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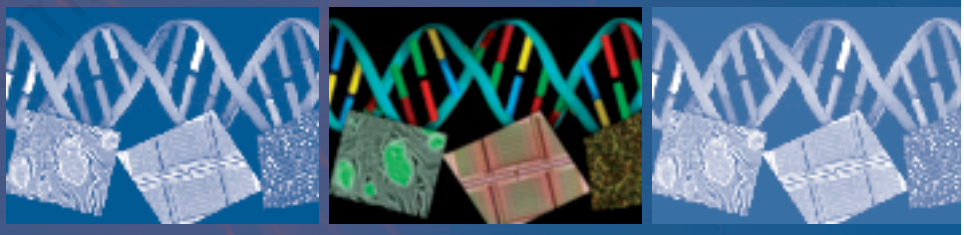

The role of 3Rs in the age of One Health: where we are and where we're going

September 13-15, 2023

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Valeria Chiono is Full Professor at the Department of Mechanical and Aerospace Engineering of Politecnico di Torino, Italy. She is lecturer of "Engineering for regenerative medicine", "Laboratory of Tissues and Physiological Processes' Models" and "Cell and tissue engineering" - Master's and Bachelor's Degree Course in Biomedical Engineering.

She earned a Master Degree cum laude in Chemical Engineering (2001) and a PhD in Chemical and Materials Engineering (2006) from the University of Pisa, Italy. From 2006 to 2012, she has been postdoc fellow in the bioengineering research group managed by Prof. G. Ciardelli, initially at the University of Pisa and, then, since 2007 at Politecnico di Torino. From 2015 to 2018, she became Associate Professor at Politecnico di Torino and in 2018 she got the position of Full Professor as a recognition for being awarded the ERC Consolidator project BIORECAR.

During her academic career, she has been the coordinator of multiple research projects, among which STARIGEN FIRB2010 project (2012-2015), on the preparation of biomimetic scaffolds for cardiac regeneration and the ERC Consolidator project BIORECAR (772168; www.biorecar.polito.it; 2018-2024) on advanced strategies for myocardial regeneration. She has been recently granted ERC-PoC POLIRNA project, where she develops transfection kits for research use. Furthermore, in 2023 she has been granted 2 additional Proof-of-Concept grants and 1 PRIN 2022 project as coordinator, and 1 PRIN PNRR 2022 project as Unit Responsible, all focused on cardiac regenerative strategies through mini-invasive approaches.

Currently, she manages a research team including 1 Associate Professor, 2 Researcher2, 2 Postdoc Fellows and 6 PhD students. Furthermore she manages BIORECAR Cell Laboratory.

In 2021, she has been appointed Deputy Director of Centro 3R, the national Interuniversity Center for the Promotion of 3Rs Principles in Teaching and Research. Prof. Chiono has been involved in the organization of several conferences and symposia at national and international level.

Her research is highly interdisciplinary aimed at the design of innovative bioengineering approaches to solve key problems in regenerative medicine and nanomedicine, and includes the development of bioactive materials and interfaces, tissue engineering, materials characterization, in vitro tissue models, drug delivery and non-viral gene therapy. One main research topic is cardiac tissue regeneration through in situ miRNA release.

Prof. Chiono has supervised several undergraduate and graduate students (including multiple PhD students), postdoc fellows and researchers and delivered more than 40 oral presentations at international conferences, and authored >200 conference abstracts. She is author of 130 publications, including 83 articles and 34 abstracts in international peer-reviewed journals and 13 book chapters (H-index: 36 Scopus; 41 Google Scholar). She is also editor of 1 book on biofabrication. She filed 5 patents in the field of biomaterials, tissue engineering and nanomedicine for RNA therapy.

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September 13-15, 2023

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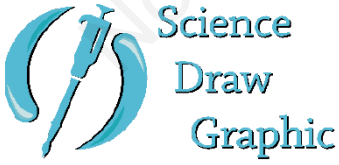

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The role of 3Rs in the age of One Health: where we are and where we're going

September 13-15, 2023

ORAL COMMUNICATIONS

Biomimetic air-liquid interface milli-bioreactor for skin tissue engineering applications

B. Masante[1,2,3], S. Gabetti[1,3], D. Baruffaldi[4], S. Villata[4], A. Sanginario[5], A. L. Audenino[1], F. Frascella[4,3], D. Massai[1,3]

[1]Department of Mechanical and Aerospace Engineering and PolitoBIOMed Lab, Politecnico di Torino; [2]Department of Surgical Sciences, University of Torino; [3]Interuniversity Center for the Promotion of the 3Rs Principles in Teaching and Research, Pisa; [4]Department of Applied Science and Technology, Politecnico di Torino; [5]Department of Electronics and Telecommunications, Politecnico di Torino, Italy

Presenting author:
B. Masante. E-mail: ✉ beatrice.masante@polito.it

Conventional approaches for testing drugs and cosmetics are based on *in vitro* monolayer culture or animal models, with clear limitations related to low biomimicry and ethical issues, respectively. Here, we developed a biomimetic milli-bioreactor for modelling *in vitro* the Air-Liquid Interface (ALI), to be used for culturing Three-Dimensional (3D) skin tissue models for drug testing and Skin Tissue Engineering (STE) applications.
The milli-bioreactor Culture Chamber (CC), made of a citocompatible and autoclavable resin (VisiJet M2S-HT250; 3D Systems, Rock Hill, USA), was designed for housing standard transwell inserts and is part of a closed-loop recirculation circuit (priming volume <1.5 mL), based on a multichannel peristaltic pump (Longer, Futian, China). The optimization of the CC geometry was guided by Computational Fluid Dynamics (CFD) simulations (COMSOL Multiphysics; COMSOL Inc., Stockholm, Sweden), performed imposing different inlet and outlet flow rates (0.1-0.5 mL/min). Performance tests for assessing the bioreactor reliability were carried out. Finally, for sterility maintenance assessment, Gelatin-Methacryloyl (GelMa) 10% w/v hydrogels laden with A540 GFP+ cells were poured in transwell inserts and cultured for 10 days in DMEM (gibco; Thermo Fisher, Waltham, USA) within the milli-bioreactor in static conditions. Cellular viability was qualitatively assessed by fluorescence analysis.
CFD results demonstrated that the flow streamlines within the CC run tangentially to the transwell membrane, preventing recirculation regions. Performance tests confirmed the ease of use and reliability of the milli-bioreactor, without air bubble stagnation. Fluorescence analysis showed high cell viability, confirming the bioreactor sterility maintenance.
The proposed milli-bioreactor is a powerful parallelizable tool for investigating the complexity of skin tissue, overcoming the limitations of conventional cell culture methods and representing a viable alternative to animal models. Biological tests with 3D skin tissue models are ongoing.

Production of a reusable micromolded microcavity insert to standardize spheroid generation for drug screening

H. Fernandes[1,2,3], V. Valeri[1], C. Degrassi[1], M. Rasponi[2], C. Mota[3]

[1]MTTlab S.r.l., Trieste, Italy; [2]Department of Electronics, Information and Bioengineering, Politecnico di Milano, Italy; [3]Department of Complex Tissue Regeneration (CTR)/MERLN Institute, Maastricht University, The Netherlands

Presenting author:
H. Fernandes. E-mail: ✉ heliacristina.debarros@polimi.it

Drug screening relies mainly on simple *in vitro* and *in vivo* models that even when combined, do not provide a perfect representation of human physiology. Likewise, due to the ethical concerns surrounding the wide use of animals for research, the improvement of *in vitro* models to support the 3R's policy is crucial. Considering the liver's pivotal role in drug metabolism and its high susceptibility to toxicity over time, better 3-Dimensional (3D) liver models are needed for efficient drug screening. We developed an approach based on micromolding to generate a reusable microcavity insert in Polydimethylsiloxane (PDMS), to facilitate spheroid generation.
An acrylic mold with small microcavities was designed and after double-casting, both a negative and a microcavity insert in PDMS were produced. The inserts were attached to the bottom of a 48-well plate, sterilized and coated to prevent cell adherence. HepG2 cells were seeded and after 72h, treatment was initiated. The efficacy of sorafenib was evaluated by assessing the drug's impact on the spheroid size, shape and viability.
The PDMS negative and microcavity insert were successfully developed, presenting the desired topography. The insert was used to seed HepG2 cells and after 48h, 1500 compact spheroids were observed per insert, similar in shape and size. Upon treatment, the spheroids showed reduced viability, loss of shape and size reduction with increasing concentration of the compound.
We were able to develop a reusable micromolded microcavity insert, in which we can generate thousands of homogeneous spheroids, in a simple and fast manner. Compact spheroids are obtained and easily retrieved. Sorafenib treatment was performed, and a dose-dependent effect was observed. The developed microcavity insert is an encouraging platform to screen drugs *in vitro*, on more reliable and physiological relevant models, reducing the need for animal research.

A multi-purpose platform for the assessment of the pro-oxidative potential of silver nanoparticles

L. Faccani[1], R. Bengalli[2], M. Gualtieri[2], F. C. Simeone[1], P. Mantecca[2], M. Blosi[1], A. L. Costa[1]

[1]CNR-ISSMC (Former ISTEC), National Research Council of Italy-Institute of Science, Technology and Sustainability for Ceramics, Faenza (RA); [2]POLARIS Research Centre, Dept. Earth and Environmental Sciences, University of Milano - Bicocca, Italy

Presenting author:
F. C. Simeone. E-mail: ✉ felice.simeone@issmc.cnr.it

With the exponential growth of engineered Nanomaterials (NMs), there is an increasing need to find abiotic, fast, predictive and reproducible methods to test the ability of these materials to induce oxidative stress in cells. In many European projects the scientific community has studied the mechanisms of action underlying the adverse responses of the organism and has created tools to predict the effects caused by new materials already in the design phase of the material itself (Safe by Design concept). We compared abiotic colorimetric tests and Cytochrome C (cytC) tests with a cellular test (DCFDA-DH in A549 cells) to evaluate the production of Reactive Oxygen Species (ROS) due to different nanoparticle design to correlate the biological responses with the pro-Oxidative Potential (OP) assessed through the evaluation of the consumed moles of Glutathione (GSH) or P-Nitroaniline (RNO), a molecular probe specific for the detection of OH•. Different design strategies for silver nanoparticles (AgNPs)(ASINA EU project) with different biopolymeric coatings: Naked (NKD), HydroxyEthylCellulose (HEC), Polyvinylpyrrolidone (PVP) and Curcumin (CUR) has been compared. From the intracellular ROS tests, results show that AgHEC an AgCUR NPs do not induce an increase in ROS levels, while the ROS production increases in the case of AgNKD and AgPVP NPs. The same trend was also found in the case of the abiotic test with the GSH assay. As regards the tests of the intrinsic OP, the trend is reversed, with AgHEC NPs with higher intrinsic OP in comparison to the other AgNP tested. This follows what was found by the consumption test of the RNO molecule. The resulting strong correlation is very promising because provides a proof of concept for the use of abiotic tests for the evaluation of one of the main relevant mechanisms related to NPs toxicity.

Study of radiation effects through an innovative and alternative biodosimetric method based on the use of a plant organism

M. G. Cascone[1], E. Rosellini[1], T. Butini[1], F. Barco[1], S. de Souza Lalic[2], F. d'Errico[1]

[1]Department of Civil and Industrial Engineering, University of Pisa, Italy; [2]Physics Department, Federal University of Sergipe – UFS, Aracaju, Brazil

Presenting author:
M. G. Cascone. E-mail: ✉ maria.grazia.cascone@unipi.it

Human beings are continuously exposed to radiation from multiple sources throughout their lives (solar radiation, background radiation, radiation from medical procedures, *etc.*). It has therefore become increasingly important to develop systems for evaluating radiation effects on cells and in particular on genetic material, using simple, rapid and easily applicable techniques to a large number of samples. These techniques are based on the identification of cytogenetic defects (endpoints), which can be generated inside the cells, due to interaction with radiation. The relationship between endpoints and absorbed dose is called biodosimetry.

