater potential of the cells for therapeutic purposes, and at the same time to bypass the controversies related to the ethical problems of ESCs use, a lot of studies are focusing on the induced pluripotent stem cell lines (iPSCs) generation. One of the objectives of this work is the characterization of a new pluripotency cell line obtained for the first time by reprogramming human gingival mesenchymal stem cells (hGMSCs-derived iPS cell line) through a non-integrating method. The characterization of the hGMSCs-derived iPS is performed through the evaluation of pluripotent markers expression by real-time PCR, confocal microscopy, and flow cytofluorimetry. The gene stability of the hGMSCs-derived iPS cell line is demonstrated by karyotype reconstruction and the genetic fingerprinting of the starting cells is reported by DNA fingerprinting technique. Morphological analysis of the hGMSCs-derived iPS colonies was done by scanning electron microscopy (SEM) in addition to light microscopy. The ability of the hGMSCs-derived iPS to differentiate into the three embryonic layers is demonstrated through the *in vitro* generation of embryoid bodies, which are evaluated by real-time PCR and confocal microscopy. Therefore, it is characterized the exosome content of both starting hGMSCs and hGMSCs-derived iPS in order to identify more potent therapeutic approaches for regenerative medicine. The second objective of this work is to obtain a new autologous primary cardiomyocytes line from hGMSCs-derived iPS cells through the only use of specific medium. This allows to give an innovative approach for personalized cardiac tissue regeneration.

PARALLEL SESSION 2

DEGENERATIVE DISEASES AND RESPONSE TO INJURY

Lysosomal storage diseases as a paradigm to study the role of autophagy lysosomal pathways in brain homeostasis and neurodegeneration

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Autophagy-lysosomal pathway (ALP) plays a major role in brain homeostasis. Indeed, disruption of ALP degradation contributes to neurodegeneration in many diseases, including age-related disorders, such as Alzheimer's and Parkinson's. Another hallmark of several neurodegenerative conditions is the CNS deposition of amyloidogenic proteins, including α-syn, tau, and Aβ, which causes cytotoxicity by interfering with various cellular functions.

Increasing body of evidence show that ALP dysfunction and amyloid aggregation are functionally interconnected and induce each other during neurodegenerative processes. We have studied this interplay and the underlying mechanisms in lysosomal storage diseases (LSDs). LSDs are a class of rare conditions often associated to a severe neurodegenerative course and caused by inherited lysosomal gene deficits, which lead to global lysosomal dysfunction and autophagy flux impairment. Moreover, we have shown that brain deposition of multiple amyloid proteins is a key contributor to neurodegenerative processes in several LSDs. Therefore, LSDs represent a paradigm of diseases in which neuropathology is driven by ALP stress and amyloid deposition.

Amyloids can be degraded by ALP. Our data support the concept that amyloid aggregation itself may affect ALP, thus triggering a vicious cycle, which boost neurodegenerative cascades. Using brain samples and neuronal cultures from mouse models mucopolysaccharidoses (MPS), a large family of LSDs, we demonstrate that amyloids accumulate in the proximity of lysosomes, causing massive lysosomal enlargement and perinuclear clustering. Cell-free data show that such effect is mediated by the direct binding of α-synuclein, a major component of amyloids, to the lysosomal membrane that induce the assembly and fusion of lysosomes in large structures. This, in turns, reduces lysosomal dynamics, thus impairing lysosomal capability to encounter and clear autophagosomes to complete autophagy.

We have also shown that amyloid deposition in MPS is also involved in the initiation of inflammatory cascades by triggering microglia and astroglia activation. Specifically, our data indicate a strong induction of A1 reactive astrocytes, a neurotoxic phenotype, which involve the loosing of normal homeostatic functions and the secretion of neurotoxic factors.

Our results uncover mechanistic insights linking amyloid aggregation to ALP-mediated brain homeostasis and neuroinflammation in severe neurodegenerative conditions.

We also present data showing how targeting these mechanisms may represent an innovative therapeutic avenue to treat neuronopathy in MPS, and in general in LSDs.

Clinical and histopathological evaluation of adipose stem cells-derived extracellular vesicles as a novel therapy in an experimental model of multiple sclerosis

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Adipose mesenchymal stem cells (ASCs) represent a promising therapeutic strategy for neurological diseases due to their ability to reach the damaged sites in the central nervous system (CNS), mediate neuroprotection and modulate the immune response. Recent findings have revealed that the positive effects of ASCs are mainly due to the production of small extracellular vesicles (EVs). In this regard, many studies indicate that ASC-EVs secrete protective factors and can modulate the activated immune response in various CNS diseases and in particular in multiple sclerosis (MS), whose pathogenesis is characterized by an immune dysregulation. In order to validate the treatment with ASC-EVs as a future therapy, we evaluate the intranasal administration as a facilitated access route to the CNS and a direct and safe way to distribute EVs in patients.
The aim of our study is to investigate the therapeutic potential of ASC-EVs in a mouse model of MS, the Experimental autoimmune encephalomyelitis (EAE), testing two different therapeutic regimens of intranasal delivery.
The administration of three doses of ASC-EVs starting from the onset of the disease every four days, displayed a significant reduction in disease clinical severity, which became evident after the end of treatment. Instead, ASC-EVs-treated animals displayed an early milder clinical course lasting until the late chronic phase of the disease when injected for ten consecutive days from disease onset.
The improvement of the clinical outcome was accompanied by morphological changes in the EAE affected mice with a significant reduction of T lymphocytes inflammatory infiltrates, a reduction of the demyelinated areas and a decrease of microglial cells number in the spinal cord. In addition, several molecules (cytokines and chemokines) related to inflammatory response in EAE disease were modulated in both brain and spinal cord.
Our results showed beneficial effects of ASC-EVs to reduce the clinical and pathological course of EAE supporting their therapeutic potential for the development of future and less invasive ASC-EVs therapies for the treatment of MS.

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TRPML1 activation improves skeletal muscle deterioration and storage pathology in Pompe Disease

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Pompe disease (PD) is a metabolic myopathy due to GAA gene mutations, causing lysosomal acid alpha-glucosidase deficiency. This leads to glycogen storage, impaired autophagy, and severe muscle dysfunction. Current treatment of PD patients is based on enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA, alglucosidase alpha) in association with supportive or palliative interventions. Despite significant success of ERT in improving survival, heart disease and motor dysfunction in PD patients, limitations of its efficacy have emerged. In this scenario alternative strategies to treat the skeletal muscle pathology are essential.
Important evidence demonstrated that the lysosomal Ca^{2+} channel TRPML1 (ML1) represents key regulator of sarcolemma resealing mechanism and the autophagy pathway. Modulation of these two mechanisms could represent a potential therapeutic target to rescue the muscle storage pathology and fibers degeneration in PD patients. Lysosome may provide a major source of membranes for repairing damaged sarcolemma, and ML1 is essential for Ca2+-dependent delivery of lysosomal membranes (i.e., lysosomal exocytosis) to damaged sites. Due to the ability of promote the cellular clearance and regeneration of muscle fibers, the TRPML1 induction may represent an alternative strategy for the treatment of PD pathology. Here we demonstrated that genetic activation of TRPML1 improves the lysosomal dysfunction by reducing the glycogen storage and ameliorate the autophagy impairment in skeletal muscle of Pompe disease mouse model with restoration of skeletal muscle organization. Collectively, the TRPML1 induction represent a novel beneficial therapeutic strategy for the skeletal muscle pathology in PD patients.

Intestinal microbiota-gut-brain axis in neurodegenerative disorders: from bench to bedside

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The microbiota gut-brain (MGB) axis is emerging as a pivotal pathway involved in the maintenance of brain physiology as well as in ~~the~~ sustaining ~~the~~ pathophysiological events underlying neurological aging and disorders, including mild cognitive impairment (MCI), dementia, multiple sclerosis (MS), Alzheimer's disease (AD) and Parkinson's disease (PD) [1]. The mechanisms underlying the MGB rely mainly on interactions between the enteric bacteria, intestinal epithelial barrier, immune system and nerve pathways. For instance, pathogenic bacterial products can translocate into the blood stream and spread upwards to the brain, where they can impair blood brain barrier integrity and influence the central circuits. In addition, bacterial products can directly activate circulating immune/inflammatory cells, which, in turn, migrate to the CNS [1-2].
Of interest, the manipulation of gut microbiota with fecal transplantation, pre- or probiotics or drugs acting on gut barrier or enteric immune system have been proposed as useful therapeutical approaches for brain diseases. Indeed, short chain fat acids (SCFAs)-derived indigestible fibers, prebiotics and multiple probiotics have been shown to exert beneficial effects in animal models and patients with CNS diseases [2].
On these bases, after providing an overview of the mechanisms underlying MGB axis in brain physiology and pathology, current preclinical and human evidence showing the beneficial effects resulting from the modulation of gut microbiota in central nervous system (CNS) disorders will be discussed [2-3]. In addition, special attention will be paid to show the results of my research on the effects of novel gut-directed anti-inflammatory drug in AD pathology, thus providing new insights about the role of microbiota gut-brain axis in CNS diseases and clarifying how this bacteria-gut-brain network can impact on clinical practice, in terms of therapeutic strategies, in the foreseeable future.

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Developing an *in vitro* model to unveil IMPG2 role in Retinitis Pigmentosa

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The retina, the innermost neural layer of the eye, is composed of six types of organized nerve cells connected by synapses. Among them, photoreceptors, divided into rods and cones, are the ones responsible for phototransduction: the conversion of light stimuli into electrical impulses that the brain processes to form a visual image (Mahabadi e Al Khalili 2024). The manifestation of retinal diseases can occur at different stages of life, in some cases severely affecting visual abilities. Retinitis pigmentosa (RP) defines a heterogeneous group of inherited disorders that cause gradual loss of vision due to degeneration of photoreceptors. Rods, followed by cones, lose their integrity and function, leading first to night blindness, then to loss of visual acuity, and finally to blindness (Bandah-Rozenfeld et al. 2010).
Mutations in 53 different genes ("RetNet - Retinal Information Network", see https://retnet.org/) have been associated with the onset of the autosomal recessive form of RP. Among them, mutations in the interphotoreceptor matrix proteoglycan 2 (IMPG2) gene have been identified (Brandl et al. 2017). IMPG2, a proteoglycan synthesized by photoreceptors and secreted in the interphotoreceptor matrix, plays a critical role in retinal adhesion, cell differentiation, and transport (Ishikawa, Sawada, and Yoshitomi 2015). However, its role in retinal organization has not been characterized.
This project aims to elucidate the location, function and relevance of IMPG2 in both healthy and diseased states using in vitro models. By transfecting the human IMGP2 gene into cells, we have created a manipulable model to study differences in proteoglycan organization and function. Site-directed mutagenesis will be used to introduce mutations in IMPG2 that cause the autosomal recessive form of RP, allowing us to study the mechanisms leading to retinal degeneration and to explore potential therapeutic targets

Role of Semaphorin 6C in remote cell death after focal brain injury

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Abstract

Axonal degeneration is recognised as a major driver of disability and disease progression in central nervous system (CNS) disorders such as amyotrophic lateral sclerosis, multiple sclerosis, traumatic brain injury and spinal cord injury.

In such disorders, the impairment is also caused by degeneration of distal regions that are functionally and/or structurally connected to the primary lesion site.

Semaphorins are a complex class of secreted or transmembrane proteins that are critical for axonal growth during development in the CNS. Their expression levels decrease in adulthood, but can be modulated after injury to the CNS.