The use of biodosimetry on human models is documented in the literature, but most of the data refers to subjects who absorbed high doses, while there is a lack of data on the effects of low doses.

This deficiency is linked to the impossibility of performing this type of studies on humans. Furthermore, the use of animals is also strongly discouraged, not only for ethical reasons, but also due to the difficulty of obtaining the large number of samples necessary to generate statistically acceptable results.

The aim of this work is the biodosimetry of high and low LET ionizing radiations (alpha particles and X-rays), and the biodosimetry of Ultraviolet radiations (UVB and UVC) through the use of apical meristematic cells of a plant organism (*Allium Cepa*). The use of this organism is of great interest, as it allows to carry out experimental work that applies the principle of the 3Rs with the aim of replacing the animal model. Studies on the genotoxicity of X, alpha, UVB and UVC radiations were carried out through the application of the micronucleus test, the analysis of the mitotic index and of chromosomal aberrations. In addition the already known procedure for the preparation of the samples was optimized and standardized through the design and construction of a device for the obtainment of meristematic cell monolayers to be analyzed.

Alginate dialdehyde-gelatin bioinks exploiting internal gelation mechanism for cardiac tissue engineering

E. Marcello[1,2], G.P. Stola[1,2], C. Paoletti[1,2], L. Nicoletti[1,2], V. Chiono[1,2]

[1]Department of Mechanical and Aerospace Engineering, Politecnico di Torino; [2]Centro 3R, Interuniversity Center for the Promotion of the 3Rs Principles in Teaching and Research, Pisa, Italy

Presenting author:
E. Marcello. E-mail: ✉ elena.marcello@polito.it

Cardiovascular diseases are the leading cause of death worldwide. New resolutive therapies are highly demanded due to heart tissue limited regenerative capabilities. Three-Dimensional (3D) bioprinted cell-laden constructs are a promising approach as *in vitro* models for new drug preclinical discovery and validation, in agreement with the 3R's principles.

Alginate (Alg)-based bioinks have been widely studied thanks to Alg cost-effectiveness and tunable features. Alg internal ionic gelation mechanism allows to obtain homogeneous self-standing 3D printed filaments without the use of support baths or post-printing crosslinking treatments. However, Alg presents no cell adhesion and poor *in vivo* degradability.

The aim of this work was to combine Oxidized Alginate (ADA), Alg and Gelatin (Gel) to produce bioinks suitable for cardiac tissue engineering.

Firstly, Alg/ADA bioink composition was tailored varying polymer weight ratio and calcium ion to achieve cardiac tissue-like viscoelastic properties. Alg-ADA hydrogels showed a time-dependent shear thinning behavior suitable for 3D bioprinting, due to the gradual pH-triggered release of calcium ions over time. Moreover, Alg-ADA samples showed higher degradation rate (40% weight loss) compared to Alg samples (25% weight loss) after 21 days in PBS.

Gel incorporation into Alg-ADA was optimized to support Adult Human Cardiac Fibroblasts (AHCF) adhesion, producing shear thin-

ning inks with tunable viscoelastic properties (G' 650-1300 Pa) and degradation profile (40-80% weight loss after 21 days in PBS) by varying Gel concentration. Alg-ADA-Gel showed good cytocompatibility *in vitro* according to ISO-10993-5. Finally, 3D AHCF-laden Alg-ADA-Gel bionks could be successfully printed and the samples with the highest gelatin content (25% w/v) allowed AHCFs adhesion after 24 hours of incubation, showing potential application for cardiac tissue modeling.

This project is supported by ERC BIORECAR- EU H2020 GA N°772168.

Dynamic 3D culture promotes lymphoid tissue maturation and allows the study of Chronic Lymphocyitc Leukemia cells dissemination *in vitro*

D. Barozzi[1,2], F. Scagnoli[1], F. Mantegazza[2], F. Barbaglio[1], D. Ribezzi[3], S. Farè[3], B. Vergani[2], V. Berno[4], P. Ghia[1] C. Scielzo[1]

[1]Division of Experimental Oncology - Malignant B Cells Biology and 3D Modelling Unit - B Cell Neoplasia Unit, IRCCS Ospedale San Raffaele, Milano; [2]Università degli Studi di Milano Bicocca, School of Medicine and Surgery; [3]Politecnico di Milano Department of Chemistry, Materials and Chemical Engineering; [4]ALEMBIC, advanced microscopy laboratory, IRCCS Ospedale San Raffaele and Università Vita-Salute San Raffaele, Milano, Italy

Presenting author:
D. Barozzi. E-mail: ✉ barozzi.dafne@hsr.it

Chronic Lymphocytic Leukemia (CLL) is a dynamic disease characterized by the accumulation of mature B cells in peripheral blood and lymphoid tissues. Circulating leukemic cells are resting and tend to home within lymphoid tissues where they acquire an activated phenotype and start to proliferate. Our aim is to establish an *in vitro* macroscale model of lymphoid tissues, in which recirculate CLL cells and study their behaviour in an *in vivo*-like environment.

We used and characterized a collagen-based scaffold on which we seeded human bone marrow stromal cells or lymph node fibroblasts with endothelial cells. The scaffolds were maintained in a millifluidic system (IVTech; Massarosa, Italy) and the dynamic settings were defined based on *in silico* computational studies. A leukemic cell line was used for circulation experiments. We analysed tissue viability and maturation by comparing static and dynamic cultures and evaluated leukemic cells immunophenotype at different timepoints.

Through the analysis of viability and specific functional markers (*e.g.* Collagen IV, CD31), we observed that the dynamic condition promotes a viable and compact tissue-like architecture, stimulating the organization of endothelial structures, thus reducing the risk of necrotic core. We then recirculate CLL cells in the matured lymph node and bone marrow tissues, comparing pre- and post-circulation conditions. Neoplastic cells efficiently home in both compartments, and preliminary data show regulation in the expression of functional markers (*e.g.* CXCR4, CD49d), resembling the *in vivo* situation.

We here demonstrated the feasibility and advantages of using a Three-Dimensional (3D) dynamic culture to obtain viable, organized, and vascularized 3D lymphoid tissues to study leukemia cells dissemination *in vitro*. Moreover, this model opens the possibility to increase its complexity by adding other relevant cell types and to interconnect the different tissues to obtain a multiorgan system for CLL and other haematological malignancies.

Design and fabrication of an intestinal phantom

P. Signorello[1,2,3], L. Cacopardo[1,2,3], N. Guazzelli[1,2,3], I. Nicolai[2], A. Ahluwalia[1,2,3]

[1]Research Centre "E. Piaggio", University of Pisa; [2]Department of Information Engineering, University of Pisa; [3]Centro 3R, Pisa, Italy

Presenting author:
P. Signorello. E-mail: ✉ paolo.signorello@phd.unipi.it

Modelling gut complexity is of particular interest for the study of intestinal dysfunctions, which are known to affect nutrient absorption. However, current systems are not able to provide a human-relevant model at the macroscale. Here, a method to create an intestinal phantom that can replicate the human gut will be presented. The aim is to engineer a physical twin replicating gut architecture, mechanical features, as well as the rheological properties of the luminal content and the inner mucus layer.

Firstly, porcine small intestine samples were tested for tensile properties (resulting in elastic modulus of 0.97±0.26 MPa). To match this, Polydimethylsiloxane (PDMS) at a 3:1 ratio was thus selected, functionalizing it with 3% v/v 3-AminoPropylTriEthoxySilane (APTES) to enhance its hydrophilicity and mucus affinity (1.298±0.09 MPa). The mucus layer was obtained with 2% w/v type 2 mucins. After 24 h incubation with PDMS, the uniformity of the mucus layer was quantified using image analysis.

Different molds, replicating different small intestine tracts, were designed (using Fusion 360; Autodesk, San Francisco, USA) and fabricated by fused deposition modelling (using Stratasys, Eden Prairie, USA). Finally, the intestinal fluid mimic was obtained by dissolving pectin in buffer solutions with pH values of 6,7 and 8 mimicking the intestinal pH range. Rheological measurements performed with a Brookfield viscosimeter demonstrated its suitability to replicate the physiological viscosity range of 0.1-10 Pa*s.

In conclusion, a phantom model replicating small intestine mechanics and anatomy, as well as the presence of an inner mucus layer and the luminal content, was designed, characterized, and fabricated. The development of human-relevant phantoms contributes to reducing the need for animal experimentation, in line with the 3Rs principles.

In vitro model of the human esophageal epithelium by tissue engineering tools

M. Spedicati[1,2], A. Zoso[1,2], I. Carmagnola[1,2], V. Chiono[1,2]

[1]Department of Mechanical and Aerospace Engineering, Politecnico di Torino; [2]Interuniversity Center for the Promotion of the 3Rs Principles in Teaching and Research (Centro 3R), Pisa, Italy

Presenting author:
M. Spedicati. E-mail: ✉ mattia.spedicati@polito.it

Different pathologies, such as reflux and cancer, negatively affect esophagus functionalities, altering the integrity of the epithelium. Surgical resection of cancer tissue represents the main clinical approach, however it often causes early mortality and morbidity.

In vitro tissue-engineered models are useful tools to support the preclinical validation of new alternative therapies. *In vitro* models of epithelia have been frequently obtained by Air-Liquid Interface (ALI) cell culture in collagen hydrogels on commercially-available tran-

swell inserts. However, commercial inserts have high cost and do not allow flexibility in the choice of composition and architecture, which are fundamental tools to counteract tissue contraction.
This work was aimed at designing a bi-layered human *in vitro* model of esophageal epithelium by *in vitro* culture of a cellularized hydrogel on custom-designed inserts, fabricated by Melt Extrusion Additive Manufacturing (MEAM) from Poly(ε-Caprolactone) (PCL). The hydrogel matrix was based on Gelatin Methacryloyl (GelMA), mimicking the stiffness and composition of the extracellular matrix of the esophageal submucosa. UV-mediated photocrosslinking time, using lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) initiator, was determined by photorheology. GelMA hydrogels showed permeability to a model dye molecule (Toluidine Blue O, TBO), and biocompatibility towards Human Esophageal Epithelial Cells (hEECs).
In vitro models were obtained by culturing: a monolayer of hEEC on GelMA hydrogel i) not cellularised), or ii) cellularized with human epithelial fibroblasts (bilayered construct), under ALI condition.
The easily microfabricated inserts could avoid or minimize the contraction of the esophageal tissue model. Cell viability and tissue model formation were demonstrated by Live/Dead assay and immunofluorescence analysis of mucosa and submucosa markers, respectively. In the future the model will be used to screen new therapies.

Global 3R approach and bioreactors in biomedical research

L. Calvillo

Department of Cardiology, Cardiology Research Laboratory; Istituto Auxologico Italiano IRCCS, Milan, Italy

Presenting author:
L. Calvillo. E-mail: ✉ l.calvillo@auxologico.it

Biomedical research needs preclinical models able to discriminate among the organs crosstalk and to distinguish mechanisms of action. A global 3Rs approach in our works led to important observations and technological spillovers, ensuring animals' welfare and their partial Replacement in certain experimental steps. In the last decade, our group applied a global 3R approach in several preclinical models, Refining techniques, Reducing the number of animals and partially Replacing some *in vivo* procedures by using bioreactors in a millifluidic system. The published results showed better analgesic strategies, described animals' behaviors associated with specific *in vivo* procedures, and highlighted correlations among brown adipose tissue temperature, stress, weight, neuroinflammation and handling. Working on Refinement, the issue of finding a model to investigate the organ crosstalks alteration activated by distress arose. Animals are too complex and cells in a petri dish too simple. Bioreactors allowed to explore crosstalks in a way impossible with classic *in vivo*/*in vitro* models. In our 2022-awarded work on PlosOne 2020 (www.aaalac.org/awards/global-3rs-winners) we assessed a simplified model of nervous-cardiovascular crosstalk in bioreactors, finding that coronary-artery and neuroblastoma cells connected under flow conditions started a dialogue triggering the activation of PKCβII/HuR/VEGF pathway after angiotensin II treatment. This activation was not present when cells were subjected to flow and treatment, but not connected with each other. Bioreactors will be used in our 2023-2024 CAAT granted project (https://caat.jhsph.edu/programs/grants/), which will deal to cardiovascular issues not addressable with current *in vivo*/*in vitro* models. Bioreactors appears a promising strategy to test new therapies without the use of animals.

Acinus-on-a-chip microfluidic device with 3D spherical air-liquid interfaces

N. Guazzelli[1,2,3], L. Cacopardo[1,2,3], P. Signorello[1,2,3], P. Singh[4], A. Ahluwalia[1,2,3]

[1]Research Center 'E. Piaggio', University of Pisa, Italy; [2]Department of Information Engineering, University of Pisa, Italy; [3]Centro 3R, Pisa, Italy; [4]Finnadvance OY, Oulu, Finland

Presenting author:
N. Guazzelli. E-mail: ✉ nicole.guazzelli@phd.unipi.it

Regulatory hazard assessment for airborne substances is still based on forced inhalation tests on animals. Therefore, there is a need for *in vitro* systems capable of producing human-relevant results. However, current lung-on-a-chip systems still have limitations, such as the use of Two-Dimensional (2D) membranes. In this work, a microfluidic platform able to reproduce the dynamic air-liquid interface by incorporating an array of Three-Dimensional (3D) transparent spherical micro-membranes is presented. The membranes were fabricated by casting a 10mg/mL agarose-50mg/mL gelatin solution in stereolithography-fabricated custom molds (Formlab, Somerville, USA). After agarose crosslinking, the array was treated overnight at 37°C with 100 U/g microbial transglutaminase, allowing gelatin crosslinking. The array was dried at 40°C for 24 hours before being Ultraviolet (UV)-sterilised and then rehydrated in deionized water. Tensile tests were performed to calculate the apparent elastic modulus of the membranes (1.07±0.35 MPa). Finally, the membranes were integrated into an Acinus-on-a-chip platform fabricated by the stereolithography process and Biomed Clear resin. Preliminary cell tests were performed using A549 cells seeded on the membranes (100.000 cell/cm^2) and in PET Transwells as a control. Transepithelial electric resistance measurements (EVOM - WPI) and confocal (Nikon A1; Nikon, Minato, Japan) images were acquired to determine the presence of an integral cell monolayer. Transcellular (Pg-p activity) and paracellular transport were investigated using FITC-labelled dextran (0.5 mg/mL) and rhodamine 128 (10μM). The membranes outperformed the controls in terms of barrier tightness and transport properties, presenting lower FITC passage and increased Pg-p activity, which indicates cell polarisation. The 3D micro-membrane array integrated in the Acinus-on-a-chip paves the door for more reliable and human-relevant inhalation studies. Further research is on-going to evaluate the device performance, investigating novel approaches for reproducing breathing dynamics.

Microfluidic 2D and 3D human organ-specific vasculature models to study circulating cancer cell adhesion in metastasis formation

C. Cerutti[1], A. Luraschi[1,2], L. Bettinelli[1,3], V. Grazioli[1,2], I. Kasioulis[4], N. Romero[5], A. Granata[4], G. Spinetti[3], M. Rasponi[2], P. Pelicci[1]

[1]Department of Experimental Oncology, IEO, European Institute of Oncology IRCCS, Milan, Italy; [2]Department of Electronics, Information, and Bioengineering, Politecnico di Milano, Italy; [3]Laboratory of Cardiovascular Research, IRCCS MultiMedica, Milan, Italy; [4]Division of

Clinical Neurosciences, Clifford Allbutt Building, Cambridge Biomedical Campus, Cambridge, UK; [5]Department of Life, Health and Chemical Sciences, The Open University, Milton Keynes, UK

Presenting author:
C. Cerutti. E-mail: ✉ camilla.cerutti@ieo.it

Interaction between cancer cells and Endothelial Cells (ECs), which line blood vessels, is an early and critical event in metastasis formation. Breast cancer in the most common cancer in women worldwide, that metastasise to the brain, lung and bone, causing 90% of cancer-related death. Although animal models have contributed significantly to the understanding of cell-cell interactions and cancer research, there is a need for new alternatives to reduce the use of animal models and provide *in vivo* validation. Here, we developed human organ-specific vasculature *in vitro* models to investigate the organ tropism of breast cancer. First, we established and characterized microfluidic human vascular models of brain, lung and bone Then, we designed, fabricated by photolithography, cultured and characterized a human microfluidic Three-Dimensional (3D) Blood-Brain Barrier (BBB)-on-a-chip featuring an *in vivo*-like cylindrical geometry with brain ECs alone or in co-culture with iPSC-derived pericytes in 3D ECM matrixes. These models were characterized for the expression of endothelial and cell junction markers like PECAM1, VE-cadherin and ZO1, as well as measuring permeability. Finally, we used the microfluidic Two-Dimensional (2D) and 3D models to study the interaction between human cancer cells and ECs under hemodynamic shear stress coupled to live-cell imaging. These models serve as valuable tools to uncover the molecular mechanisms underlying the interaction of cancer cells with organ-specific vasculatures, and they offer new targets for the prevention and reduction of breast cancer metastasis.

A comparative approach to recapitulate intestinal physiological absorption *in vitro*, using a novel, modular and versatile MicroPhysiological platform

L. Coppadoro[1], A. Rando[1], A. Marchesini[1], M. Poppa[1], C. Russo[1], M. Lombardi[2], S. Nicolò[2], C. Foglieni[2], G. Fiore[1], M. Soncini[1]

[1]Politecnico di Milano; [2]Ospedale San Raffaele, Milano, Italy

Presenting author:
L. Coppadoro. E-mail: ✉ lorenzopietro.coppadoro@polimi.it

The absorption of orally administered drugs through the intestinal barrier is crucial for determining their bioavailability. However, current *in vitro* models have limited reliability, primarily because they are based on static, 2D cultures. To address this issue, we have developed True Tissue On Platform (TTOP), a cartridge-based, modular and versatile *in vitro* platform. Initially, we placed the cartridge in an "open-well" static module and cultured CACO-2 intestinal epithelial cells using standard protocols. We monitored the Trans-Epithelial Electrical Resistance (TEER) during the cultures and obtained differentiated and polarized cell monolayers after 7 days, as confirmed by the expression of Human Epithelial Antigen (HEA) and Junction Adhesion Molecule (JAM). In parallel, we incubated CACO-2 cells at day 12 with or without 100µM Lucifer Yellow (LY) to evaluate cell permeability. Similarly, we integrated and cultured EpiIntestinal™ samples, which are human 3D Small Intestinal Models (SMI) from MatTek™ (Ashland, MA, USA), for 12 days in TTOP static devices. We evaluated and compared TEER, absorption of 10mM caffeine (2h), and LY paracellular passage with MatTek™ controls and CACO-2 data. SMI samples were stained with HEA and DAPI at different time points. Confocal microscopy, made possible by the controlled retrieval of the cartridge, demonstrated preserved Three-Dimensional (3D) villi-like tissue morphology at all time points. The controlled retrieval of the cartridge also allowed us to perform sequential treatments. Specifically, after 7 days of static preparation, CACO-2 cartridges were plugged into a "closed-well" perfusion module, and recirculating flows were applied for 24 hours. The versatility of TTOP enabled us to compare Two-Dimensional (2D) immortalized and 3D primary intestinal cell cultures, thereby reducing inter-device artifacts. Moreover, the introduction of controlled flows will pave the way for more relevant intestinal models, aiming at reducing the need for animal testing in drug absorption studies.

Development of green approaches based on solvent-free nanoparticles and *in vitro* models for the management of metastatic melanoma

C. Mattioda, C. Mattu, G. Ciardelli

PolitoBIOMed Lab, DIMEAS, Politecnico di Torino, Italy

Presenting author:
C. Mattioda. E-mail: ✉ carlotta.mattioda@polito.it

Nanoparticles (NPs) brought many advantages in cancer therapy, but their synthesis process results still environmentally unsustainable, due to the amount of organic solvent involved.
The aim of this project is the development of green NPs synthesis technique to deliver hydrophobic drugs. Two green NPs platforms were prepared i) antibody-loaded Chitosan (CS) NPs obtained by ionic gelation and ii) siRNA-loaded phosphate-Poly(Allylamine-Hydrochloride) (PAH) NPs, obtained through electrostatic self-assembly.
Small size NPs with low polydispersity index, and positive Z potential were obtained. No sign of cytotoxicity caused by NPs was observed against melanoma and fibroblasts cell lines. NPs-induced platelet activation was tested to investigate NPs safety after sistemic injection. Platelet activation was evaluated through SEM microscopy and by FACS analysis. PAH NPs did not trigger platelet activation, at any of the tested concentrations, while CS NPs did not induce activation at low concentrations.
NPs showed capacity to load model payloads and to release it in a controlled fashion. FACS analysis and confocal microscopy showed that PAH NPs were able to significantly enhance siRNA delivery to cells, as compared to free siRNA administration.
According to 3R principles, a Three-Dimensional (3D)-printed metastatic melanoma model is under development as a NPs testing device, representing an alternative to animal tests.
Skin fibroblasts (Hff-1) were embedded in a collagen/hyaluronic acid-based hydrogel, and allowed to grow up to four weeks. To recreate the vasculature, a channel was obtained within the hydrogel and seeded with Endothelial Cells (hUVECs). The model will be inoculated with melanoma cells and used to investigate NPs extravasation towards the primary tumor and their ability to target metastatic melanoma cells present in the channel.
C. Mattioda acknowledges PON "Ricerca e Innovazione" 2014-2020 Azione IV.R "dottorati su tematiche green" for co-financing her Ph.D scholarship.

New non-invasive, label-free monitoring approach for 2D and 3D cell culture

A. Jötten[1,2], L. Kunze[1,2], L. Gleiter[2], P. Paulitschke[1,2]

[1]Ludwig Maximilian University, Munich; [2]PHIO scientific GmbH, Munich, Germany

Presenting author:
P. Paulitschke. E-mail: ✉ philipp@phio.de

Two major issues of cell-based toxicological and drug response assays are the lack of the temporal component of endpoint assays, and the strong dependency of reproducibility and significance on the quality and condition of the cells used. Thus there is a tremendous need to provide insight into the usually inaccessible processes inside the incubator. We developed a novel lensfree imaging method exploiting the optical properties of the cell itself for imaging inside the incubator, which allows non-invasive, super compact, label-free, live-cell monitoring. By applying Artificial Intelligences (AI) to determine key cell culture parameters such as confluence, proliferation, and cell motility,[1] high-quality, automated, objective, and real-time data can be collected. Applying our Lensfree Microscopy (LM) method, we find that memory effects from heterogeneous cell culture conditions lead to an increase of variance during subsequent assays like *e.g.* omics-readouts[2] or other cell based assays, like wound healing assays, motility and proliferation assays significantly. Furthermore, our LM is also suitable for Three-Dimensional (3D) applications and will enable quantification of organoid growth dynamics and interactions. Our approach dramatically increases control and processing speed. In the context of the reproducibility crisis, we hope to make a contribution in the direction of standardization of cell-based research in the future.

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In vitro human-relevant glioblastoma models as the novel frontier of nanomedicine screening

A. Bezze[1,2,3], G. Ciardelli[1,2,3], C. Mattu[1,2,3]

[1]PolitoBIOMed Lab - Politecnico di Torino; [2]Department of Mechanical and Aerospace Engineering - Politecnico di Torino; [3]Centro 3R, Interuniversity Center for the Promotion of the 3R Principles in Teaching and Research, Pisa, Italy

Presenting author:
A. Bezze. E-mail: ✉ andrea.bezze@polito.it

The highly heterogeneous Tumor Microenvironment (TME), the stiff Extracellular Matrix (ECM) and the Blood Brain Barrier (BBB) hinder treatment efficacy against Glioblastoma (GBM). Hence, the preclinical evaluation of novel drug delivery platforms is key in GBM management. Currently, drug screening relies on animal studies or *in vitro* models, which do not fully replicate GBM complexity. To fill this gap, this study aims to develop a human-relevant GBM model to investigate the efficacy of Nanoparticles (NPs)-based drug delivery systems.