This study addresses the function of semaphorin-6c (Sema6c) up-regulation on the fate of distal axotomized precerebellar neurons in a mouse model of focal brain injury known as hemicerebellectomy (HCb). We provide morphological, functional and biochemical evidence that HCb upregulates Sema6c in axotomized neurons and glial cells. By time course analysis, we determined its parallel progression with the apoptotic cascade in neurons. In addition, we found that Sema6c induction was clearly detected in microglia with a pro-inflammatory phenotype, consistent with its role in influencing the progression of remote degeneration. By modulating Sema6c, we were able to demonstrate its effective role in neuronal damage and in the worsening of functional recovery. These findings have therapeutic implications, demonstrating the potential of Sema6c modulation for the treatment of acute brain pathologies such as traumatic brain injury and spinal cord injury.

PARALLEL SESSION 2
CANCER THERAPY

Dissecting macrophage diversity in cancer to unveil distinct contributions to therapeutic resistance

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The efficacy of immune checkpoint inhibitors (ICIs) in cancer relies on the presence of an effective T cell-mediated immune response. However, multiple features of the tumor microenvironment constrain T cells activation thus contributing to therapy resistance. Importantly, emerging studies have shown that ICIs efficacy is in part dependent on tumor associated macrophages (TAMs). On this line, we recently discovered that lipid laden macrophages (LLMs) infiltrate tumors, including prostate cancer, ovarian cancer and melanoma. Here we applied single cell RNA sequencing to the immune infiltrate of prostate cancer and melanoma. We then implemented a multiparametric flow cytometry strategy to confirm transcriptional findings. In vivo, we set up transgenic and transplantable models of prostate cancer and melanoma to investigate the immune composition of tumors. Finally, we isolated tumor infiltrating lipid-loaded macrophages and we performed bulk RNA sequencing and mass spectrometry to dissect the transcriptional features and the proteome profile of LLMs. We demonstrated that the abundance of LLMs correlates with tumor size and we discovered that LLMs promote cancer progression in association with immune evasion and poor response to chemotherapy. Lipid intake in TAMs is partially dependent on scavenging by MARCO and MARCO neutralization in vivo deplete LLMs both in prostate cancer and melanoma models. Mechanistically, we also found that TAMs display a dysfunctional autophagy provoked by a deregulation of the TFEB-dependent CLEAR signaling pathway that is responsible of lipid accumulation. Finally, we demonstrated that LLMs infiltrate melanoma and ovarian tumors in patients that are refractory to ICIs. Importantly, this enrichment is even observed prior to treatment initiation, suggesting that LLMs may be predictive of ICIs response. Together, our findings identify a heterotypic crosstalk involving LLMs and cancer cells that drives tumor aggressiveness and is implicated in resistance to immunotherapies.

Spatial and molecular control of tumor-associated macrophages

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Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with high resistance to therapies. Inflammatory and immunomodulatory signals co-exist in the pancreatic tumor microenvironment (TME), leading to dysregulated repair and cytotoxic responses. Tumor-associated macrophages (TAMs) are key players in PDAC, but their diversity prevented therapeutic exploitation. Here, we combined single-cell and spatial genomics with functional experiments to elucidate macrophage functions in pancreatic cancer. We uncovered an inflammatory loop between tumor cells and interleukin (IL)-1b^{+} TAMs, a subset of macrophages elicited by a local synergy between prostaglandin E_2 (PGE_2) and tumor necrosis factor (TNF)-a. Physical proximity with IL-1b^{+} TAMs was associated with inflammatory reprogramming and acquisition of pathogenic properties by a subset of PDAC cells. This occurrence was an early event in pancreatic tumorigenesis and led to persistent transcriptional changes associated with disease progression and poor patient outcome. Blocking PGE_2 or IL-1b elicited TAM reprogramming and antagonized tumor cell-intrinsic and -extrinsic inflammation, leading to PDAC control *in vivo*. Targeting the PGE_2-IL-1b axis may enable preventive or therapeutic strategy to reprogramming of immune dynamics in pancreatic cancer.

Selective Endocytic Uptake of different decorated Liposomes in intestinal cancer cells

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Colorectal cancer (CRC) is the third most common cancer and the cause of cancer-related death in both men and women worldwide (Siegel et al., 2020). The human epithelial cell line Caco-2 and intestinal goblet cell line HT-29 have been widely used as a model to study the effects of different antitumour drugs on CRC cells. Previous research has observed that L-fucose is highly required by various cancer cells, particularly in CRC cell models (Osuga et al., 2016). This study reports two different liposomal preparations, particularly the production of fucose-decorated liposomes, called sweetosomes, and their functional uptake, intracellular trafficking and behaviour towards endosome membrane, merging to them and/or destabilizing them: all processes of great importance for the effective drug intracellular release. Sensitive and versatile tools, such as Flow Cytometry (FC) and Confocal Microscopy (CM), were mainly employed in building a useful, not very expensive protocol that could be applied to monitor new Drug Delivery System (DDS), particularly liposomes but also Nanoparticles (NP), as our group work for, since 2021 (Sola et al., 2022, 2021). The two different liposomal formulations were labelled by Rhodamine, and FC was requested to evaluate the Mean fluorescence intensity (MFI) for labelling, since it impacts the uptake quantification. Indeed, to investigate the main mechanisms of endocytosis involved in liposome internalization, including clathrin-dependent, caveolae, and/or cholesterol-rich lipid rafts mechanisms, we employed several inhibitors (4°C; Chlorpromazine hydrochloride, Nocodazole, Genistein, EIPA, Dynasore, Hypertonic Sucrose): A combination of the different pathways are involved, but a reduced impact of clathrin-dependent endocytosis is evident for sweetosome uptake. Finally, a in house panel, based on CD81, CD63 (LAMP3) and CD107a (LAMP1) labelling, together with Lysotracker Deep Red and LysoSensor Green staining, enables to follow the intracellular journey of liposomes and sweetosomes, giving important information on endosomal escape of DDSs, also without a specific payload.

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A novel combination of anti-Trop-2 monoclonal antibodies shows enhanced antitumor efficacy in vivo

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ABSTRACT

The Trop-2 transmembrane signaling molecule is a cancer driver and a clinically-relevant therapeutic target (Trerotola et al, 2013; Bardia et al., 2021; Guerra et al., 2023a). We discovered that Trop-2 undergoes ADAM10-mediated proteolytic activation that is specific for cancer cells (Trerotola et al., 2021; Guerra et al., 2021), and we generated the 2G10 monoclonal antibody (mAb) that targets this activated form of Trop-2 (Guerra et al., 2023b). To further improve Trop-2- targeted anticancer treatment we sought additional recognition of Trop-2 that could penetrate densely packed tumor sites. We designed immunization and screening procedures based on Trop-2 deletion mutants and obtained the 2EF mAb that recognizes a novel epitope in the cysteine-rich N-terminal region of Trop-2. The 2EF mAb proved able to bind Trop-2 at cell-cell junctions in MCF-7 breast cancer cells and in internal sites in prostate cancer, which could not be reached by benchmark anti-Trop-2 mAbs. 2EF was able to inhibit the growth of HT29 colon tumor cells in vitro, with the highest activity at high cell density. In vivo, 2EF showed anticancer activity against HT29, Colo205, HCT116 colon, DU-145 prostate, and SKOv3 ovarian tumors, with the highest efficacy on densely packed tumor sites, whereby 2EF performed better than benchmark anti-Trop-2 mAbs. Since 2EF and 2G10 display different recognition patterns, we hypothesized that a combination of the two mAbs could boost anti-tumor efficacy in vivo. Indeed, the 2EF mAb was shown to enhance the activity of 2G10 against tumor xenotransplants, providing novel approaches for Trop-2-targeted therapy. We humanized the 2EF by CDR grafting and 3D-modeling for targeted residue substitution and obtained the Hu2EF for use in patients bearing Trop-2-expressing tumors.

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Emerging role of CTLA4 and PD-L1 in atezolizumab bevacizumab response in human hepatocellular carcinoma

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Background: Advanced hepatocellular carcinoma (HCC) has a dismal prognosis, although the introduction of the atezolizumab-bevacizumb combination has improved overall survival and novel immune checkpoint inhibitors are entering clinical use. Despite more therapeutic options are available, no biomarkers currently guide treatment selection. Indeed, tissue-based analyses and complex analytical procedures present hurdles to clinical translation. We explored the informativeness of a simple, non-invasive, repeatable flow cytometric assay on peripheral blood to predict response and survival in HCC patients treated with atezolizumab-bevacizumab.

Methods: 25 cirrhotic patients, 50 HCC patients under atezolizumab-bevacizumb therapy, and an independent validation cohort of 25 HCC patients underwent flow cytometric analysis of peripheral white blood cells to assess baseline percentages of PD-L1+ and CTLA4+ cells in different cell populations, as well as their early on-treatment variations. Immunophenotypes were evaluated against treatment response. RNAseq followed by RT-PCR validation was used to elucidate the molecular correlates of immunophenotypic observations. In vitro and ex-vivo experiments were performed to dissect the role of PD-L1 in cancer cells.

Results: While the percentage of PD-L1+ cells does not predict response either at baseline or when evaluating treatment-induced changes, the percentage of CTLA4+ cells showed predictive significance for both baseline lymphocytes and early on-treatment changes in lymphocytes, monocytes, and granulocytes. The predictive significance in terms of TTP and OS was even stronger for early changes in the entire white blood cell population. The immunophenotypic findings correlated with the transcriptional modulation of CTLA4 target genes and other immune response-related genes. In contrast to what has been observed on white blood cells, atezolizumab induced alterations in cell cycle and apoptosis in cancer cells, calling for further investigation.

Conclusion: A repeatable, simple, and non-invasive blood test can predict response to immunotherapy in HCC patients, in terms of both TTP and OS. The observed increase in CTLA4+ cells in non-responders suggests a potential resistance mechanism, highlighting CTLA4 as a possible druggable target. Considering the results from ex vivo and in vitro, extensive studies are needed to fully understand the role of PD-L1 in cancer cells, which may help refine patient stratification.

MONESI-RIZZOLI AWARD
SESSION 1

The C-C Chemokine-Receptor 2 (CCR2)/CCL2 axis contribution in the development of Myelofibrosis

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The Philadelphia negative Myeloproliferative Neoplasms (MPNs) are clonal disorder caused by the JAK/STAT hyperactivated signalling. Myelofibrosis (MF) is the most severe myeloproliferative disorder, occurring as primary MF (PMF) or post – Essential Thrombocytopenia (ET) or Polycytemia Vera (PV). The pro-inflammatory milieu in the bone marrow (BM) microenvironment favors the disease evolution, by sustaining fibrosis, hematopoietic failure and consequently extramedullary hematopoiesis.

The vicious circle that fuels the MPNs progression starts from the hematopoietic stem cell (HSC), which is both triggered by the increased level of inflammatory molecules in blood and bone marrow as well as the aberrant expression of chemokine receptors on HSC/HPC surface. The role of proinflammatory cytokine in MF are subject to extensive investigation, as novel prognostic elements and new pharmacological target for therapies. The C-C Chemokine 2 (CCL2) is an immune-modulatory cytokine highly expressed in MF, exerting its biological function engaging the CCR2 receptor, by regulating the MAPK/ERK, PI3K/Akt and JAK/STAT pathways.

The CCR2 expression was increased on hematopoietic progenitor CD34+ cells isolated from MF patients, and poorly expressed on CD34+ cells from healthy subjects and PV or ET patients.

By confocal microscopy, we recognized most of the c-Kit positive cells also expressing CCR2 in overt MF vs pre-fibrotic PMF patients. The activated Akt downstream pathway by CCL2, increased in MF subjects, also supported clonal proliferation. To sustain the hypothesis that CCR2-CCL2 axis favors the BM microenvironment rearrangement, we analyzed the BM reticular deposition that positively correlates with the percentage of CCR2 positive cells.

As the RPS14 ribosomopathy reduces the GATA1 content in MF, a transcription factor that transcriptionally regulate CCR2, we confirmed that in vitro cultured megakaryocytes (MKs) obtained from isolated MF HSC were hypomorphic for GATA1. Moreover, bone marrow from MF patients showed the increased CCR2 expression also in MKs.