Multicellular Tumor Spheroids (MTS) were prepared by mixing different cell types at varying ratios (*e.g.*, tumor cells, microglia, and GBM-Stem Cells) to model GBM composition and embedded in polymeric hydrogels resembling the main properties of GBM ECM. MTS infiltration capacity and viability were assessed on the model following treatment with polyurethane NPs for the controlled release of Bortezomib (BTZ), a proteasome inhibitor. The results confirm that BTZ can reduce tumor proliferation and infiltration in ECM-like gels, with the effect depending on the cellular composition.

To verify NPs extravasation across brain capillaries, a vascular network was inducted into the MTS through a commercial microfluidic platform, using brain capillary endothelial cells. Immunostaining and perfusion assays were performed to analyze microvessels properties. CD31-staining showed the homogeneous presence of endothelial cells forming tight junctions (confirmed by ZO-1 staining). Fluorescent NPs injected in the channels were retained without extravasation, confirming previous *in vivo* observations.

This model represents a prototype for a 3R-compliant replica of GBM microenvironment, combining key cell actors, biomimetic materials, and an *in vitro* brain microvasculature. The promising results suggest the possibility to increase model complexity, *e.g.*, by including pericytes and astrocytes, to provide a reliable tool for nanomedicine screening.

Application of reduction and refinement principles in the evaluation of prodromal markers of Parkinson's disease in a progressive neurotoxic mouse model using multi-tracer PET imaging

M. Tassan Mazzocco[1,2], S. Belloli[2,3], A. Pinna[4], M. Serra[5], M. Morelli[4,5], R. M. Moresco[2,3,6]

[1]PhD Program in Neuroscience, School of Medicine and Surgery, University of Milano-Bicocca; [2]Nuclear Medicine Department, San Raffaele Scientific Institute (IRCCS), Milan; [3]Institute of Molecular Bioimaging and Physiology (IBFM), CNR, Milan; [4]Institute of Neuroscience (IN), CNR, Cagliari; [5]Department of Biomedical Sciences, University of Cagliari; [6]School of Medicine and Surgery, University of Milano-Bicocca, Italy

Presenting author:
M. Tassan Mazzocco. E-mail: ✉ m.tassanmazzocco@campus.unimib.it

Positron Emission Tomography (PET) is a non-invasive technique used to image metabolic processes *in vivo* with different radiotracers, allowing repeated measurements over time in the same animal. One of the advantages of PET in preclinical research is the possibility to follow the 3Rs principle, especially Reduction and Refinement. Indeed, by setting up longitudinal studies, it is possible to reduce the number of animals by using the same experimental subject for each time point and process investigated. Refinement is ensured by the use of small animal-dedicated instruments that allow the translation to preclinical research of non-invasive diagnostic imaging procedures already in use in clinical practice.

Here, we characterized the prodromal stage of Parkinson's disease using a mouse model obtained by treatment with the neuro-

toxin MPTP and the clearance inhibitor probenecid (MPTPp), by combining *in vivo* PET imaging and immunohistochemistry.
A group of 10 mice were injected with 100 mg/kg of probenecid followed by 25 mg/kg of MPTP, twice a week, for a total of 5 weeks. They were monitored longitudinally with PET before treatment and after 1, 3 and 10 MPTPp injections using two radiotracers: [18F]-FP-CIT, a marker of Dopamine Transporter (DAT) and [18F]-FDG to assess brain glucose metabolism. They were then sacrificed and brains collected for post-mortem immunohistochemical analysis.
We found that both striatal DAT-binding in-vivo assessed with [18F]-FP-CIT PET and the density of striatal DAT-positive fibers observed post-mortem started to decrease significantly after 3 MPTPp injections. [18F]-FDG uptake was significantly decreased in the striatum and thalamus already at the first administration, while at 10 MPTPp injections [18F]-FDG uptake was increased in the somatosensory and somatomotor cortex. Our results suggest that glucose metabolism is an earlier marker than DAT-binding in detecting neurodegeneration.

The fantastic voyage of solid lipid nanoparticles from the lung to the brain: non-invasive tomographic imaging as a feasible refinement process

G. Terribile[1], S. Di Girolamo[1,2], E. Donzelli[3], F. Re[4], P. Gasco[5], G. Sancini[4]

[1]School of Medicine and Surgery, University of Milano-Bicocca, Monza (MB); [2]PhD Program in Neuroscience, University of Milano-Bicocca; [3]Experimental Neurology Group, School of Medicine and Surgery, University of Milano-Bicocca, Monza (MB); [4]Nanomedicine Center, Neuroscience Center, School of Medicine and Surgery, University of Milano-Bicocca, Monza (MB); [5]Nanovector S.r.l., Torino, Italy

Presenting author:
G. Sancini. E-mail: ✉ giulio.sancini@unimib.it

Solid Lipid Nanoparticles (SLN) are colloidal drug delivery systems characterized by higher entrapment efficiency, good scalability of the preparation process and increased sustained release of the payload. Surface functionalization of SLN with ligands to achieve a site specific targeting makes them attractive to overcome the limited Blood-Brain Barrier (BBB) penetration of therapeutic compounds. SLN are prepared for brain targeting by exploiting the adaptability of warm microemulsion process for the covalent surface modification with an Apolipoprotein E-derived peptide (SLN-mApoE). Furthermore, the influence of the administration route on SLN-mApoE brain bioavailability is here evaluated by means of Fluorescence Molecular Tomography, an advanced optical imaging technology that uses the Near-Infrared Spectrum (NIR) (600–900 nm) for non-invasive *in vivo* imaging and Three-Dimensional (3D) quantification of the fluorescent probes. Fluorescent labelled SLN-mApoE are able to cross intact a BBB *in vitro* model. The pulmonary administration of SLN-mApoE is related to a higher confinement in the brain of Balb/c mice compared to the intravenous and intraperitoneal administration routes, without inducing any acute inflammatory reaction in the lungs. These results promote the pulmonary administration of brain-targeted SLN as a feasible strategy for improving brain delivery of therapeutics as well as the FMT's ability of quantitative assessment *in vivo*-bio-distribution studies.

Nebuloid: a novel *in silico* agent-based cell model

P. Mancini[1,2,3], E. Botte[1,2,3], F. Biagini[1], C. Magliaro[1,2,3], A. Ahluwalia[1,2,3]

[1]Research Center "E. Piaggio", University of Pisa; [2]Department of Information Engineering, University of Pisa; [3]Interuniversity Centre for the Promotion of 3R Principles in Teaching and Research (Centro 3R), Pisa, Italy

Presenting author:
P. Mancini. E-mail: ✉ piera.mancini@phd.unipi.it

Proliferation and resource consumption of cells are predicted using a classic continuum approach in *in silico* models. For instance, in a Three-Dimensional (3D) cell-laden spheroid consuming oxygen, the construct is represented as a unique finite domain through which oxygen flux is governed by the diffusion and consumption equation. Although this approach is widely used for several applications, it has some limitations. As a matter of fact, encapsulated cells in 3D structures are composed of discrete consuming units within extracellular non consuming space. Thus, *in silico* models assume consumption in all the nodes of the mesh (*i.e.*, the domain where the physics applies) using an estimated cell density. Moreover, they do not take into account the real arrangement of the cells within the construct or consider any regions occupied by extracellular matrix and do not attribute cell-specific metabolic parameters, which do in fact change with phenotype. Here, we propose an *in silico* model developed with the COMSOL (COMSOL Inc., Stockholm, Sweden) Livelink environment for Matlab, where cells within the construct were modelled as a point cloud with a homogeneous spatial distribution. In the simplest model, the cells consume oxygen following the Michaelis-Menten equation, with the same metabolic parameters (sOCR and Km). The metabolic rate (B) was calculated as the inward flux at spheroid surface for spheres with different radius and same cell density (5.14e12 [cell/m^3]). Preliminary results show discrepancies in the values of B between the bulk continuum model and the one obtained in the Nebuloid model developed here. Nebuloid allows control of the spatial position and the metabolic parameters for each cell: this is crucial for developing more relevant and predictive models for 3R approaches.

From data exploration to predictive models: advanced Machine Learning and Artificial Intelligence techniques for cardiotoxicity analysis

E. L. Viganò[1], A. Roncaglioni[1], D. Ballabio[2]

[1]Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan; [2]Milano Bicocca University, Italy

Presenting author:
E. L. Viganò. E-mail: ✉ edoardo.vigano@marionegri.it

In recent years, cardiovascular toxicity has attracted considerable attention from scientists and clinicians since Cardiovascular Disease (CVD) is one of the leading causes of mortality worldwide. However, except for drugs, the evaluation of the potential cardiotoxic effects of chemicals is poorly addressed and regulated. *In silico* methodologies are rapidly emerging as an essential tool in toxicology and pharmaceutical research. These approaches comprise a series of methodologies, which can play an important role in the reduction,

and replacement of *in vivo* experiments that are much more laborious, time-consuming, and expensive.
Our work focuses on developing predictive models using state-of-the-art Artificial Intelligence (AI) and Machine Learning (ML) techniques to assess the cardiotoxicity of drugs, pesticides, and industrial products. These models can help identify potential safety concerns early in the drug discovery process. As AI approaches are "data-hungry," we have collected thousands of data points for different molecular initiating events and key events related to cardiotoxicity, following the concept of the Adverse Outcome Pathway (AOP) network.
To effectively represent chemical information for AI/ML, we curated the collected data and conducted an analysis to evaluate different types of chemical representations such as Quantitative Structure-Activity Relationships (QSARs) descriptors, fingerprint, embeddings, graph, and combinations of these in more complex AI architectures. Our aim was to determine the most effective descriptors to represent chemical information in a way that enables high-performance models. Indeed, we tested various AI architectures to consider multiple aspects that could lead to potential toxic effects, such as multimodal and multitask approaches. By considering these different approaches, we aim to develop models that can accurately predict potential hazard cardiotoxic effects of drugs, pesticides, and industrial products.

A computational platform to assess the metabolic-electrophysiological behaviour of neurons cultured in monolayers

R. Fabbri[1,2], A. Ahluwalia[1,2,3], C. Magliaro[1,2,3]

[1]Research Center "E. Piaggio", University of Pisa; [2]Department of Information Engineering (DII), University of Pisa; [3]Interuniversity Center for the Promotion of 3R Principles in Teaching and Research (Centro 3R), Pisa, Italy

Presenting author:
R. Fabbri. E-mail: ✉ rachele.fabbri@phd.unipi.it

In vitro models of neural tissues are invaluable tools for the study of the human brain. However, they are associated with high costs and poorly reproducible results. In this scenario, computational model-based solutions can support a better characterization of neural behavior *in vitro*. However, current *in silico* models of neurons and neuron networks do not consider oxygen concentration, which is a crucial parameter for *in vitro* constructs since they lack vascularization.
Here we present a computational platform where an oxygen-dependent model of neuron firing is implemented. It allows modelling *in vitro* monolayers of neurons reproducing their spatial arrangement and connections. Oxygen diffuses through the aqueous medium over the monolayer and is consumed by the cells, for sustaining both neuron firing and cell functions not directly related to electrophysiological activities. Input conditions are cell density, medium height, spatial arrangement of neurons and boundary oxygen concentration. The outputs of the simulation are the neuron membrane potential and the oxygen concentration at the cell level over time.
To validate the platform, we implemented *in silico* networks reproducing those observed *in vitro* and monitored via commercial micro electrode arrays and simulated their firing. To emphasize the crucial role of oxygen, the same networks were also simulated without considering oxygen dynamics. The outputs of both the models were compared with experimental data. The results highlight statistically significant differences with the oxygen-independent model, while the outcomes of the experimental data and the oxygen-dependent model are similar. These results suggest that our platform more accurately replicates the electrophysiological behavior of neuronal monolayers and that oxygen is a key variable to be considered for describing their firing dynamics. The computational platform represents a powerful tool useful to optimize - or even replace - *in vitro* experiments.