The morphological observation on BM sample from MF patients highlighted the increased CCR2 expression in the cytoplasm of MKs, mostly arranged in aggregate-like structures, which likely resemble platelet aggregation, suggesting that CCR2 may play an important role in the residual platelet function in the MF context.

Consistently with the aberrant MK phenotype in MPNs, recent evidence demonstrates that myeloproliferative disorders are characterized by the high incidence of arterial and venous thrombotic evets, potential progression of post PV/ ET and PMF.

In conclusion, our result shed in light that, in MF, with the selective expression of CCR2, the CCR2-CCL2 chemokine axis might exert several biological function in the MF progression as well as contributing on secondary complications.

Abstract

Vito Amodio - IFOM, Milano

Title: **Immunohistochemical and flow cytometric characterization of epithelial tumors harbouring alterations in DNA repair mechanisms**

Authors:
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Despite the great advancements in cancer immunotherapy, a large fraction of colorectal cancer (CRC) remains ineligible for immune-based therapy due to its immune-cold phenotype.
We previously established syngeneic preclinical models of colorectal cancer harbouring molecular alterations in mismatch repair system and we characterized, by immunofluorescence and flow cytometry, their impact on the composition of the immune infiltration, mainly focusing on the adaptive immune T cell compartment. We observed that complete or partial mismatch repair deficiency in murine subcutaneous tumour affects the composition of the immune microenvironment. Moreover, immunohistochemical staining of chemotherapy-treated murine subcutaneous CRC suggested that these treatments can perturb tumor immune infiltration.
However, a more comprehensive description of innate and adaptive immune subpopulations that infiltrate tumors is a crucial step for understanding the interplay between cancer cells and the immune system. This is required to identify new players, other than T cells, that are involved in antitumor immune surveillance and in response or resistance to immune-based therapies.
In order to cover this gap, we optimized a spectral flow cytometry multi-colour panel for mouse tumors that allows to simultaneously identify more than 30 immunological markers, which recognize innate and adaptive immune compartments including T cells, B cells, NK cells, macrophages, gamma-delta T cells and dendritic cells. To better define the functional states of each immune cell population, we have also included activation markers such as interferon-gamma, granzyme B, perforin and exhaustion markers like PD1, CTLA4, TIM3, and LAG3. We applied this approach to a syngeneic mouse model of CRC upon treatment with clinically relevant chemotherapy regimens, including FOLFOXIRI.
Initial results indicate that this workflow captures differences in the composition of the immune microenvironment induced by chemotherapy treatment, providing a functional tool for characterizing tumors with different molecular and immunological features.

Inhibiting WEE1 kinase to overcome acquired resistance to targeted therapies in colorectal cancer

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Molecular therapies targeting the EGFR-MAPK pathway have markedly improved the treatment of colorectal cancer (CRC) [1]. However, the emergence of acquired resistance remains a major challenge in clinical management [2-4]. The DNA damage response (DDR) plays a crucial role in enabling cancer cells to survive the stress induced by therapy [5]. In CRC, the DDR pathways are often dysregulated, allowing cancer cells to withstand DNA damage caused by treatment, ultimately contributing to therapy resistance. This phenomenon presents a potential therapeutic avenue for targeting DDR effectors [6, 7]. To investigate acquired resistance mechanisms, we developed a series of CRC models- the Ares platform - representing diverse genetic backgrounds, including KRAS and BRAF wild type, BRAF mutant, and KRAS G12C mutant genotypes. To assess the potential efficacy of treatments targeting DDR, we evaluated key histological markers such as γ-H2AX (as a proxy of DNA damage), RAD51 (an essential player in homologous recombination repair pathway), and pRPA32 (to measure replication stress). Each of these markers plays a distinct role in the cellular response to DNA damage, a critical feature of cancer progression and treatment resistance. Our analysis revealed that resistant cells exhibited a constitutively active MAPK pathway, along with elevated basal levels of DNA damage due to high replication stress. Increased expression of RAD51 in resistant cells appeared to mitigate this stress, facilitating cellular adaptation to elevated replication stress. By testing several DDR inhibitors, we identified in both *in vitro* and *in vivo* settings that resistant cells were particularly sensitive to inhibition of WEE1, a pivotal component in the replication stress response pathway. Mechanistically, this heightened sensitivity was attributed to pronounced replication stress induction and to the disruption of RAD51 foci formation, leading to rapid accumulation of DNA damage and mitotic catastrophe. Notably, this detrimental effect persisted even at sublethal doses of WEE1 inhibitor, suggesting the potential for combinatorial therapies with DDR inhibitors or other DNA damaging agents to enhance treatment effectiveness. In conclusion, our findings highlight WEE1 as a valuable therapeutic target in the acquired

resistance context, providing new treatment avenues for those patients who become refractory to targeted drugs.

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Human adipose stem cells transfer their mitochondria to breast cancer patient derived organoids increasing drug resistance

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Mitochondrial transfer (MT) from mesenchymal stem cells showed to restore damaged cell function in inflammatory disease models. We showed that breast cancer (BC) cells acquire mitochondria from human adipose stem cells (hASCs) via tunneling nanotubes (TNTs), promoting multidrug resistance. Here we aimed at evaluating if MT occurs in patient-derived organoids (PDO) co-cultured with hASC.

We generated PDOs and matched hASCs from consenting BC patients. Fresh tissues were mechanically and enzymatically digested and cultured with proper medium. Organoids were characterized by immunocytochemistry and hASCs by flow cytometry (FC). A 2D-3D co-culture was set up, plating hASCs with PDOs with/without insert: hASCs mitochondria were stained with MitoTracker Red CMXRos ® and PDOs cytoplasm with Cell Tracker Blue®. MT was analyzed by immunofluorescence microscopy and FC. Moreover, a TNT inhibitor, Cytochalasin B, was added to the co-culture to evaluate if TNTs are involved in MT. PDOs were subjected to Mitoception (MCP) and treated with chemotherapeutic drugs. Cell viability was assessed with CCK8® assay.

We successfully generated and characterized PDOs from luminal BC patients, and showed that they maintained the same hormone receptor profile and showed cell heterogeneity. Furthermore, we generated primary hASCs from the same patients which showed a FC CD45-CD324-CD34-CD29+CD44+CD73+CD90+CD105+ pattern. We set up a hybrid co-culture model with 3D PDOs and 2D hASCs, showing that MT occurs massively in direct co-culture, but also (at lower level) with insert. Indeed, when treating with Cytochalasin B MT was not blocked, indicating that it occurs with additional mechanisms than TNTs. To validate the effect of MT on drug resistance, we forced hASCs-mitochondria internalization into PDOs via MCP, and treated them with chemotherapeutic drugs, observing an increase in PDOs viability with respect to those not subjected to MCP

We confirmed that MT occurs in a more physiological model such as PDOs and matched hASCs, in which it reduces drug response. It appears as a key process that could drive tumor aggressiveness, whose better understanding could help to design more effective treatment strategies to overcome drug resistance.

Biological properties of cholangiocytes when exposed to inflammatory microenvironment: activation of IL-6 and PD-L1 pathways

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Cholangiopathies (CP) include a spectrum of chronic intrahepatic and extrahepatic biliary tract disorders strongly associated with inflammatory bowel disease and characterized by cholestasis, immune infiltration, and progressive fibrosis of the bile ducts. These events can cause the occurrence of cholangiocarcinoma which represents the leading cause of CP-associated mortality. Cholangiocytes play a pivotal role in the pathogenesis of these disorders leading to the release of a plethora of inflammatory and fibrotic mediators. In this context, we aimed to investigate the immunomodulatory properties of human cholangiocytes (H69 cells) when exposed to an inflammatory microenvironment, mimicked in vitro by an indirect co-culture system with Peripheral Blood Mononuclear Cells activated with anti-CD-3/CD-28 antibodies (aPBMCs). Data showed that H69 cells expressed the immunomodulatory molecule PD-L1 as well as IL-6 after exposure to the inflammatory microenvironment, while NF-kB/p65 expressed in basal conditions was up-regulated in H69 after PBMCs co-culture earlier than IL-6 and PD-L1 ($n=3$, $p<0.001$ vs H69 alone). Moreover, H69 cells underwent a cell cycle arrest and showed a fibroblast-like morphology when exposed to inflammatory conditions. Subsequently, a significant decrease of pro-inflammatory cytokines was detected in aPBMCs after H69 co-culture ($n=3$, $p<0.001$ vs aPBMCs alone), probably due to the compensatory pathways that synergistically allow H69 to modulate the inflammatory microenvironment and support fibrosis establishment and neoplastic progression of CP. The IL-6, PD-L1, and NF-kB/p65 levels were restored to basal levels when H69 cells were treated with an IL-6 receptor inhibitor (100 μg/mL), suggesting a possible synergic role between IL-6, PD-L1, and NF-kB/p65 pathways in promoting the immunomodulatory effects of H69 under inflammatory conditions. Finally, to strengthen the hypothesis on the role of IL-6 in driving PD-L1 expression, H69 cells were treated with IL-6/sIL-6R complex (120 ng/mL). Data confirmed the activation of the IL-6 trans-signaling pathway and showed that IL-6/sIL-6R treatment induced an increased expression of PD-L1 ($n=3$, $p<0.001$). Our results confirm that a) the PD-1/PD-L1 pathway is an immunomodulatory checkpoint expressed by cholangiocytes when exposed to an inflammatory milieu whose activation is driven by the IL-6 pathway; b) the NF-kB axis promotes the IL-6 production that acts in an autocrine manner on cholangiocytes to drive their immunomodulatory properties. These data pave the way for future insights into pathophysiological mechanisms involved in the cascade of cholangiopathies aiming to guide further research to find new potential therapeutic strategies.

Human Cytomegalovirus infection induces adaptive reconfiguration of NK cells resulting in increased atherosclerotic plaque instability

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Purpose: Human Cytomegalovirus (HCMV) infects a broad range of host cells, including monocytes/macrophages, fibroblasts, epithelial cells, endothelial cells, muscle fibers, and remains latent upon primary infection. Recent works detected HCMV in atherosclerotic plaque, suggesting that the virus may contribute to atherosclerotic processes (1,2). In some patients (pts), HCMV promotes a marked reconfiguration of the Natural Killer (NK) cell compartment characterized by adaptive immune features, long-term persistence, distinct phenotype and antibody-dependent enhanced functional capabilities, including Antibody-Dependent Cell-mediated Cytotoxicity (ADCC), and cytokine production (3,4). IFN-γ producing NK cells have been implicated in atherosclerosis progression, apparently in association with HCMV infection (5,6). Yet, the precise role of NK cells in atherosclerotic plaque destabilization and the mechanisms underlying HCMV-associated atherosclerosis progression remain open issues. This study aims to investigate the potential impact of HCMV in the atherosclerotic micro-environment, as well as how the HCMV-induced reconfiguration of NK cell compartment is involved in the pathogenic mechanisms underlying the instability of atherosclerotic plaque. **Methods**: A total of 64 pts were enrolled in a follow-up protocol for carotid artery stenosis. Pts were classified according to conventional criteria as bearing high-risk plaques (High-risk pts- HR pts) or low-risk plaques (Low-risk pts- LR pts). High-risk pts underwent carotid endarterectomy according to the European Society for Vascular Surgery guidelines. Carotid plaques and preoperative blood samples were obtained from all pts, processed and examined through flow cytometric and histological analyses.
Results: Immunohistochemical analysis reveal that NK cells ($NKp46^+$ cells) and macrophagic cells ($CD\ 68^+$ cells) infiltrate the arterial wall containing the atheromatous plaque of high-risk HCMV-seropositive pts, and that HCMV is localized within cells of macrophage origin ($CD\ 68^+$ cells). Remarkably, through multiparameter flow cytometric analysis we observed that adaptive-non-conventional NK cells are enriched in high-risk atherosclerotic HCMV seropositive pts compared to low-risk pts and display an increased expression of NKG2D (activating receptor). Curiously, adaptive $Fc\varepsilon R1\gamma^-$ NK cells increase upon plaque destabilization in peripheral blood and accumulate in carotid atherosclerotic plaques (CAP) of symptomatic high-risk seropositive pts. Moreover, NK cells from high-risk HCMV seropositive pts have enhanced antibody-dependent effector functions compared to low-risk pts, and antibody-dependent IFN-γ release correlates with the frequency of $FC\varepsilon R1\gamma^-$ in high-risk seropositive pts. Surprisingly, masking of NKG2D receptor significantly reduces antibody-dependent IFN-γ release by $FC\varepsilon R1\gamma^-$ NK cells, suggesting that this receptor might trigger NK cells activation. Interestingly, we found an enhanced expression of Matrix Metalloproteinase-9 (MMP-9) within $CD68^+$cells in CAP of symptomatic high-risk seropositive pts, the same pts who shown an increased expression of activated adaptive $Fc\varepsilon R1\gamma^-$ NK cells in CAP, suggesting a possible correlation with plaque instability. In conclusion, all together our data indicate the importance to detect the presence of adaptive NK cell subsets in atherosclerotic pts as well as HCMV as a triggering pathway. Both could become potential markers to identify pts who may suffer from plaque destabilization and require early surgery and/or closer follow-up.