Insights from computational studies in drug design and toxicity assessment

S. Motta, L. Callea, L. Bonati

Università degli Studi di Milano-Bicocca, Italy

Presenting author:
S. Motta. E-mail: ✉ stefano.motta@unimib.it

In recent years, computational approaches have emerged as powerful tools to complement and advance the principles of the 3Rs. Among these approaches, molecular dynamics simulations have proven particularly valuable in the field of computational chemistry. Molecular dynamics simulations involve the computational modeling of biomolecular systems, providing insights into their behavior and interactions at the atomic level. The application of molecular dynamics within the 3Rs framework offers numerous advantages, allowing for the reduction in the number of animals required for specific studies. By employing computational models, researchers can decrease the need for a large number of experimental animals by partially replacing animal testing with in-silico modeling. These methods can provide reliable predictions of molecular behavior, drug interactions, and toxicity assessments, offering alternatives to traditional animal experiments.
In this context, the present work aims to highlight the contribution of molecular dynamics simulations to the principles of the 3Rs. Several applications of molecular dynamics will be discussed where in-silico methods complement in-vitro experiments, providing an enhanced understanding of the interactions between proteins and small molecules. Specifically, we will explore the dynamic behavior of protein-ligand complexes, including the HIF-2α:ARNT target for tumor therapy, the loading mechanism of drugs onto functionalized TiO_2 nanoparticles as drug carriers, a structure-based approach to design drugs targeting ALKBH2 for the treatment of glioblastoma and the binding modes of various chemicals to the Ah receptor (AhR) and the Pregnane X Receptor (PXR). These case studies showcase the power of molecular dynamics simulations in elucidating key molecular interactions, offering valuable insights for rational drug design and toxicity assessment.

Study of bio-based nanomaterials inflammation potential in a zebrafish embryo model

C. Bragato, R. Bengalli, A. Persico, R. Mazzotta, P. Bonfanti, M. Gualtieri, P. Mantecca

POLARIS Research Center, Department of Earth and Environmental Sciences, University of Milano-Bicocca, Italy

Presenting author:
C. Bragato. E-mail: ✉ cinzia.bragato@unimib.it

The increasing development of new Bio-Nano-Materials (Bio-NMs) requires animal models experimentation to demonstrate their biocompatibility and their interactions in biological *milieu*. To reduce or avoid the extensive use of higher vertebrates, the zebrafish embryos can be used as a well-suited model for testing materials at nanoscale and to support a safe-by-design strategy for new chemicals and materials.

Zebrafish embryos are considered a promising bridge model between *in vitro* and *in vivo* research, achieving the requirements of reduction and replacement in conducting experiments using full-grown animals.

The Bio-NMs developed need to be examined to evaluate their safety on humans and environmental organisms, which implies the absence of adverse effects such as acute toxicity and inflammation. To test biological interactions and bioactive effects of Bio-NMs we used different methods: the classic Fish Embryo Toxicity acute (FET) test characterized by the presence of chorion, the natural barrier that envelopes and protect a fish embryo, and a modified FET test, in which the embryos are treated without the chorion.

By the use of classic and modified FET, we compared the effects of novel bio-based nanoparticles developed within the BIOMAT project (*i.e.* SiO_2-NPs from rice husk), taking into consideration the inflammation as main adverse effect.

The inflammatory process is investigated by qPCR, analyzing the level of genes as il8, il6, il1β, tnfα, nfkbia, nfk2, known to have an important role within this pathway, and by the evaluation of neutrophils recruitment, taking advantage of the Sudan Black staining.

The high level of genetic homology to humans, besides the simplicity of structures, makes the zebrafish embryos model a powerful system to predict and translate the effects observed on human beings, but at the same time, the model flexibility makes the results relevant also for environmental toxicology purposes.

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Stress-induced premature senescence in hiPSC-derived cardiomyocytes recapitulates aging-induced cardiac remodelling

E. Lazzarini[1], A. M. Lodrini[2,3], M. Arici[2], S. Bolis[1,4], S. Vagni[2], S. Panella[1], A. Rendon-Angel[1,5], M. Saibene[6], A. Metallo[2], T. Torre[5], G. Vassalli[4,5], P. Ameri[8,9], C. Altomare[1,*], M. Rocchetti[2,*], L. Barile[1,5,10,*]

[1]Cardiovascular Theranostics, Istituto Cardiocentro Ticino, Laboratories for Translational Research, Ente Ospedaliero Cantonale, Bellinzona, Switzerland; [2]Department of Biotechnology and Biosciences, Università degli Studi di Milano-Bicocca, Italy; [3]Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, Netherlands; [4]Cellular and Molecular Cardiology, Istituto Cardiocentro Ticino, Laboratories for Translational Research, Ente Ospedaliero Cantonale, Bellinzona, Switzerland; [5]Faculty of Biomedical Sciences, Università della Svizzera Italiana, Lugano, Switzerland; [6]Department of Earth and Environmental Sciences, Università degli Studi di Milano-Bicocca, Milano, Italy; [7]Department of Cardiac Surgery Istituto Cardiocentro Ticino, Ente Ospedaliero Cantonale, Lugano, Switzerland; [8]Cardiovascular Disease Unit, IRCCS Ospedale Policlinico, Genova, Italy; [9]Department of Internal Medicine, University of Genova, Italy; [10]Institute of Life Science, Scuola Superiore Sant'Anna, Pisa, Italy

Presenting author:
M. Arici. E-mail: ✉ martina.arici@unimib.it

Aging of the heart involves adverse remodeling in Cardiomyocytes (CMs), resulting in heart failure. This study exploits CMs differentiated from Human Induced Pluripotent Stem Cells (hiPSC) as a tool to reproduce and characterize mechanisms involved in cardiac aging. A stress–induced premature senescence was induced by short exposure to Doxorubicin (Dox) at the Sub-Lethal Concentration (Sen-CMs). We explored Sen-CMs in comparison to untreated CMs, correlating them with the results obtained in CMs isolated from young (7 weeks, y-mCMs) and old (18 months, o-mCMs) C57BL/6 mice. Dox treatment induced expression of cyclin-dependent kinase inhibitors and increased positivity to senescence-associated β-galactosidase, typical markers of senescence. Moreover, Sen-CMs showed increased oxidative stress and a depolarized mitochondrial membrane potential, which resulted in decreased ATP production. Functionally, Sen-CMs showed altered electrical activity in terms of prolonged QTc interval and Action Potential Duration (APD). This was ascribable to increased I NaL and reduced I Kr. In parallel, o-mCMs in comparison to y-mCMs, showed APD prolongation and INaL enhancement, thus reproducing Dox-induced abnormalities. Moreover, in both Sen-CMs and o-mCMs, pCAMKII level was increased in comparison to untreated CMs and y-mCMs respectively.

Overall, Sen-CMs largely recapitulate the phenotype of aged primary CMs and thus they can be considered a novel *in vitro* platform to study aging mechanisms.

Development of *in vitro* intestinal barrier model for predictive pre-clinical evaluations

A. M. A. Rando[1], L. P. Coppadoro[1], M. Poppa[1], C. Russo[1], S. Nicolò[2], M. Lombardi[2], C. Foglieni[2], G. B. Fiore[1], M. Soncini[1]

[1]Department of Electronics, Information and Bioengineering, Politecnico di Milano; [2]Cardiovascular Research Area, IRCCS San Raffaele Scientific Institute, Milano, Italy

Presenting author:
A. M. A. Rando. E-mail: ✉ alessandramaria.rando@polimi.it

Intestinal drug absorption represents a pivotal step for drug candidates to enter in clinical trials. However, pre-clinical evaluations are mainly performed in oversimplified conditions enabled by standard Two-Dimensional (2D) culture systems, failing in replicating the physiological environment, or in animal models. Therefore, the translational potential of intestinal *in vitro* models to humans is limited.

We developed a novel cartridge-based bicompartmental platform, named True Tissue on Platform (TTOP), which allows to host various culture substrates and to retrieve the biological sample in a controlled manner for endpoint analyses.

The platform was validated with endothelial (EA.hy 926) and intestinal epithelial (Caco-2) cell lines. Comparative studies were carried out to evaluate the effects on the cultures of coatings and substrates, such as gelatin-based and silk fibroin scaffolds, with respect to bare polycarbonate membranes. Co-culture experiments of these cell lines were conducted to replicate the Gut-Vascular Barrier (GVB). To assess barrier functions, Trans Endothelial/Epithelial Electric Resistance (TEER) was measured throughout the cultures. At endpoint, samples were fixed, stained (DAPI, ZO-1) and imaged.

Validation experiments confirmed the suitability of the platform both for endothelial and epithelial models, by assessing proliferation and barrier function (TEER, ZO-1) for up to 21 days. The addi-

tion of ECM-like substrates significatively promoted proliferation and differentiation, enabling the formation of Three-Dimensional (3D) cellular constructs after 7 days. An easy co-culture protocol was developed, and good tissue maturation was observed.
By hosting different substrates in the same support, we demonstrated, in an unbiased manner, the importance of the extracellular environment in promoting the formation of 3D constructs in static conditions. Preliminary results on co-culture experiments will enable the development of a 3D GVB model to better mimic intestinal drug absorption.

A novel human iPSC-based co-culture model to study neurocardiac interaction *in vitro*

G. Cattelan[1,2], G. Gentile[1,2], C. Volani[1,3], L. S. Frommelt[1,4], A. Lavdas[1], L. Foco[1], M. De Bortoli[1], C. Altomare[5,6,7], L. Barile[5,6,8], S. Zacchigna[4], P. P. Pramstaller[1], I. Pichler[1], A. Zanon[1], A. Rossini[1]

[1]Eurac Research, Institute for Biomedicine (Affiliated Institute of the University of Lübeck), Bolzano, Italy; [2]Faculty of Science and Technology, Free University of Bolzano, Italy; [3]The Cell Physiology MiLab, Department of Biosciences, Università degli Studi di Milano, Italy; [4]Cardiovascular Biology Laboratory, ICGEB Trieste, Italy; University of Trieste, Department of Medicine, Surgery and Health Sciences, Trieste, Italy; [5]Cardiovascular Theranostics, Istituto Cardiocentro Ticino, Ente Ospedaliero Cantonale, Lugano, Switzerland; [6]Laboratories for Translational Research, Ente Ospedaliero Cantonale, Bellinzona, Switzerland; [7]Euler institute, Università Svizzera italiana, Lugano, Switzerland; [8]Faculty of Biomedical Sciences, Università Svizzera italiana, Lugano, Switzerland

Presenting author:
G. Cattelan. E-mail: ✉ Giada.Cattelan@eurac.edu

The cardiac autonomic nervous system is involved in many cardiac disorders. However, the neuronal regulation of the heart in these diseases remains poorly understood mainly due to the lack of proper human cell models. To overcome this limitation, we have created an *in vitro* neurocardiac model uniquely based on human Induced Pluripotent Stem Cell (iPSC)-derived cells, namely iPSC-Cardiomyocytes (iPSC-CMs) and iPSC-Sympathetic Neurons (iPSC-SNs). iPSC-SNs in monoculture were characterized for MAP-2 (neuronal marker), for TH and DBH (adrenergic lineage markers), and for peripherin (peripheral nervous system marker) by immunofluorescence and western blot analyses. Quantification of TH+/DBH+ double positive cells at day 30 using flow cytometry showed 71-90% of positivity. iPSC-SNs exhibited spontaneous firing and burst activity measured using the Maestro Edge Multi-Electrode Array (MEA). iPSC-CMs and iPSC-SNs were co-cultured in two chambers of a silicon insert and, after insert removal, iPSC-SNs formed axons projecting towards the CMs. The beat amplitude of iPSC-CMs was measured using the MEA system and was significantly increased after 7 days of co-culture (monoculture 0.65%±0.04 *vs* co-culture 2.20%±0.14; $p<0.0001$), although the beat rate was stable. Of note, a significant increase in the beat rate of iPSC-CMs in co-culture was observed after nicotine treatment (baseline 53 BPM±8 *vs* nicotine 79 BPM±12; $p=0.0034$), that had no effect on iPSC-CMs in monoculture. On the contrary, after treatment with α-bungarotoxin, a toxin binding to nicotinic receptors and blocking neural transmission, the beat rate of iPSC-CMs in co-culture was unaffected thus confirming the capability of iPSC-SNs to establish functional connections with iPSC-CMs. The proposed neurocardiac system provides a promising modelling tool for a wide range of cardiac pathologies, as well as for drug screening and personalized medicine approach.
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The role of 3Rs in the age of One Health: where we are and where we're going