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Beta-3 adrenoceptor agonist protects the intestinal enteric nervous system against the hyperoxia-induced alterations.

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Organogenesis occurs in the uterus under low oxygen levels (4%). Preterm birth exposes immature newborns to a relatively hyperoxic environment, potentially affecting the maturation process of organs, including the intestine, that complete their development after birth. Postnatal development of the enteric nervous system (ENS) is especially crucial for establishing the proportions of different enteric neuronal subtypes, forming neuronal connections, and facilitating glial maturation.
The β3-adrenoreceptor (β3-AR) has an oxygen-dependent regulatory mechanism and it is expressed in myenteric and submucosal cholinergic neurons of both human and rat colon. To investigate whether β3-AR could protect the postnatal maturation of the ileal ENS from the detrimental effects of high oxygen levels, Sprague–Dawley rat pups were raised under normoxia (21%) or hyperoxia (85%) for the first 2 weeks after birth and treated or not with BRL37344, a selective β3-AR agonist, at 1, 3, or 6 mg/kg. Hyperoxia reduced the number of neurons in the submucosal plexus and altered the neuro-chemical coding in both the myenteric and submucosal plexuses. Specifically, the percentage of nitrergic subpopulation in the myenteric plexus decreased, while it increased in the submucosal plexus. In contrast, the proportion of cholinergic neurons in the myenteric plexus rose under conditions of high oxygen exposure. Notably, the administration of BRL37344 at 3 mg/kg, but not at 1 mg/kg, significantly prevented these alterations. The analysis of the glia/neuron ratio revealed that hyperoxia exposure significantly reduced both myenteric and submucosal glial cells, indicating an altered maturation of the plexuses. The treatment with BRL37344 at 3 mg/kg effectively prevented this leakage, while the lower 1 mg/kg dose was ineffective. Our findings suggest that β3-AR agonism could provide a new therapeutic strategy to counteract the hyperoxia-induced alterations in enteric neurotransmission and, more generally, the disorders of prematurity associated with exposure to supra-physiologic levels of oxygen.

Epigenetic regulation of human dermal fibroblasts: the role of hsa-miR-210-3p on cell proliferation and differentiation

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The role of microRNAs (miRNAs) in regulating the functions and phenotype of dermal fibroblasts has been partially elucidated. Hsa-miR-210-3p is a key regulator of epithelial cell proliferation and differentiation. We recently identified hsa-miR-210-3p expression in dermal cells. In this study, the role of hsa-miR-210-3p was investigated in human dermal fibroblast cells. Transfection experiments with hsa-miR-210-3p mimic and inhibitor were performed in human dermal fibroblast cells (HDFa) to evaluate the microRNA function on cell proliferation, colony formation and migration abilities. The expression of hsa-miR-210-3p target genes (n=84) were evaluated by quantitative PCR array in miR-overexpressed HDFa cells and compared to untransfected cells. Forced hsa-miR-210-3p expression in HDFa cells increased cell proliferation, colony formation and migration abilities, while the opposite effects were determined in miR-inhibited cells. HIF3A and CHN1 were the most significantly downregulated genes in HDFa cells overexpressing hsa-miR-210-3p, compared to untransfected cells. HIF3A is known to inhibit cell survival and proliferation as a negative response mechanism to hypoxia, while CHN1 positively regulates cell differentiation and inhibits cell migration. Enrichment analyses for biological processes on the entire set of differentially expressed miR-target genes indicated the downregulation of positive regulators of cell differentiation, maturation and axonogenesis in HDFa cells overexpressing hsa-miR-210-3p, compared to untransfected cells. These data suggest that hsa-miR-210-3p is a regulator of cell proliferation and differentiation, independently of the cell type, either epithelial or fibroblast. The activity of hsa-miR-210-3p may be used in RNA-based therapies of different dermatological diseases to restore skin layers.

Developmental defects on zebrafish (*Danio rerio*) after exposure to fossil-based and bio-based plastic derivates

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Plastic pollution has become uncontrollable, to the point of defining the present era the PLASTICENE. The advent of biobased plastics, such as polylactic acid-based ones, seems to have opened a glimmer of hope to stem the effects of this pollution, mostly produced by conventional fossil-based plastics. However, the safety and accumulation of bioplastics have not yet been elucidated, *in vitro* and *in vivo*. In this work, the experimental zebrafish (*Danio rerio*) model was used to test and compare the effects of polylactic acid nanoplastics (PLA-NPs) with polystyrene microplastics (PS-MPs) on embryonic development, bioaccumulation and morphological alterations. To this scope, zebrafish embryos have been exposed to two different concentrations (0,1 and 1 mg/L) of heterogenous-in-size (275±75 nm) biodegradable rhodamine-labeled PLA-NPs and fluorescent PS-MPs (1 μm), up to 120 hours after fertilization (hpf). Distribution and bioaccumulation of PLA-NPs and PS-MPs was evaluated at 24, 48, 72 and 120 hpf by *in vivo* fluorescence microscope observation. The Zebrafish Embryo Acute Toxicity Test (ZFET) was used to assess developmental alterations at the same time points. Furthermore, histological analyses and the evaluation of cell stress and inflammation markers using RT-PRC were conducted at 72 and 120 hpf. Overall, a bioaccumulation in models treated with PLA-NPs and PS-MPs was noticed mainly in the digestive tract starting from 96 hpf; discordant alterations in heartbeat rate in PLA-NPs and PS-MPs treated zebrafish were recorded; histological analysis of fish exposed to PLA-NPs and PS-MPs suggests a damage of the digestive canal wall, probably impairing the esophageal sphincter; at last, gene expression of cellular stress markers resulted in differential entity of inflammation and oxidative stress following the PLA-NPs and PS-MPs exposure. To our knowledge, several studies reported in the literature have shown the effects of PLA-MPs and PS-MPs, however, this is the first (or one of the first) study on PLA-NPs also using a heterogenous-in-size sample of plastic debris which we retain may be more reliable and closer to the real biodegradable process of bio-based plastic.
Further studies are under progress in our laboratories, nevertheless, with these preliminary data we suggest that the toxicological effects of any plastic may be harmful to the marine environment and, in turn, because of the biomagnification process, may be hazardous to human beings.

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MONESI-RIZZOLI AWARD
SESSION 2

Loading-related changes affect FNDC5/Irisin in the musculoskeletal system.

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Long-term exposure to microgravity, induced by spaceflight or hypomobility condition on earth, results in severe osteoporosis and muscle atrophy, while mechanical loading by exercise or artificially produced hypergravity allows bone and muscle mass to recover. Therefore, research in the space biomedical field is focusing on identifying biomarkers of microgravity damage and possible protective effects of hypergravity.

The purpose of this study was to investigate the involvement of the molecule irisin and its precursor FNDC5 under hypogravity and hypergravity conditions. The first was performed on a rodent model of mechanical unloading, the hind limb unloaded (HU) mice for 4 weeks, to mimic microgravity-induced osteoporosis and muscle atrophy. Hypergravity condition was performed through a unique experiment conducted at the European Space Agency (ESA) in which mice were maintained at a gravitational acceleration of 3g for 4 weeks, using a large-diameter centrifuge (LDC) equipped with an appropriate payload (MDS) for the animals' cages. Histological and biomolecular analysis of the Vastus Lateralis (VL) and Gastrocnemius (GN) muscles were performed.

Histological analysis of muscle from HU mice showed that muscle fibers became atrophic in the first 2 weeks of unloading, also evidenced by up-regulation of *Atrogin* and *Murf-1*, and by downregulation of the myosin isoform *MyHCIIx*. Moreover, HU mice showed a decrease in *FNDC5* expression and irisin serum levels at 4 weeks, while irisin treatment prevented the unloading-dependent decline of myosins. Additionally, in femurs from HU mice, we observed reduced trabecular thickness and increased number of apoptotic osteocytes in cortical bone.

Conversely, the hypergravity condition increased cross-sectional area (CSA) of muscle fibers in VL of mice in MDS cages at 3g compared with mice kept in same size cages but at terrestrial gravity (TC cages) which displayed an atrophic phenotype. At molecular level, hypergravity increased the expression of *FNDC5*, *MyHCI* and *MyHCII*, and markers of early stages of myogenesis, such as *PAX7* and *MYF5*, compared to TC cages.

Overall, these data suggest that the FNDC5/Irisin system is significantly affected by mechanical loading with opposite effects in hypo- and hypergravity.

miR-486 overexpression improves skeletal muscle function in two different mouse models of RyR1-related myopathies

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Mutations in the *RYR1* gene are the most frequent cause of non-dystrophic congenital muscle disorders and are causative for a group of diseases collectively known as RYR1-related myopathies. Among these, central core disease (CCD), which is the most common, presents with variable clinical symptoms and is histologically characterized by large areas devoid of mitochondria and oxidative enzyme activity, known as cores. Moreover, some mutations in the *RYR1* gene are causative for malignant hyperthermia susceptibility (MHS), a rare and potentially life-threatening pharmacogenetic disorder that manifests as a hypermetabolic state with uncontrolled muscle contractions and elevated body temperature triggered by exposure to halogenated anesthetics or depolarizing muscle relaxants.

Despite advances in understanding these conditions, there are no approved treatments for RYR1-related myopathies. Previous studies have shown that skeletal muscle-specific overexpression of miR-486, a muscle-enriched microRNA, ameliorates the dystrophic phenotype of mdx mice, a model of muscular dystrophy. Based on this evidence, we aimed to investigate whether a similar strategy could be beneficial in mouse models of RYR1-related myopathies. For this purpose the effects of skeletal muscle-specific miR-486 overexpression were evaluated in two established knock-in mouse models of RYR1-related myopathies: $Ryr1^{Y524S/+}$ and $Ryr1^{I4895T/+}$. These models were crossed with transgenic mice that selectively overexpress miR-486 in skeletal muscle tissue ($Tg^{miR-486}$) generating two novel lines: $Ryr1^{Y524S/miR-486}$ and $Ryr1^{I4895T/miR-486}$. The phenotype of these novel lines was assessed by functional tests to evaluate *in vivo* muscle performance, and by histochemical and biochemical analyses to characterize muscle morphological and molecular features.

The results obtained indicate that miR-486 overexpression significantly enhances muscle performance in both $Ryr^{Y524S/miR-486}$ and $Ryr1^{I4895T/miR-486}$ mouse models. Moreover, histological evaluations revealed that overexpression of miR-486 also results in a mild increase of muscle fibers cross-sectional area. Additional studies are currently ongoing to identify the pathways whereby miR-486 exerts the observed beneficial effects. Collectively, these results suggest that skeletal muscle-specific overexpression of miR-486 improves muscle function of mice affected by RYR1-related myopathies, likely due to a hypertrophic effect.

Mitochondrial alteration in Mesenchymal Stem Cells: a crucial factor in osteoporosis development in Cushing's Syndrome

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ABSTRACT

Cushing's Syndrome (CS) is an endocrine disorder resulting from prolonged exposure to high levels of glucocorticoids (GCs), either produced in excess by the adrenal glands (endogenous) or due to long-term GC therapy (exogenous). Both forms of CS are linked to various comorbidities, including osteoporosis. Osteoporosis occurs when bone resorption by osteoclasts exceeds bone deposition by osteoblasts, and recent researches suggest that mitochondrial dysfunction in bone cells may contribute to its development by compromising energy production and cellular processes essential for bone maintenance. Mesenchymal stem cells (MSCs) play a crucial role in bone health, as they can differentiate into osteoblasts; however, the effects of GCs on MSCs remain poorly understood, warranting further investigation into their role in osteoporosis. To better understand the osteoblasts/osteoclasts imbalance in CS, MSCs and PBMCs were isolated from skin biopsies and blood of healthy subjects and CS patients and differentiated into osteoblasts and osteoclasts, respectively. The results showed that osteoclast differentiation was more pronounced in patients with exogenous CS than in control subjects, confirming an increased bone resorption activity. Conversely, patients with endogenous CS showed lower osteoclast differentiation and activity, probably due to the chronicity of the disease. Both endogenous and exogenous CS patients displayed delayed MSC differentiation into osteoblasts, with lower collagen production, reduced mineralization, and a delayed expression of markers involved in osteoblastogenesis. Additionally, MSCs from CS patients showed mitochondrial dysfunction, characterized by a more fragmented mitochondrial network, increased ROS production, higher calcium uptake, and decreased mitochondrial membrane potential. All alterations observed in MSCs from CS patients were fully reversed in those undergoing steroid-sparing therapy, confirming that these changes were directly related to GC excess. In conclusion, this research demonstrated that high GC levels detrimentally impact MSCs, leading to mitochondrial dysfunction and delayed osteoblastogenesis, thus confirming the crucial role of MSCs in the onset and maintenance of osteoporosis in CS patients.

Exploring transcriptomic variability in induced Pluripotent Stem Cells derived from Amyotrophic Lateral Sclerosis patients carrying different mutations.

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Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterized by the loss of neuronal function, primarily affecting central and peripheral motoneurons, with phenotypic and genetic heterogeneity. Mutations were found in familial and sporadic cases, including mainly *C9ORF72, SOD1, TARDBP* and *FUS*, and over 30 genes associated to rare ALS form. Although these mutations affect different molecular pathways, all of them result in motor neuron degeneration, highlighting the need to understand the molecular basis of these disruptions for developing targeted therapies. For this study, we used Induced Pluripotent Stem Cells (iPSCs) as a cellular model to investigate transcriptomic differences associated with various ALS genetic mutations and identify pathways related to ALS. iPSCs were generated from 4 patients carrying mutations in *C9orf72* (G_4C_2 expansion), *TARDBP* (c.1144G > A), and *KIF5A* (c.2753+1G>A) genes and 2 healthy donors by reprogramming peripheral blood CD34+ cells with the Sendai virus system. The iPSCs showed typical morphology and expression of stem-cell markers at RNA and protein levels, without residual of Sendai virus. Embryoid bodies were generated, demonstrating the expression of ectodermal, mesodermal, and endodermal markers. This pluripotent capacity is essential for modeling ALS, as it can allow for the differentiation of iPSCs into motor neurons and other relevant cell types affected by the disease. To explore the potential of these iPSCs in disease modeling, we successfully differentiated both healthy and ALS iPSCs into neurospheres, obtaining high levels of neural progenitor cell markers. Importantly, iPSCs transcriptomic analysis has been performed, identifying several differentially expressed genes (DEGs) ($|log2FC| > 2$; $padj \leq 0.05$) in each patient compared to the healthy control. Subsequently, an enrichment analysis has been conducted by Metascape. Fifty –two genes are shared across all patients, belonging to intracellular transport regulation pathways. No common GO terms were found across all mutations, while genes belonging to extracellular matrix (ECM) (GO:0031012) or extracellular matrix organization (GO:0031012) have been found to be enriched in all patients, underlying a potential role of ECM in the pathological process. In conclusion, iPSC technology is a powerful tool for modelling ALS, enabling the study of disease-specific mutations and their effects on cellular function. The identified transcriptomic differences provide valuable insights into ALS progression and genetic variability, supporting the development of novel targeted therapies.

Dual Function Strategy for Type 1 Diabetes: Insulin-Producing and Immune-Modulating Perinatal Cell Spheroids.

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Abstract

Type 1 diabetes mellitus (T1DM) is an autoimmune condition where the immune system attacks pancreatic beta cells, leading to insulin deficiency. Conventional treatments focus on insulin replacement, not addressing the root autoimmune cause. This study aims to develop a cellular therapy for T1DM using perinatal cell-derived spheroids capable of insulin release and immune modulation. This dual approach seeks to restore insulin production while protecting newly formed beta cells from autoimmune destruction.

We first evaluated the immunomodulatory properties of undifferentiated perinatal spheroids made of Wharton's jelly mesenchymal stem cells (WJ-MSCs) in combination with amniotic epithelial cells (AECs). Perinatal spheroids were co-cultured with activated peripheral blood mononuclear cells (PBMCs) from healthy donors to assess changes in pro-inflammatory and anti-inflammatory cell populations. Following this, we induced endocrine differentiation in AECs to produce insulin-secreting cells, verifying pancreatic differentiation via immunofluorescence for specific markers. The differentiated AECs were then combined with undifferentiated WJ-MSCs to form cohesive spheroids with enhanced immunomodulatory potential.

The undifferentiated perinatal cell spheroids showed significant immunomodulatory capacity, reducing the activation of pro-inflammatory cells and promoting anti-inflammatory responses. Spheroids containing differentiated AECs successfully formed cohesive structures and exhibited potential for insulin production. Further studies will assess the therapeutic efficacy of these perinatal cell spheroids in immune modulation and insulin production for antidiabetic therapy.

Perinatal cell spheroids present a promising dual strategy for T1DM treatment, combining insulin production with immune modulation. This approach may address the symptoms and underlying immune dysregulation in T1DM, offering a more comprehensive cellular therapy beyond traditional insulin replacement.

Targeting Endo-Lysosomal autophagy pathway in GLUT1 Deficiency Syndrome

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The GLUT1 Deficiency Syndrome (GLUT1DS) is an autosomal dominant rare neurological disorder. This syndrome is caused by heterozygous mutations in the *SLC2A1* gene, encoding the Glucose Transporter 1 (GLUT1). GLUT1 is a plasma membrane(PM)-bound glycoprotein particularly abundant in endothelial cells of the Blood-Brain Barrier (BBB). The localization of GLUT1 at PM of BBB-endothelial cells ensures the supply of glucose, the main source of energy for the brain. However, the haploinsufficiency of the wild-type allele of the *SLC2A1* gene results in defects in protein production, trafficking, and function. Patients affected by GLUT1DS experience early-onset drug-resistant epilepsy, motor/mental retardation, and acquired microcephaly with no therapy. The molecular mechanisms underlying the GLUT1 shuttling within the cell and its exposure at PM are currently understudied. For this reason, we started to manipulate GLUT1 trafficking, both metabolically and pharmacologically, to elucidate those molecular events. Both treatments modulate the internalization of GLUT1 in the endo/lysosomal compartment. We performed LC-MS approaches highlighting the downstream affected pathways and adaptor proteins involved in subcellular trafficking. Another unexplored field is the use of small molecules able to modulate the GLUT1 transport as a putative pharmacological approach. Then, we tested the efficacy of an FDA-approved library of compounds in reducing GLUT1 segregation at endo/lysosomal vesicles. To pursue this aim we performed an HCS-based drug-screening*in vitro*. Based on preliminary results, 42 compounds were identified to rescue GLUT1 subcellular segregation at lysosomes. Moreover, through a Drug Set Enrichment Analysis (DSEA), we demonstrated that the most effective compounds shared as the first affected pathway the autophagy. Therefore, we conclude that autophagy could be a valuable target pathway to manipulate the trafficking of GLUT1 between endo/lysosomal vesicles and PM.

YOUNG INVESTIGATORS
PARALLEL SESSION 1

Neuropilin1-dependent paracrine signaling of cancer cells mediated by miRNA exosomal cargo

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Abstract

Background. Neuropilin-1 (NRP1) is a transmembrane protein involved in surface receptor complexes for a variety of extracellular signals. NRP1 expression in human cancers is associated with prominent angiogenesis and advanced progression stage. However, the molecular mechanisms underlying NRP1 activity in the tumor microenvironment remain unclear. Notably, diffusible forms of NRP1 in the extracellular space have been reported, but their functional role is poorly understood.

Methods. Extracellular vesicles (EV) were isolated from conditioned media of diverse cancer cells. The quality of exosome-enriched preparations was validated by the presence of specific markers in western blotting, as well as by light scattering and nanoparticle tracking analysis. Wound healing and transwell assays were carried out to assess the activity of cancer cell-derived exosomes in the regulation of endothelial cells (EC). RNA interference was applied to obtain NRP1 knock-down, and cDNA transfer to achieve its overexpression, in exosome-releasing cells. The micro-RNA profile carried by exosomes was investigated by Next Generation Sequencing. miRNA-Scope in situ hybridization was used to assess the transfer of miRNA exosome cargo to target cells, and immunofluorescence analysis revealed expression regulation of targeted proteins. miRNA activity was blocked by the use of specific antago-miRs.

Results. In this study, we show that diverse human cancer cells release NRP1 embedded in exosome-like small extracellular vesicles, which mediate a previously unknown NRP1-dependent paracrine signaling mechanism regulating endothelial cell migration. By transcriptomic analysis of the cargo of NRP1-loaded exosomes, we found a significant enrichment of miR-210-3p, known to promote tumor angiogenesis. Gene knock-down and overexpression experiments demonstrated that the loading of miR-210-3p into exosomes is dependent on NRP1. Data furthermore indicate that the exosomes released through this NRP1-driven mechanism effectively transfer miR-210-3p to human endothelial cells, causing paracrine downregulation of the regulatory cue ephrin-A3. The mechanistic involvement of miR-210-3p in this pathway was confirmed by applying a specific antago-miR.

Conclusions. In sum, we unveiled a previously unknown NRP1-dependent paracrine signaling mechanism, mediated by the loading of pro-angiogenic miR-210-3p in exosomes released by cancer cells, which underscores the relevance of NRP1 in controlling the tumor microenvironment.

Leukemic cells adherence to mesenchymal stromal cells: a new mechanism for stem cell differentiation in hematopoietic cells?