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POSTER

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Comparison between a dynamic millifluidic and a static culture system to study 3D brain tumor co-culture

T. Barra[1], J. Pisano[2], M. Prisco[2], S. Valiante[2]

[1]Department of Pharmacy, School of Medicine, University of Naples Federico II; [2]Department of Biology, University of Naples Federico II, Italy

Presenting author:
T. Barra. E-mail: ✉ teresa.barra@unina.it

Advanced *in vitro* techniques are a very important issue to study possible therapeutic agent in toxicology and biological research. In the last years many pharmaceutical companies have been focused on advanced *in vitro* model since they resemble more closely the *in vivo* situation for the predictive study of molecular toxicity. The use of these powerful tools can also permit to study physiological processes often overcoming the limits of traditional *in vitro* tests, providing better correlation with *in vivo* results. Fluid-dynamic models allow a higher oxygen supply to cultured cells thanks to the presence of continuous flow. They facilitate liquid-liquid interface between chambers and spheroids avoiding air formation. Static models are not nutrient recycling. The aim of this study is to analyze the main physiological differences of conventional *in vitro* model compared to innovative dynamic millifluidic system by studying Three-Dimensional (3D) brain co-culture made by neuroblastoma (SH-SY5Y) and glioblastoma cell lines (U-87MG) cultured in both conditions. 3D co-culture spheroids were formed by hanging drop method. Once formed 3D co-cultures were transferred in the two different systems: one static Ultra-Low-Attachment plates (ULA) and one fluid-dynamic bioreactor (Livebox1; IVTech, Massarosa, Italy). We carried out morphological analyses, evaluated pH changes, cell viability and toxicity, reactive oxygen species and the proliferation status for each culture condition. Comparison of the results determined both advantages and disadvantages of different culture conditions, providing pivotal reasons to implement advanced *in vitro* models as routine cell analysis tool, helping to replace and reduce animal laboratory use.

P02

Development of an advanced culture system to mimic *in vivo*-like behaviour for vascular tissue engineering

E. Pederzani[1], C. E. Campiglio[2], M. Ripamonti[3], C. Milani[1], M. Fuso[1], A. Rizzo[1], M. Esposito[1], A. Caldiroli[4], S. A. Riboldi[4], G. B. Fiore[1], A. Remuzzi[2], M. Soncini[1]

[1]Department of Electronics, Information and Bioengineering, Politecnico di Milano; [2]Department of Management, Information and Production Engineering, University of Bergamo; [3]Department of Biomedical Engineering, Istituto di Ricerche Farmacologiche Mario Negri-IRCCS; [4]Dialybrid S.r.l., R&D, Italy

Presenting author:
E. Pederzani. E-mail: ✉ elia.pederzani@polimi.it

Vascular tissue engineering aims to regenerate vessels "at the target site" using scaffolds able to induce endogenous regeneration. Despite encouraging *in vivo* proof-of-concept studies, intimal hyperplasia and early stenosis remain prevalent complications. Regrettably, the underlying biomechanisms remain elusive. With the aim of investigating these phenomena, *in vitro* and ex vivo models are becoming increasingly more relevant as alternatives to *in vivo* animal tests. In this scenario, we succeeded in developing an innovative and versatile culture system able to perform cell seeding and mimic *in vivo*-like stimuli (pre-tensioning, wall shear stress and dynamic pressure) for long-term experiments.
The developed system main components are: a culture chamber, a rotating mixer for semi-automatic cell seeding, a pinch-valve to generate a pulsatile pressure stress, and two fluid dynamic circuits (luminal and extraluminal compartments). Through a custom wireless user interface, it is possible to set an experiment by specifying the duration, the pumps flow rate, the pressure regime, and the culture chamber rotation speed for the cell seeding.
Three-layered electrospun grafts (diameter 6 mm, length 60 mm), composed of a nanometric mesh of Silk Fibroin (SF) and Polyurethane (PU) enclosed within SF layers, were manufactured and used. Human Umbilical Vein Endothelial cells (HUVECs) were seeded performing an ad hoc semi-automated procedure which was optimized through *in silico* simulations.
We observed that HUVECs completely covered the graft and establish a complex cell-graft and cell-cell interaction network, resulting in a compact endothelial cells monolayer. Furthermore, we found a good correspondence between *in silico* analysis and biological results.
In perspective, through the developed advanced culture system, it will be possible to establish an accurate culture model that allows to investigate the complex biological interactions that occur in vascular tissue engineering.

P03

High resolution GelMA bioprinting in Carbopol-based supporting bath

D. Baruffaldi[1], S. Villata[1], C. G. Gaglio[1], G. M. Nastasi[1], M. Petretta[3], C. F. Pirri[1,2], F. Frascella[1]

[1]DISAT - PolitoBIOMed Lab – Politecnico di Torino, Italy; [2]Center for

Sustainable Future Technologies, Italian Institute of Technology, Turin, Italy; [3]REGENHU SA, Villaz-St-Pierre, Switzerland

Presenting author:
C. G. Gaglio. E-mail: ✉ cesare.gaglio@polito.it

Nowadays, Three-Dimensional (3D) *in vitro* models have acquired importance in the field of bioengineering since they have the possibility to spatially pattern cells and their matrix. To improve the reproducibility of this models, many studies have been focused on the use of 3D bioprinting, especially the extrusion-based one. The main component of this technique is the bioink, defined as a formulation of cells and an acellular biomaterial component, which is loaded into a cartridge equipped with a needle. In this scenario, Methacrylated Gelatin (GelMA) has gained an incredible success, but its weak mechanical properties before the photopolymerization lead to poor spatial resolution and have reduced its use in bioprinting. To overcome this limitation, the potentialities of a supporting bath consisting of Carbopol ETD 2020 NF, which is high molecular weight poly (acrylic acid) polymers, have been assessed. Particularly, differently from previous studies, Carbopol was dissolved in cell culture medium rather than water to minimize its toxic effect on cells loaded into the bioink. Rheological analysis confirmed its potentialities thanks to its shear thinning behavior and fast recovery after shear stress application allowing the deposition and the support of the printed ink and, most importantly, the maintenance of the printed shape. Surprisingly, with this strategy, good resolution was obtained also with larger nozzles which are preferable when cells are present to reduce shear stress related death. Further, it was possible to print shapes that could not be obtained with standard approaches because of the presence of holes, lumens or non-planar surfaces. As expected, this new approach did not show any toxic effects towards cell embedded in GelMA, maintaining high cell viability also after two weeks of culture. At the end, empty channels inside the printed scaffold were obtained leading to the possibility to seed cell inside the lumen to mimic physiological vessels.

P04

Acute toxicity and inflammatory effect of asbestos fibres in an *in vitro* model of human gastrointestinal tract

S. Mirata[1,4], V. Almonti[1,4], S. Tirendi[1,4], S. Vernazza[1,4], D. Di Giuseppe[2], A. F. Gualtieri[2], A. M. Bassi[1,4], S. Scarfì[3,4]

[1]Department of Experimental Medicine (DIMES), University of Genova; [2]Inter-university Centre for the Promotion of the 3Rs Principles in Teaching & Research (Centro 3R), Pisa; [3]Department of Chemical and Geological Sciences, University of Modena and Reggio Emilia, Modena; [4]Department of Earth, Environment and Life Sciences (DISTAV), University of Genova, Italy

Presenting author:
S. Mirata. E-mail: ✉ serenamira94@gmail.com

The inhalation of asbestos fibers is notoriously associated with the insurgence of pulmonary diseases (*i.e.*, asbestosis, malignant mesothelioma, lung cancer). Furthermore, increasing evidence suggests that gastrointestinal malignancies may arise from the oral ingestion of asbestos fibers, since they may be released from asbestos-cement pipes resulting in the contamination of drinking water supplies. Nonetheless, the potential adverse effects associated with the ingestion of asbestos fibres are yet to be completely understood.

In this regard, a very useful experimental model is represented by the human intestinal Caco-2 cell line, which has been extensively employed for *in vitro* toxicology studies on the intestinal barrier. Indeed, when cultured to confluence onto porous membrane inserts, Caco-2 cells undergo a spontaneous differentiation which promotes the formation of a polarized cellular monolayer with functional features of small intestinal enterocytes.

Thus, we set up an *in vitro* model based on differentiated intestinal Caco-2 cells expressing mature enterocyte markers to study the potential damaging effects of commercial Russian chrysotile asbestos fibres divided into two different length fractions (> or <5-µm). The cytotoxic and inflammatory effects of these chrysotile fibres were evaluated in comparison to UICC crocidolite, which is an amphibole asbestos commonly employed as a positive carcinogenic standard. Indeed, both chrysotile fibre fractions induced a significant degree of acute cytotoxicity, with results comparable to the well-known damaging effects of crocidolite. Furthermore, the <5-µm chrysotile fraction also promoted the transcriptional upregulation of several pro-inflammatory cytokines (*i.e.*, IL-1β, IL-6, IL-8 and TNF-α). Overall, these preliminary results suggest that the adverse effects caused by the unwanted ingestion of asbestos fibres could be suitably investigated using the intestinal barrier model obtained from Caco-2 cells.

P05

HDAC inhibitors as antineoplastic and neuroprotective drugs: *in vitro* assessment

A. Squarzoni[1,2,3], P. Alberti[1,2], E. Donzelli[1,2], A. Scuteri[1,2], G. Cavaletti[1,2]

[1]Experimental Neurology Unit (ENU group), School of Medicine and Surgery, University of Milano-Bicocca; [2]Milan Center for Neuroscience (NeuroMI), Milan; [3]PhD in Neuroscience, curriculum Experimental Neuroscience, 37° cycle, University of Milano-Bicocca, Italy

Presenting author:
A. Squarzoni. E-mail: ✉ a.squarzoni@campus.unimib.it

Histone Deacetylases Enzymes (HDACs) are a class of enzymes involved in the regulation of gene expression. HDACs have action on both histone and non-histone proteins, including some proteins and factors involved in malignancies. In 2006 the first HDACs inhibitor (HDACi), SAHA, was approved by FDA for the treatment of cutaneous T-cell lymphoma. Since then, more and more HDACi have been produced and tested. However, HDACi are approved for hematological malignancies and little is still known about their application for the treatment of solid tumors. Moreover, new studies suggested the involvement of HDACs in neuropathological conditions.

With more and more studies on HDACi application, a new generation of HDACi has been developed and is currently tested to inhibit specific HDACs, possibly improving the action towards certain pathological conditions.

To reduce the *in vivo* experiments, the *in vitro* phase has a larger role in our project. This decision has a double aim: the reduction of the animals used for the following experiments and the easier initial screening and identification of the most promising drug combinations.

To do that, we are currently using different colon cancer cell lines in order to assess the IC50 of different HDACi, both alone and in combination with Oxaliplatin (OHP), the gold standard current therapy. Since OHP is well known for its neurotoxicity towards the peripheral nervous system, inducing OHP-Induced Peripheral Neuropathy (OIPN) in patients, we assess the effect of our drugs on dorsal root ganglia, the target OHP neurotoxicity.
The effect of the combination on the cancer cell line varies according to the different cell lines used for the screening. Interestingly, current data show that the combination of HDAC6 specific inhibitors (like SW-100) is very promising as a protective agent towards OHP-induced neurotoxicity.
For these reasons, we suggest that this kind of combination could be a possible advancement for the treatment of OIPN patients.