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Mesenchymal stromal cells (MSCs) are believed to regulate hematopoietic stem cell (HSC) behavior, especially within the bone marrow microenvironment, which plays a pivotal role in leukemic progression.[1]
To analyze the influence of MSCs on leukemic cells, we set up both direct contact and transwell in vitro models using MSCs obtained from different sources (HS-5 cell line and dental pulp-derived MSCs), co-cultured with leukemic cells (THP-1 cell line). Cellular behavior was assessed via optical and scanning electron microscopy (SEM) examination, while cytofluorimetric analyses evaluated CD11b and CD14 expression in suspended HSCs and in HSCs adherent to MSCs (in direct-contact co-cultures) and HSCs in transwell insert (in non-direct contact co-cultures).
Our findings show that MSCs in direct contact with HSCs may stimulate cell adhesion and induce leukemic cells to divide in two sub-populations: suspended and adherent to MSCs. Interestingly, cytofluorimetric analyses revealed a progressive increase in CD11b and CD14 expression on both suspended and MSCs-adherent THP-1 cells up to 144h after direct-contact co-culture, suggesting that MSCs may affect myeloid differentiation. Even though transwell co-cultures showed increased CD11b and CD14 expression in THP-1 as well, the increase was significantly lower compared to direct-contact co-cultures. This difference suggests the probable role of MSCs secreted factors in inducing myeloid differentiation, but highlights the prominent involvement of direct cellular contact. This hypothesis was further supported by the observation that THP-1 cells already induced to macrophage differentiation by phorbol myristate acetate (PMA) are less adherent to MSCs in direct-contact co-cultures and tend instead to adhere to the free areas of the flask.
SEM analysis highlighted distinct morphological differences among leukemic cells in various culture conditions: single culture, suspended cells in direct-contact co-cultures, and cells in co-cultures using transwell inserts. Specifically, leukemic cells in single culture displayed a rounded shape with pseudopod-like structures. In direct-contact co-cultures, leukemic cells showed a notable loss of their rounded morphology and pseudopod structures, while leukemic cells in transwell co-cultures have an intermediate rounded shape with elongated pseudopod-like extensions. Ongoing immunolabeling experiments will allow us to better detect and localize differentiation markers within the samples.
All in all, our findings may further elucidate the effect of MSCs on HSCs during myeloid differentiation or leukemic progression, possibly leading to novel therapeutic strategies targeting the bone marrow microenvironment.

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Morphological study of bone marrow microenvironment in Multiple Myeloma patients

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Multiple myeloma (MM) is a hematological disorder due to the abnormal proliferation of clonal Plasma Cells (PCs) characterized by the increase of monoclonal immunoglobulins. MM is a heterogeneous malignancy able to promote extra medullary lesion.

Our knowledge about the bone marrow (BM) myeloma microenvironment are poorly defined as well as the cellular interaction between PCs. We exploited Bone marrow biopsies of MM patients and according to morphological alteration of PCs, we differentiated them in two populations: one that has clustered PCs that appear increased in dimensions, with vacuolated cytoplasm, other group is characterized by the increase of smaller and morphologically recognizable PCs, straggler in the microenvironment.

By Morphological observation on this last subgroup, we recognized the increased number of neutrophils that are localized close in contact with PCs. These cells were founded close to plasma cells and lymphocytes (T subset), with atypical morphology. Based on these results we focus our attention on the interaction between neutrophiles and the PCs.

Confocal microscopy observation confirm the increase of Neutrophils Elastase and Ly6b positive cells, which release chromatin strand in the microenvironment (NETtosi).

As consequence of neutrophilic chemotaxis, we tested the IL-8 expression, the pro-inflammatory cytokine involved in the neutrophilic chemotaxis, which was manly expressed by Plasma Cells.

In MM, the Neutrophil to lymphocyte ratio combines a marker of inflammation and reduced cell turnover to reflect are relate alteration in the immune system. Our result provides new elements about the interaction occurring in MM between neoplastic PCs and neutrophilic intervention in the bone marrow microenvironment.

Given the complexity and the heterogeneity of MM this study provides new elements for clinicians to find different therapeutic strategies in accordance to the morphological alteration observed in MM microenvironment.

Impact of a PLCG2 point mutation on proliferation and apoptosis of hematopoietic cells

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Myelodysplastic neoplasms (MDS) are hematopoietic tumors defined by clonal proliferation and a variable risk to evolve into acute myeloid leukemia (AML)[1]. Inositide-specific enzymes, such as Phospholipase C (PLCs), are currently known to be deregulated in MDS[2]. In particular, we identified a common cluster of point mutations affecting three inositide-specific genes (PLCG2, AKT3 and PI3KCD) that was significantly associated with loss of response to Azacitidine (AZA) and Lenalidomide (LEN) therapy in patients with MDS at higher risk of leukemic evolution[3].

To investigate the functional role of that PLCG2 mutation, the CD34+ KG1 cell line was transduced using a lentiviral system, carrying either the wild-type (WT) or the mutated (Q548R) form of the PLCG2 gene, to ensure stable expression of each variant within the cells.

Molecular analyses confirmed the high expression levels of PLCG2 in the transduced KG1 cells, validating the success of the lentiviral transfection. Further experiments revealed that only the cells expressing the Q548R PLCG2 variant showed a reduced CD33 and CD14 expression, suggesting an impairment in myeloid differentiation and potentially indicating that these cells are unable to mature properly within the myeloid lineage. Additionally, the decreased expression of the pro-apoptotic protein PUMA in the mutated cells could imply a reduced susceptibility to apoptosis, which may allow these cells to resist cell death triggered by stress or therapeutic interventions.

Considering that the Azacitidine and Lenalidomide combination therapy has now been largely replaced by the Azacitidine and Venetoclax (AZA+VEN) treatment[4], we are conducting experiments treating WT or mutated KG1 cells with the AZA+VEN combination to investigate whether the PLCG2 point mutation may correlate with loss/lack of response even with this therapeutic approach, thus possibly offering new insights into leukemic transformation mechanisms.

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GASTRIC ORGANOIDS AS A TOOL TO MODEL MICROSATELLITE UNSTABLE GASTRIC CANCER

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Microsatellite instability (MSI) is a genetic condition commonly found in sporadic solid tumors, particularly in gastrointestinal, ovarian and endometrial malignancies. MSI results from defects in the DNA mismatch repair (MMR) system, such as *MLH1* epigenetic silencing or mutations in other MMR genes (e.g., *MSH2, PMS2, MSH6, MSH3*). In gastric cancer (GC), the MSI subgroup represents a significant portion of patients (22-23%) and it is generally associated with a good outcome compared to the other GC molecular subtypes. Nevertheless, some reports highlighted the existence of a subpopulation of MSI GC patients with worse prognosis, hampering accurate stratification. Additionally, while in recent years immune checkpoint inhibitors emerged as a valid therapeutic option for MMR deficient/MSI tumors, key challenges remain in GC. A high degree of molecular heterogeneity, the lack of reliable predictive biomarkers and a limited efficacy in advanced stages are still hindering their full therapeutic potential in this tumor type.

Organoid cultures are widely recognized as valuable resources for translational research in oncology, as they enable advanced disease modelling and drug testing. However, since organoids can be easily manipulated in controlled environments, they can also be an essential tool for functional studies, potentially providing insights into tumor progression mechanisms. In a published study, for example, organoids from human gastric mucosa were used to model *Helicobacter pylori* infection *ex vivo*, shedding light on the host-pathogen interactions through which this major risk factor contributes to GC development.

Leveraging a similar approach, we applied organoid technology to mimic MSI GC neoplastic transformation *ex vivo*. We generated non-transformed organoids from gastric mucosa of BALB/c mice and, since MMR impairment is considered an early event in MSI GC tumorigenesis, we used CRISPR/Cas9 genome editing to inactivate either *Mlh1* or *Msh2* genes. Importantly, we did not introduce any other alteration in known oncogenes and tumor suppressor genes, thus allowing the natural emergence of driver mutations within the context of genetic instability.

After 3-6 months in culture, MMR-deficient organoids, unlike the wild-type controls, exhibited MSI and a significant increase in their mutational burden. Additionally, after being cultured under stringent conditions (two-dimensional culture, growth factor deprivation), only the MMR-deficient cells formed tumor masses following subcutaneous injection into immunodeficient NOD SCID mice. Histological evaluation of the tumors revealed higher necrosis and lower E-cadherin expression (IHC H-score) in *Msh2 KO*, compared to *Mlh1 KO* tumor tissues. These findings were consistent with the epithelium-to-mesenchymal transition (EMT) phenotype observed in a *Msh2 KO* cell line retrieved from a tumor. Notably, both *Mlh1 KO* and *Msh2 KO* cells isolated from tumor masses (n = 8) displayed mutations in a substantial proportion of genes previously associated with the MSI subgroup (21 out of 35), including *Kras*, *Arid1a*, *Erbb3*, and *Tp53*, thus providing a satisfying representation of the human MSI GC mutational landscape.

In conclusion, we took advantage of organoid technology to generate MSI GC mouse models. The use of organoids derived from an immunocompetent mouse strain (BALB/c) will allow us to investigate the molecular mechanisms underlying the clinical heterogeneity of this subtype, including prognostic and predictive factors of response to immunotherapy.

Downmodulation of PD-1 receptor in NK cells using siRNA technology: a novel approach for immune-checkpoint blockade therapy

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Recently, we explored the potential application of small interfering RNA (siRNA) technology as an adjunct to immunotherapy to enhance Natural Killer (NK) cell antitumor activity. siRNAs, small non-coding RNAs, play a crucial role in gene regulation by silencing or downregulating the expression of specific genes. Our focus was to modulate the expression of immunecheckpoints in NK cells, in particular PD-1, to restore their functionality against tumor cells. To achieve efficient transfection of human NK cells with siRNAs, we developed a new protocol based on a cell transfection, with a smart pool of siRNAs specifically targeting the PD-1 receptor. In our experiments we employed both a human NK cell line called, YT, as they express PD-1 and PD-1+ NK cells from healthy donors. After 72
hours of incubation with siRNAs, we conducted phenotypic, molecular, and functional analyses on the collected
cells. Phenotypic analyses demonstrated a significant down-modulation of PD-1 expression compared to the control conditions. This down-modulation was also observed at the PD-1 mRNA level. Preliminary functional
experiments provided promising results, indicating that siRNA-mediated PD-1 down-modulation significantly increased NK cell cytotoxicity against tumor cells expressing PD-1 ligands. These findings suggest a potential avenue to enhance the antitumor activity of NK cells through siRNAs technology, specifically targeting immune checkpoints such as PD1. In conclusion, our study introduces a novel approach involving siRNA technology to modulate immune-checkpoints in
NK cells, highlighting its potential in enhancing antitumor responses. Further research and clinical validation
are needed to explore the full therapeutic potential of this approach.

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Semaphorin 4C-dependent regulation of ferroptosis in ovarian cancer cells: implications for therapy responsiveness

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Introduction. The dismal prognosis of ovarian cancer is largely due to the paucity of available therapeutic options in the advanced stage, and especially to the frequent onset of resistance to main stand platinum-based chemotherapy. Notably, alterations in redox balance and deregulated redox signaling are common hallmarks of cancer progression and resistance to therapy, including in ovarian cancer. Our lab has previously demonstrated the importance of Semaphorin 4C (SEMA4C) in breast cancer progression; indeed, SEMA4C is highly expressed in high grade advanced ovarian cancers, although its putative mechanistic role in this context has not been assessed.
The aim is to elucidate the functional role and underlying signaling mechanism of SEMA4C in ovarian cancer cells, and its potential relevance as molecular target to improve therapy responsiveness.
Material and Methods. In order to investigate the role of SEMA4C in therapy responsiveness a panel of ovarian cancer cell lines were transduced by lentiviral vectors and transfected with siRNA to achieve the overexpression and the silencing of SEMA4C, and then subjected to platinum-based treatments. The mechanisms of therapy responsiveness were also validated in primary cultures of cancer cell spheroids derived from the ascitic fluids of patients that experience high grade serous ovarian carcinoma.
Results and Discussions. Our data indicate a so far unknown role of SEMA4C in the regulation of NRF2, which is a key player of the antioxidant response, controlling refractoriness to platinum therapy. NRF2 is known to be involved in the modulation of ferroptosis regulating proteins, indeed the depletion of SEMA4C impacts on ROS and lipid ROS production, and, consequently, on viability of cells subjected to platinum-based treatments. In this context, the strengthening of oxidative stress and then, ferroptosis, can make ovarian cancer cells more susceptible to chemotherapy.
Conclusion. The discovery of SEMA4C as a new player in oxidative stress-controlling chemoresistance could pave the ground for new approaches, improving ovarian cancer responsiveness to therapy.

HDAC1 inhibition drives chondrosarcoma differentiation promoting anti-tumor effects

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Chondrosarcomas (CS) are heterogeneous bone tumors, that account for one-third of skeletal system tumours, with limited treatment options, primarily involving surgery, as chemotherapy and radiation therapy provide limited benefit. Given the poor prognosis and frequent recurrence of CS, especially in cases that progress to dedifferentiated forms, there is an urgent need for novel therapeutic approaches. Indeed, epigenetic regulation arise as a promising therapeutic strategy in CS [10]. Among these mechanisms histone acetylation is targeted by a broad compound screen including histone deacetylases (HDACs) inhibitors since several studies linked HDAC inhibitors to anti-tumoral development. Among these, it has been demonstrated that Valproic Acid (VPA) functions as an antitumoral effector by inhibiting HDAC class 1 (HDAC1, HDAC2) molecules.

This study investigates the impact of VPA, an FDA-approved HDAC inhibitor, on chondrosarcoma cell lines Sarc119 and Sw1353. Through quantitative RT-PCR, we observed that VPA significantly downregulated HDAC1 gene expression in both cell lines. To further explore VPA's mechanism, HDAC1 was silenced in the cells, and assays for colony formation, wound healing, and migration were conducted. VPA treatment and HDAC1 silencing both led to a marked decrease in colony formation efficiency, cell motility, and migration, suggesting a potential reduction in cell stemness and invasiveness. Additionally, RT-qPCR and Western Blot analyses showed reduced expression of stemness-associated genes Oct3/4, Sox2, and Nanog in treated cells. Chondrogenic differentiation assays further revealed increased expression of differentiation markers Sox9, Comp, Aggrecan, and Collagen II in cells treated with VPA and silenced for HDAC1.

These findings suggest that VPA, via HDAC1 inhibition, may reduce the malignancy of CS cells by decreasing stemness and invasiveness, making it a promising candidate for chondrosarcoma therapy.

DNA Methylome profiling to assess the Merkel cell carcinoma originating cell

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The limited molecular knowledge of the Merkel cell Polyomavirus (MCPyV)-positive/-negative Merkel cell carcinoma (MCC) subtypes (MCCP/MCCN) prevents the identification of the MCC originating cell. Recent studies outlined the presence of epigenetic dysregulations, including improper DNA methylations at target genes, in both MCC tumors and cell lines. However, a comprehensive evaluation of the methylomic changes characterizing the two MCC subtypes is still lacking. In this study, the DNA methylome profile was investigated in MCCP and MCCN cells to elucidate the molecular mechanisms of tumor onset. Analyses were performed *via* Infinium MethylationEPIC v2.0 array in two MCCP and two MCCN cell lines. Hierarchical clustering and Principal component (PCA) analyses were conducted to categorize cells according to the DNA methylation patterns. Chromosomic distributions of methylation patterns were evaluated. Moreover, enrichment analyses were performed to identify distinctive genes and pathways. Epigenome-wide analysis revealed that MCCP and MCCN cells were clusterizable from each other according to the methylation patterns. The average methylation level was higher in MCCP cells compared to MCCN cells. Differentially methylated positions between MCCP and MCCN were similarly distributed across chromosomes and chromosomic regions. The top-most differentially methylated positions were identified at neural retina leucine zipper (NRL) and survival of motor neuron 1 (SMN1) gene loci, which are involved in neuronal differentiation. Enrichment analyses indicated that MCCP-associated differentially methylated genes are involved in neuronal differentiation and neurogenesis. Methylomic changes at neurogenesis-associated genes might play a role in the Merkel cell transformation process. MCCP cells might therefore originate from either a neuroendocrine cell or a neural precursor cell.

YOUNG INVESTIGATORS
PARALLEL SESSION 2

The involvement of Sorcin in the alveolarization process during lung development

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Sorcin is one of the most important calcium sensor proteins that regulates the concentration of calcium in the Endoplasmic Reticulum (ER), inducing resistance to apoptosis and preventing ER stress. Sorcin controls the epidermal growth factor receptor (EGFR) downstream signaling pathways, both in physiological and pathological processes, affecting cellular migration and invasion in non-small-cell lung carcinoma (NSCLC) cell lines.

The present work aims at investigating the relation between Sorcin and EGFR expression in lung development in Sorcin knockout (KO) mouse model. As a result, we observed that Sorcin KO compared to wild-type mice showed: 1) an impairment of alveolarization process and an alteration of bronchi and bronchioles development, by histology sections; 2) a reduction of the expression of genes of branching morphogenesis markers (as FGF10) and of surfactant proteins (as SP-B, SP-C and ABCA3), by Real time PCR analysis; 3) an increase of glycogen content and a decrease of lipid droplets, resulting, respectively, in type II pneumocyte immaturity and impairment of lipid surfactant; 4) a reduction of EGFR, RAS and RAB5C proteins, which are correlated with their roles in defects in lung maturation and protein surfactant recycling, by Western blot analysis. Accordingly, *in vitro* analysis supported evidence showing that the downregulation of Sorcin alters EGFR localization and internalization. Altogether, these data reveal a novel role of Sorcin in the mammalian development, regulating lung alveolarization and pulmonary surfactant maturity, whose alteration are associated to many human diseases, such as respiratory distress syndrome (RDS).

Modelling *in vitro* the foetal-maternal interface

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Understanding the physiological processes governing the early stages of pregnancy is a challenge of reproduction research. Implantation of the embryo is a crucial event, where migration and invasion of fetal extravillous trophoblast (EVTs) into the endometrium is regulated by several cells of the maternal environment, such as endothelial, immune, and stromal cells [1]. Alteration of trophoblast migration and invasion strongly affects implantation and placental development, leading to miscarriages or pathological pregnancy.
In the last decades, the use of engineered nanomaterials (ENMs) in consumer products has increased leading to increased human exposure. This may have a particular impact in vulnerable populations, such as pregnant women. Indeed, it has been already demonstrated that several nanoparticles, to which the mother is exposed during pregnancy, can interfere with trophoblast migration and invasion, and hence placental formation [2-4].
In this respect, we developed a model based on organ-on-chip (OOC) technology to reproduce the implantation process by modeling the foetal-maternal interface using Human Extravillous Trophoblast Cells (HTR8/SVneo) and Human Endometrial Stromal Cells (HESCs) embedded in reconstituted extracellular matrix containing or not TiO_2 nanoparticles. In this model HTR8 migration and invasion were timely studied by mean of Time Lapse Microscopy and were confirmed by immunofluorescence analysis.
Our results show an increased ability of HTR8 to invade the ECM containing HESC when TiO_2 NPs were present. To better understand this crosstalk, we studied the expression of pro-inflammatory cytokines released by HESCs and observed that their levels increased in the presence of TiO_2, suggesting that the nanoparticles enhanced the inflammatory environment present during the implantation, further stimulating trophoblast migration and invasion.

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Intestinal organoids as a tool to study intestinal mucosal barrier alterations in digestive and extra-digestive diseases

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Background. Alterations in intestinal mucosal barrier integrity and permeability represent the common pathological feature in digestive and extra-digestive diseases, including inflammatory bowel disease, obesity and neurodegenerative disorders (1). Indeed, gut barrier impairments contribute to alterations of both secretory and bowel motor functions in such disorders (2). In this context, three-dimensional (3D) culture systems, including organoids and gut-on-chip, represent a better and more realistic model than 2D *in vitro* ones for studying the pathophysiological mechanisms underlying the gut barrier alterations as well as for testing new drugs in such pathological contexts.
Methods. In this study, we generated intestinal organoids from adult stem cells deriving from murine ileal tissues. Intestinal organoids were long-term cultivated and characterized with several specific markers by whole-mount immunofluorescence. In addition, we present a novel gut-on-chip microsystem device designed to explore intestinal mucosal barrier alterations.
Results. Intestinal organoids displayed the microscopic aspect of the ileal tract of origin, showing crypt-villus structures. In particular, the presence of MUC-2-positive cells (goblet cells), Ki-67-positive cells (proliferating cells) and lysozyme-positive cells (Paneth cells) have been observed in free-floating organoids, indicating the achievement of specific phenotype differentiation. In addition, organoids displayed a correct distribution of cell-cell junctions, such as E-cadherin (adherens junction) and Zonulin-1 (tight junction). In our gut-on-chip, pre-coated with extracellular matrix, intestinal organoids were able to adhere and differentiate, thus recreating a 3D epithelial layer.
Conclusions. Our results suggest that intestinal organoids obtained in our lab and the gut-on-chip device presented here represent a suitable 3D culture system, that well resembles the physiological structure of the intestine. Therefore, our experimental 3D model could be used to study the molecular mechanisms underlying intestinal mucosal barrier alterations in digestive and extra-digestive diseases as well as to test new drugs in these pathological contexts.

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IL-1 in the microenvironment impairs response to EGFR-neutralization

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The epidermal growth factor receptor (EGFR) signaling pathway plays a pivotal role in the development and progression of colorectal cancer (CRC), providing a well-established target for therapeutic intervention. Cetuximab (CTX), a monoclonal antibody against EGFR, is routinely used to treat metastatic colorectal cancer (mCRC) in all-RAS wild-type patients. Despite its initial efficacy, resistance to CTX frequently emerges, limiting long-term treatment success. While genetic mutations account for some cases of secondary resistance, there is growing evidence that non-genetic mechanisms, such as cytokine-driven modulation of the tumor microenvironment (TME) and activation of alternative pathways, significantly contribute to therapeutic failure. This study aims to investigate the histopathological alterations induced by interleukin-1 (IL-1) signaling in the context of EGFR activation. Specifically, we evaluated how tumor-derived IL-1α and IL-1β influence cellular responses that compromise CTX efficacy. At molecular level, IL-1 promotes increased EGFR expression and activation of compensatory signaling pathways. Data from both 2D and 3D models demonstrate that IL-1 signaling interception—achieved using a soluble recombinant decoy capable of sequestering both IL-1α and IL-1β —combined with EGFR blockade, restores CTX sensitivity in CTX-resistant cells. This effect is accompanied by reduced tumor invasiveness and slower cancer cell growth in vitro. In an *in vivo* preclinical model of CTX resistance using immunocompromised mice, the treatment led to a robust anti-tumor response, significantly reducing tumor growth and increasing IL1-R1 levels in the tumor tissue. Notably, in immunocompetent mice, chronic sequestration of IL-1 resulted in complete tumor growth abrogation and long-term tumor-free survival. These findings suggest that IL-1 neutralization may enhance CTX efficacy, particularly when combined with immunotherapy, supporting the idea that IL-1 plays a role in promoting an immunosuppressive tumor microenvironment. Mechanistically, CTX treatment triggered a strong upregulation of IL-1R1, IL-1α, and IL-1β at both the mRNA and protein levels in CRC cell lines.Elevated IL-1 levels exert cellular senescence within the tumor—characterized by G0-phase arrest—followed by post-senescence reprogramming marked by increased SNAIL expression. In TME of C57BL/6 mice, elevated IL-1 levels may contribute to sustaining an immunosuppressive environment, possibly by promoting the recruitment and survival of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T_{regs}), both known for their potent immunosuppressive functions. In line, recent studies also suggest that IL1R1$^+$ Treg cells might be more effective than their IL1R1$^-$ counterparts in suppressing the proliferation of CD8$^+$ and CD4$^+$ responder cells. In conclusion, our study identifies IL-1 as a key driver of CTX resistance through its multifaceted roles in modulating EGFR signaling. Targeting IL-1 in combination with EGFR holds promise as a potential strategy to overcome resistance and enhance therapeutic responses in mCRC patients.