P06

Development of bioreactors for repopulation of porcine liver scaffolds

M. S. Massaro[1], L. Bolek[2], J. Dejmek[2], Y. Maléterová[3], G. Kuncová[3], R. Pálek[1,4], J. Rosendorf[1,4], L. Červenková[1,5], J. Ševčík[1], S. Šarčević[1,4], V. Liška[1,4], V. Moulisová[1]

[1]Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen; [2]Department of Biophysics, Faculty of Medicine in Pilsen, Charles University, Pilsen; [3]Institute of Chemical Process Fundamentals, Czech Academy of Sciences, Prague; [4]Department of Surgery, Faculty of Medicine in Pilsen, Charles University, Pilsen; [5]Department of Pathology, Third Faculty of Medicine, Charles University, Prague, Czech Republic

Presenting author:
M. S. Massaro. E-mail: ✉ Maria.Massaro@lfp.cuni.cz

Since 2008, the development of biologically derived matrices for regenerative medicine has been intensely studied. A growth-friendly environment for cells of different species is represented by decellularized tissues from a xenogeneic donor. Their main characteristic is the lack of immunogenic material. Decellularized porcine liver resembles the human counterpart and could be considered for reconstruction of human liver tissue *in vitro*. Thus, there is a high need to develop bioreactors for *in vitro* tissue engineering.
Firstly, porcine livers were decellularized with 1% Triton X-100 and 1% sodium dodecyl sulphate solutions. We designed a perfusion cell culture system and developed two types of custom-made bioreactors. Peristaltic pumps enabled oxygenated medium circulation. Adhesion and spread of both HepG2s and human endothelial cells on the scaffold pieces were tested in the bioreactors up to one week of cultivation. Haematoxylin-Eosin (HE) staining was used to verify both, the decellularization and the repopulation efficacy.
HE staining confirmed the good decellularization of the scaffold. Viable cells were detected at the injection sites at all tested time points, with cells migrating in the area surrounding the injection site at longer time points.
Both bioreactors can support cell adhesion and proliferation and are suitable for the repopulation of small pieces of pig liver scaffolds. Moreover, our model can be adapted to different types of tissues to verify the initial potential of the matrix to support cell growth. The ability to repopulate these small pieces is the first step to proceed with liver lobes and then with the entire organ.

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P07

The influence of gut microbiota on bone remodelling and repair: development of an innovative 3D platform

D. Lamanna[1], C. Daddi[2], F. Montemurro[2], M. Calvigioni[4], M. Di Vincenzo[1], N. Dhaouadi[1], E. Russello[3], I. Nunzi[1], E. Ghelardi[4], G. Vozzi[2,3], M. Mattioli Belmonte[1]

[1]DISCLIMO, Università Politecnica delle Marche, Ancona; [2]Research Center "E. Piaggio", University of Pisa; [3]Department of Information Engineering, University of Pisa; [4]Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Italy

Presenting author:
D. Lamanna. E-mail: ✉ d.lamanna@pm.univpm.it

The Human Gut Microbiota (HGM) plays a crucial role to maintain the physiological functions of the host's tissues, including bone. As the mechanism behind the crosstalk between the HGM and bone remains unclear, it's important to create multi-tissue culture systems representing a valid alternative to conventional animal models. To this aim, we intend to develop a modular bioengineered *in vitro* platform with 3 independent modules fluidically connected to resemble HGM-gut-bone axis. We have already created an *in vitro* HGM model, and here we present preliminary data for the intestinal and bone modules.
For the intestine, we fabricated Electrospun Gelatine (EG) starting from a gelatin solution crosslinked with GPTMS, evaluating mechanical and physical proprieties. EG were then used to support the growth of Caco-2 human intestinal epithelial cells. SEM and immunocytochemistry were used to analyse the development of the intestinal layer. For bone, type A gelatin from porcine, nanohydroxyapatite and genepine mixture was used in a 3D bioprinter to build a wood-pile scaffold with interconnected pore network. MTT and SEM were used for MG63 osteoblast-like cell viability assessment and cell-material interactions evaluations.
Results showed how gelatin structures were suitable to build up the intestinal model. Caco-2 cells spontaneously differentiate *in vitro* after reaching confluence. Columnar morphology, the presence of cell junction and the brush border with microvilli were detected. MG63 seeded on appropriate scaffolds and evaluated at different time points, displayed good viability and cell-material interaction.
In conclusion, our preliminary morphofunctional analyses on the development of intestinal barrier and bone models are encouraging for the development of a bioengineered platform evaluating the crosstalk between microbiota and bone. The steps forward will be the improvement of both models also considering the fluidical connection with the HGM model.

P08

Gelatin Methacryloyl (GelMA) sources and synthesis comparison for 3D bioprinting

C. G. Gaglio[1], D. Baruffaldi[1], S. Villata[1], L. Napione[1], C. F. Pirri[1,2], F. Frascella[1]

[1]Dipartimento di Scienza Applicata e Tecnologia, Politecnico di Torino; [2]Center for Sustainable Futures technologies @Polito, Istituto Italiano di Tecnologia, Turin, Italy

Presenting author:
C. G. Gaglio. E-mail: ✉ cesare.gaglio@polito.it

Gelatin Methacryloyl (GelMA) is one of the most used biomaterials in bioengineering for a wide range of applications, such as drug delivery, disease modeling and tissue regeneration.
GelMA is obtained from gelatin, which can be derived from different sources (*e.g.* bovine, porcine), through substitution of reactive amine and hydroxyl groups with Methacrylic Anhydride (MAA). The Functionalization Degree (DoF) can be tuned by varying the MAA amount used; thus, different protocols, with different reaction efficiency, have been developed, using different alkaline buffers (*e.g.*, phosphate-buffered saline, PBS, or carbonate-bicarbonate solution). Obviously, the modulation of the DoF has an impact of the final GelMA properties, so a deeply investigation on the features of the obtained hydrogel must be carried on.
The purpose of this study is to investigate how different gelatin sources and synthesis methods affect GelMA's properties, as literature lacks direct and systematic comparisons between these parameters. The final aim is to facilitate the choice of the source or synthesis method according to the needs of the considered application. Hence, in this work chemical and physical properties of GelMA formulations were assessed, determining the DoFs, mechanical and viscoelastic properties by rheological analysis, water absorption by swelling capacity and enzymatic degradation rates. Biological tests with two different cell lines, *i.e.*, lung adenocarcinoma cells (A549) and normal lung fibroblasts (MRC5) were performed.
Moreover, since 3D bioprinting is a rapidly evolving technology thanks to the possibility of precise deposition of cell-laden biomaterials (bioinks) to mimic the 3D structures of several tissues, the potential of different GelMA formulations as bioinks have been tested with a multi-material approach, revealing its printability and versatility in various applications.

P09

Preliminary results of evaluating effects of phytoestrogens on the skin according to NGRA

F. Rispo[1], L. Dondero[1], G. De Negri Atanasio[1], G. Allaria[1], F. Tardanico[1], M. Zanotti-Russo[2], F. Robino[2], J. Markus[3], S. Letasiova[3], E. Grasselli[1]

[1]Department of Earth, Environment and Life Science, University of Genoa, Italy; [2]Angel Consulting S.a.s., Milano, Italy; [3]MatTek *In Vitro* Life Science Laboratories, Bratislava, Slovakia

Presenting author:
F. Rispo. E-mail: ✉ francesca.rispo@edu.unige.it

According to the European Commission, an Endocrine Disruptor (ED) is an "exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations". Increasing scientific concern exists about the nature and the safety of the ingredients used by the cosmetics industry regarding their endocrine-disrupting effects. Phenolic compounds present in soy (genistein and daidzein) are considered the most powerful phytoestrogens due to their ability to interact with estrogen receptors; this ability is associated with the structural similarity to 17β-estradiol, a steroid hormone. These two are the only natural compounds included in the call for data published by EU regarding the concern of the presence of ED in cosmetic formulations (https://ec.europa.eu/newsroom/growth/items/651201/en). Due to their daily use, the presence of an ED can strongly impact human health. The beneficial or harmful effects are still under debate. We have designed an innovative method based on the Next Generation Risk Assessment (NGRA) approach that requires the examination of the exposure scenario.
In the simulated exposure scenario, the phytoestrogens were applied onto the Three-Dimensional (3D) reconstructed skin models (EpiDerm) followed by evaluation of viability using MTT assay. Follow up analyses included evaluation of the effects of phytoestrogens on tissue reconstitution and gene expression analysis, to evaluate the safety according to the NGRA.
We have designed a tiered approach to evaluate effects of potential ED than involves exposure of target organ (skin) and evaluation of multiple parameters including viability, regeneration and gene expression. In the future we plan to incorporate a reporter system capable to detect endocrine pathways activation.

P10

Cosmetic application of hydrolized marine collagen on skin models

G. De Negri Atanasio[1], L. Dondero[1], F. Rispo[1], G. Allaria[1], F. Tardanico[1], S. Ferrando[1], R. Boggia[2], F. Turrini[2], F. Robino[3], M. Zanotti Russo[3], E. Grasselli[1]

[1]Department of Earth, Environmental, and Life Sciences (DISTAV), University of Genoa; [2]Department of Pharmacy, University of Genoa; [3]Angel Consulting, Milano, Italy

Presenting author:
G. De Negri Atanasio. E-mail: ✉ Giulia.denegriatanasio@edu.unige.it

Collagen is a structural protein that constitutes a large part of the connective tissue, particularly in bones, tendons, and skin. Collagen in human skin is synthesized by fibroblasts as a procollagen, which is then converted into collagen molecule.
It represents a primary component in many cosmetic formulations because it is a natural humectant and moisturizer.
Due to the insolubility of collagen, hydrolyzed collagen is mainly applied in cosmetic formulations. Small peptides and short polypeptides are soluble in water, and they can be added easily to several cosmetic formulation, the hydrolysed collagen can promote the penetration in deeper layers of the skin giving good effect on the looks of the skin.
Collagen can be extracted from different animal source; marine organisms represent an interested option due to the possible valorisation of marine byproducts as asset of collagen in terms of a circular economy and the one health concept.
The aim of this study as part of the EcoeFISHent project (grant No 101036428) is the biological characterization of hydrolysed marine collagen.
The cell viability of Keratinocyte (HaCat) was investigated at different concentration in compliance with collagen percentage in cosmetic formulation using the MTT assay.
The results obtained from the first preliminary data on Two-Dimensional (2D) cells models were employed to investigate the

physiological behaviour of marine hydrolysed collagen on Three-Dimensional (3D) tissue model.
The EpiDerm full thickness 3D tissue model (from MatTek) was employed due to the mimicking *in vivo*-like morphological and growth characteristics, which are uniform and highly reproducible.
The morphological structure of the tissue model was evaluated through histological analysis and the moisturising capacity were investigated.

P11

Live monitoring of cell aggregation and spheroid morphology through a custom-made milli-fluidic device

P. Mancini[1,2], F. Biagini[1], E. Botte[1,2], C. Magliaro[1,2], A. Ahluwalia[1,2]

[1]Research Centre "E. Piaggio", University of Pisa; [2]Department of Information Engineering, University of Pisa, Italy

Presenting author:
F. Biagini. E-mail: ✉ francesco.biagini@phd.unipi.it

Live imaging *in vitro* is useful for evaluating the physiological variability of cell cultures of three-dimensional (3D) constructs, for example, in terms of construct morphology, but also for studying how morphology evolves over time during its differentiation, *i.e.*, during organoid generation. But label-free time-lapse imaging in Three-Dimensional (3D) is beset by challenges such as low resolution, the necessity of low light levels, condensation and focus drift among others. In this regards, lens-less tools, such as the PHIO Cellwatcher M (PHIO Scientific GmbH, Munich, Germany), purposely designed for being compatible with the incubator environment have been developed. However, PHIO is designed for imaging standard culture wells which are not suitable for 3D constructs, that can move and roll around. Solutions based on conic wells have been developed, but they are usually very small, therefore not directly compatible with the PHIO and the oxygen supply to cells may be limited.
To overcome these issues, in this work, we designed and fabricated a mill-fluidic device for live imaging of cell-laden spheroids. The device is a well-plate designed as an add-on of the PHIO Cellwatcher M. Firstly, its design was optimized via finite element analysis, to assess the shear stress under different well geometries – compatible with the PHIO - and flow conditions. After identifying its layout, the well plate was fabricated in polycarbonate, chosen for its optical availability and the ease of sterilization, through a milling process, while external ad hoc connectors were fabricated though stereolithography. The morphometrics were extracted from the images collected and the end point compared with those obtained with standard 3D optical techniques (*e.g.*, confocal microscope). Our results show that the device is suitable for long term non-destructive monitoring of spheroids.