BOC-FLFLF And Its Derivatives as A Novel Strategy to Overcome VEGF Drug Resistance in Diabetic Retinopathy

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Diabetic Retinopathy (DR) is the most common microvascular complication of diabetes. New therapies are eagerly required due to the limited effectiveness and resistance issues of current anti-Vascular Endothelial Growth Factor (VEGF) treatments. In this frame, we have previously demonstrated that the pentapeptide BOC-FLFLF (BOC2) acts an antagonist of bioactive heparin-binding factors involved in the pathogenesis of DR. Due to its multi-target activity, BOC2 is more effective than anti-VEGF drugs in both *in vitro* and *in vivo* disease models.
Here, to enhance the physicochemical properties of BOC2, we synthesized seven derivatives of BOC2. In addition, we used a novel experimental model of hyperglycemia-induced vascular dysfunction, using human umbilical vein endothelial cells from women with gestational diabetes (GD-HUVECs). Our results indicate that GD-HUVECs exhibit enhanced angio-inflammatory properties than healthy controls and may be further stimulated by different angio-inflammatory mediators, providing a valuable platform for preclinical drug testing. Moreover, our preliminary findings on BOC2-derivatives identify BOC-LLFLY and BOC-FLFLHser as lead candidates, based on their ability to inhibit VEGF-induced activation of VEGF Receptor 2 as well as endothelial cell sprouting.
The ongoing characterization of these compounds aims to improve the pharmacological treatment of DR, paving the way for innovative approaches that overcome the limitations of current therapies.

Beneficial effects of a novel gut-directed NLRP3 inflammasome inhibitor in experimental models of Alzheimer's disease

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Background. Increasing evidence suggests that microbiota-gut-brain axis is involved in the maintenance of brain homeostasis and in the pathophysiology of major neurological and psychiatric disorders, including Alzheimer's disease (AD). In particular, gut dysbiosis together with impaired gut barrier and enteric inflammation may represent early events in AD development thus contributing to brain pathology. In this context, the nucleotide-binding oligomerization domain leucine-rich repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome has been found to be involved in shaping central and systemic (including the gut) immune/inflammatory responses in AD[1].

Methods. In order to evaluate the effect of a novel gut-directed locally acting NLRP3 inhibitor (INF176), two different animal models of AD were employed: SAMP8 mice that develops spontaneously deficits in early learning and memory, and the transgenic model 5xFAD, characterized by an extremely aggressive amyloid pathology. SAMR1 and C57BL/6 were used as control groups respectively. Animals were treated with INF176 (50 mg/kg/day), MCC950 (used as a standard comparator, 20 mg/kg/die) or vehicle for two months (n=6/group), to evaluate the effects of a gut-directed therapy in the early stage of AD. During the last week of treatment, mice underwent the Morris water maze test to evaluate the effects of drugs on cognitive functions. Upon sacrifice, brain and colonic tissues were excised and processed for the evaluation of: 1) AD-related protein deposition (western blot for phosphorylated (p)-tau and ELISA for β-amyloid, Aβ1-42); 2) activation of inflammasome signalling (western blot of NLRP3, ASC and caspase-1 and ELISA for interleukin-1beta (IL-1β) levels); 3) expression of GFAP and Iba-1 for microgliosis and astrogliosis (western blot and immunofluorescence).

Results. SAMP8 and 5xFAD mice displayed cognitive dysfunctions together with p-tau and Aβ1-42 accumulation in the brain, as well as enteric inflammation characterized by increased active caspase-1 and IL-1β levels. In addition, brain tissue samples of AD mice displayed an increase in both GFAP and Iba-1 deposit. Treatment with INF176 counteracted cognitive impairment of SAMP8 and 5xFAD mice with effects comparable to MCC as well as decreased significantly AD-related protein accumulation in their brain as compared with AD untreated mice. INF176 also decreased NLRP3 signalling activation in both brain and colonic tissues and counteracted the increase of microgliosis and astrocytosis in the brain of AD mice.

Conclusions. The novel gut-directed NLRP3 inflammasome inhibitor INF176 exerts beneficial effects on AD mice, suggesting that the pharmacological modulation of NLRP3 inflammasome in the gut could represent a promising strategy to develop novel classes of drugs, targeting the gut-brain axis, for the treatment of AD.

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Sex-specific effects of Ube3a overdosage on Autism: Insights from Connectomic, Behavioral, and Transcriptomic Alterations

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Autism spectrum disorder (ASD) is a complex neurodevelopmental condition characterized by impairments in social communication and the presence of restrictive or repetitive behaviours. One of the most striking epidemiological features of autism is its pronounced sex bias, with males being affected approximately three to four times more frequently than females. While several factors have been proposed to explain this male predominance, including both genetic and hormonal influences, the exact molecular mechanisms underlying this disparity remain largely unknown. Numerous theories suggest that males may carry an enhanced burden of genetic and environmental risk factors, whereas females may be protected through mechanisms such as the "female protective effect." However, the specific genetic determinants and molecular pathways that contribute to this sex bias in autism are still the subject of ongoing investigation. Among the leading candidates implicated in autism is the ubiquitin protein ligase E3A (Ube3a) gene, located on chromosome 15q11-13. Copy number variations involving duplications or triplications of this chromosomal region are known to cause neurodevelopmental syndromes such as dup15q syndrome, which is strongly associated with autism. Increased Ube3a dosage resulting from these duplications has been shown to account for approximately 1-2% of autism cases, making it one of the most potent genetic risk factors identified to date. Ube3a is primarily known for its role in protein degradation via ubiquitination, but it also acts as a transcriptional coactivator for steroid hormone receptors, positioning it as a key regulator of processes that may be influenced by sex hormones. This dual functionality of Ube3a suggests it could serve as a bridge between genetic and hormonal influences on brain development, thereby contributing to the observed sex differences in autism. In this study, we sought to further explore the potential sex-differential effects of Ube3a overdosage on autism-relevant phenotypes. Using the Ube3a2X mouse model, which mimics maternally inherited triplications of the 15q11-13 region, we investigated the impact of Ube3a overexpression on neural connectivity, behaviour, and gene expression. Our results revealed significant sex-specific alterations in both neural connectomes and behaviours relevant to autism, suggesting that increased Ube3a dosage exacerbates autism-like traits more strongly in males. Furthermore, these phenotypic effects were linked to dysregulated transcription of key autism-associated genes, including those located on the X chromosome, and genes regulated by sex steroid hormones, which are known to play critical roles in neurodevelopment. Our findings underscore the importance of Ube3a as a pivotal modulator of sex-differential risk in autism, with overdosage influencing a range of molecular, cellular, and behavioural processes in a sex-specific manner. By shedding light on how Ube3a interacts with sex-differential genetic and hormonal pathways, this study contributes to a deeper understanding of the molecular underpinnings of the sex bias in autism, highlighting the need for further research into sex-specific mechanisms in neurodevelopmental disorders.

Crosstalk among neurons, muscle and bone: *in vitro* model of triple culture

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Osteosarcopenia is a complex pathology characterized by the simultaneous presence of osteoporosis and sarcopenia, two widespread conditions in elderly. Since the close influence of muscle on nerve, and *vice versa*, several studies highlighted age-related changes in biochemistry and morphology of neuromuscular junctions (NMJs). Oxidative stress is a central player in the pathogenesis of many clinical conditions and aging, as well as osteosarcopenia and NMJ impairment, therefore a treatment with a biological system, containing antioxidant active, could be useful to counteract this multi-tissues pathology[1]. Mesenchymal stromal cell-derived extracellular vesicles (EVs) are under investigation as a potential cell-free tool for the treatment of the age-related diseases, thanks to their anti-oxidant, anti-inflammatory, and cytoprotective effects[2]. Furthermore, amniotic fluid-derived MSCs (hAFSCs) have been introduced as an interesting and potent stem cell source for clinical application due to their easy, safe, and painless collection procedures with minimized ethical issues.

To study cell interactions in healthy and pathological conditions occurring in neuromuscle-skeletal apparatus, we developed a three-culture system in which osteoblasts could be treated to induce osteoporosis before the co-culture with both myotubes and neurons, treated or not with EVs. Preliminary results showed that the previous induction of osteoporosis modulated the secretome of myotubes, i.e. myostatin, bFGF, IL15, IL7, and IL6. Osteokines secreted by these cells, i.e. FGF-23 and OCN, were regulated in osteoporotic condition and can be implicated in bone-muscle crosstalk. Beside the paracrine communication, modifications in morphology and in protein expression have been investigated. The neurites reach the myotubes passing through the insert membrane; when the co-culture was exposed to osteoporotic osteoblasts, the number of healthy myotubes and of neurites contacting myotubes was affected. Indeed, the expression of typical markers of sarcopenia and neurodegeneration were analyzed. The EVs treatment reverted at least in part all these events, suggesting a possible role in slowing down the alterations induced in osteoblasts during bone disorders determining a cascade in the muscle and neuron parts.

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Cortical bone loss and fragility precedes amyloid deposition in a triple transgenic mouse model of Alzheimer's disease

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Alzheimer's disease (AD) and osteoporosis often coexist in the elderly. However, the pathophysiological link between these two diseases has been poorly investigated so far.

We examined the skeletal phenotype of a triple transgenic model of AD (3xTg-AD) harboring three mutant genes: β-amyloid precursor protein (βAPP_{Swe}), presenilin-1 ($PS1_{M146V}$), and tau_{P301L}. Extracellular Aβ plaques in 3xTg-AD mice are absent in 2-month-old mice and develop incrementally from 6 months of age, with a progressive cognitive decline that worsens significantly as the mice age, showing a specific profile that closely mimics the human AD disease.

To evaluate the skeletal phenotype associated with AD neuropathology, we performed microCT on femurs and tibiae of 2-month-old male non-Tg (n= 9) and 3xTg-AD (n= 9) mice. Our results indicated profound cortical bone loss in 3xTg-AD mice compared with littermates. Specifically, we found significantly lower bone surface (BS), periosteal and endosteal perimeters (Ct.Pm), total (Tt.Ar) and bone (B.Ar.) cross-sectional area. These data were consistent with altered long bone geometry, confirmed by lower polar moment of inertia (pMOI) (-40%), an index of resistance to fracture upon torsional forces. To determine the mechanical properties of long bones, we performed 3-point-bending test, demonstrating that bone strength, load-to-fracture, and stiffness were lower in 3xTg-AD mice than littermates.

In addition, we measured the serum concentration of irisin, whose levels are known to be low in both AD and osteoporosis. Despite the result borders on statistical significance (3xTg-AD vs non-Tg, *p=0.068*), irisin serum levels correlated positively with cortical bone parameters, suggesting that this myokine may represents an early marker of cortical bone fragility in AD.

Overall, our results demonstrate that although plaque development in 2-month-old 3xTg-AD mice is still absent, cortical bone loss occurs before the accumulation of these distinctive pathological lesions, suggesting that skeletal fragility is an early event in the pathogenesis of AD.

Regulation of ribosome activity by nutrients

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Macroautophagy delivers substrates to lysosomes through either bulk or selective mechanisms. In selective autophagy, cargo receptors facilitate the elongation of autophagosome to encircle specific cargo for degradation. However, how cargo selection and autophagosome biogenesis are coordinated during starvation-induced (bulk) autophagy remains unclear. Here, we demonstrate that the Transcription Factor EB (TFEB), activated by starvation, enhances autophagy by upregulating the autophagy receptor SQSTM1/P62. TFEB induces the formation of SQSTM1 bodies, which recruit the autophagy machinery and promote autophagosome biogenesis. We found that through this process, TFEB activation promotes ribophagy, the process of ribosomal degradation via autophagy. Mechanistically, TFEB enhances ribosomal protein ubiquitination and sequestration by SQSTM1 through the transcriptional activation of the E3 ligase ZNF598. We demonstrated the physiological relevance of the TFEB-SQSTM1 axis during starvation induced ribophagy in vivo using mice expressing a ribophagy reporter in liver. These results underscore the role of transcriptional regulation in ubiquitination during autophagy cargo selection, uncovering a novel interplay between selective and bulk autophagy.