P12

From *in vivo* to *in vitro* relative potency assays to characterize multicomponent vaccines for low- and middle-income countries: the StrepA vaccine case

C. Muzzi[1], X. Ferhati[1], F. Citiulo[1], A. Acquaviva[1], A. de Felice[1], F. Pippi[1], E. Cappelletti[1], G. M. Massantini[1], L. Rovetini[1], M. Carducci[1], L. Massai[1], D. Pasqui[2], O. Rossi[1], D. Gomes Moriel[1], A. M. Colucci[1], F. Necchi[1]

[1]GSK Vaccines Institute for Global Health (GVGH), Siena; [2]GSK, Siena, Italy

Presenting author:
C. Muzzi. E-mail: ✉ chiara.x.muzzi@gsk.com

GSK Vaccines Institute for Global Health (GVGH) is currently developing multicomponent vaccines against antimicrobial resistance threats for low- and middle-income countries. Among them, a candidate vaccine against Group A Streptococcus (StrepA) is under development. The vaccine is composed of 3 recombinant proteins (Slo, SpyAD and SpyCEP) and 1 glycoconjugate (Group A carbohydrate conjugated to recombinant CRM carrier protein) formulated on aluminium hydroxide (Alum).
A competitive-ELISA based method was developed using antigen-specific mouse monoclonal antibodies (mAbs) to identify and specifically quantify the 3 recombinant proteins in the final formulations. The aim is to use this method as an *in vitro* Relative Potency (IVRP) assay to replace the *in vivo* test which is currently used to evaluate the vaccine potency.
An accelerated stability study was performed on the formulated Drug Product to correlate *in vivo* results with the *in vitro* ones. Regarding SpyAD and SpyCEP, results did not show significant differences between the *in vitro* and *in vivo* potency, with no impairment of the immune response. Two anti-Slo protein mAbs were tested. No significant differences between the *in vivo* and the *in vitro* assay were seen with one of mAbs, while the second mAb led to an increase potency readout in the *in vitro* assay. Investigations are ongoing with the aim to identify the Slo epitopes which are targeted by each of the two mAbs and to better understand the differences in potency. Moreover, more stressful conditions are going to be evaluated to mimic subpotent vaccines.
Results obtained with these experiments allow us to measure the potency of the StrepA vaccine ensuring the quality is maintained, together with the possibility to replace the *in vivo* studies for potency monitoring.

P13

Self-assembling nanomicelles as a versatile new nano-formulation

G. Farruggia[1], L. Galassi[1], L. Anconelli[1], I. Orienti[1], P. Blasi[1], S. Iotti[1], P. Lodeserto[2]

[1]Department of Pharmacy and Biotechnology, Alma Mater Studiorum-University of Bologna; [2]Section of Endocrinology and Metabolic Diseases, Department of Systems Medicine, University of Rome Tor Vergata, Rome, Italy

Presenting author:
G. Farruggia. E-mail: ✉ Giovanna.Farruggia@unibo.it

The pharmaceutical nanoformulations on the market are liposomes, lipidic or polymeric nanoparticles, and represent the more recent evolution of nanomedicine. In comparison to the conventional formulations, nanoformulations transport active molecules specifically to target tissues (such as tumours, inflamed or infarcted tissues) providing a localized release which increases the therapeutical efficacy and decreases toxicity. This decreases the administration dose. Currently the nanoformulations need to be tailored specifically on the molecules to be incorporated. Complex prepara-

tive procedures, requiring organic solvents, make them economically disadvantageous, strongly impacting on environment.
We propose a new class of nanoparticles able to incorporate and transport several types of molecules, starting on the same basic mixtures of phospholipids, triglycerides, fatty acids and cyclodextrins. Drugs or nutraceuticals can be added to these mixtures. Nanomicelles are obtained in water by spontaneous self-assembling of the mixture components, triggered by thermodynamic balance of dissolution-solubilization. These nanomicelles are characterized by a hydrophilic-co-hydrophobic core that is able to incorporate with high efficiency both hydrophilic and hydrophobic molecules. These nanomicelles have efficiently carried fenretinide, a derivative of Vitamin A, in tumour cells of neuroblastoma, lung cancer, colon cancer, melanoma and promyelocytic leukemia. Natural substances such as spermidine and berberine have also been delivered in neuroblastoma and osteosarcoma cells. The results of *in vitro* and *in vivo* studies indicated higher antitumor efficiency of the nanoencapsulated drugs than the free molecules. On the contrary, no toxicity was observed in normal cells. Finally, we have demonstrated optimal storage stability of these nanomicelles both at -20°C, and as a freeze-dried product to be rehydrated for use.

P14

Exploring the potential of aligned electroconductive nanofibrous membranes for culturing peripheral nervous system cells

M. Bortolameazzi[1], M. M. E. A. M. El Soury[2], G. Ciardelli[1], S. Raimondo[2], G. Gambarotta[2], C. Tonda-Turo[1]

[1]Department of Mechanical and Aerospace Engineering and PolitoBiomed Lab, Politecnico di Torino; [2]Department of Clinical and Biological Sciences, Neuroscience Institute Cavalieri Ottolenghi, Università di Torino, Italy

Presenting author:
M. Bortolameazzi. E-mail: ✉ matteo.bortolameazzi@polito.it

A large variety of substances (*i.e*. pollutants and therapeutics) have been reported to cause peripheral demyelinating neuropathies that result from the injury to Schwann cells and sensory/motor neurons, by direct toxic effect or immune-mediated cytotoxicity. *In vitro* myelination/demyelination assays are carried out using organotypic cultures of dorsal root ganglia rat explants, so far.
In this work, we proposed the development of an *in vitro* model reassembling the key features of the peripheral nerve composition by co-culturing Schwann cells (SC) and neurons on an engineered substrate having instructive cues to steer cell assembling. Instructive substrates were produced by electrospinning technology using a blend of Polycaprolactone (PCL) and Polyaniline (PANI) doped with Camphor-10-Sulfonic Acid (CSA). PCL, chosen as the core polymer of fibers, is characterized by high cytocompatibility and processability, whereas, PANI doped with CSA was added to enhance the conductive properties of the mats.
Random or aligned oriented fiber were produced and characterized in terms of physicochemical (hydrophilicity, stiffness, electroconductivity and composition) and structural (topography, alignment and diameter of the fibers).
Finally, preliminary *in vitro* experiments were carried out by seeding rat-derived SC onto our membranes to assess cell viability and proliferation by CellTiter-Blue® assay and cell organization by DAPI/Phalloidin staining.
Thanks to these preliminary tests, we can state that PCL-PANI electrospun membranes represent a promising system for culturing peripheral nervous system cells. In particular, the aligned mats helped the cells to have a more physiological morphology and organization, replicating the anisotropic structure of *in vivo* nervous tissue.
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P15

Electron microscopy studies of the bio-interactions of novel nanomaterials and *in vitro* models: the contribution of PMiB

M. Saibene, P. Mantecca, G. Capitani

Piattaforma di Microscopia di Milano – Bicocca, Italy

Presenting author:
M. Saibene. E-mail: ✉ melissa.saibene@unimib.it

Electron Microscopy (EM) represents a powerful tool in biological studies to better understand how new Nanomaterials (NMs) are able to interact with *in vitro* models and studying their effects.
Transmission Electron Microscopy (TEM), with is high-resolution power (0.1 nm), allows to investigate bio-interactions between cells and NMs at the ultrastructure cellular level. Thanks to TEM analysis, it possible to describe how NMs affect the cells and if they are uptaken by cells entering the cellular membrane. Once inside the cell, NMs can be accumulated at cytoplasmic or nuclear level, causing possible structural damages bringing also to organelle malfunctions or even DNA damages.
Scanning Electron Microscopy (SEM), differently to the TEM, does not allow an investigation at the cellular ultrastructure level, but it can give information regarding the cell surface. With SEM analysis, it is possible to investigate damages at the cell membrane level and if NMs interact with cellular surface structures (*e.g*. filopodia) giving a different and complementary perspective of the bio-interactions from TEM studies.
Especially in nanotoxicology studies, knowing the structure and the composition of the NMs can be a relevant information. For these reason, Scanning Transmission Electron Microscopy (STEM) coupled with elemental analysis (*e.g*. Energy Dispersive x-rays (EDS) detector) can be used. It can univocally confirm the NM presence in the *in vitro* model both in TEM and SEM samples.
In addition, EM can be useful even to first characterize alone the NM to be tested. The novel NMs can be described in term of dimension, morphology, crystalline structure (diffraction pattern) and chemical composition (elemental analysis, *e.g.* EDS).
We report some cases of EM studies involving different *in vitro* models and several innovative nanomaterials.

P16

Evaluation of the biological effects of metal nanoparticles in an *in vitro* lung system at doses representative of environmental concentrations

G. Motta[1,2], M. Gualtieri[2], M. Saibene[2], R. Bengalli[2], J. Cabellos[3], F. Belosi[4], P. Mantecca[2]

[1]University of Milano-Bicocca, Department of Biotechnology and

Biosciences, Italy; [2]Research Centre POLARIS, Department of Earth and Environmental Sciences, University of Milano-Bicocca, Italy; [3]Leitat Technological Center, Barcelona, Spain; [4]CNR-ISAC, Institute of Atmospheric Sciences and Climate, National Research Council of Italy, Bologna, Italy

Presenting author:
G. Motta. E-mail: ✉ g.motta15@campus.unimib.it

Silver (Ag) and Titania (TiO_2) Nanoparticles (NPs) are widely used due to their respective antimicrobial properties and photocatalytic activity. We report a novel framework to identify and assess the hazard of new Ag and TiO2 based NMs designed with different coatings according to a Safe and Sustainable by Design approach, and considering the potential human exposure during NMs production. To assess the potential hazard of these NPs it was defined the most likely internal dose of a person exposed during the production of Ag and TiO2 nano-enabled products starting from a monitoring campaign in a manufacturing site. Applying the MPPD model to determine the lung retained dose of NPs, it was possible to estimate the doses representative of a chronic human exposure considering an 8-hour workweek over 1 month, 6 months, and 1 year. As *in vitro* model, we used a human cell contact co-culture (A549 and THP-1 cells differentiated in macrophages) representing the alveolar space and exposed at the air-liquid interface using the Vitrocell® Cloud Alpha 12 system. This approach allows for more reliable results than by submerged culture systems due to a closer replication of the human physiology. We determined the Deposition Efficiency (DE) of each NP using the quartz crystal microbalance available with the system. Our results show that different NPs have a different DE depending on their characteristic and that this step is critical for properly defining the concentration of NPs to be nebulized in order to obtain the final dose of exposure. An Adverse Outcome Pathways-oriented strategy was employed to connect the NPs physicochemical properties and the biological reactivity to the potential health effects. Physicochemical properties were evaluated using TEM and DLS. Cell viability and cytokine release were assessed after 24 hours. The study's preliminary results indicate no significant hazards for chronic inhalation exposure doses, confirming the safety of the developed NPs.

P17

Evaluation of the antioxidant power of the different cosmetic formulations

F. Tardanico[1], G. De Negri Atanasio[1], L. Dondero[1], F. Rispo[1], G. Allaria[1], M. Zanotti-Russo[2], E. Grasselli[1]

[1]Department of Earth, Environment and Life Science, University of Genoa; [2]Angel Consulting S.a.s., Milano, Italy

Presenting author:
F. Tardanico. E-mail: ✉ tardanico.francesca@gmail.com

Oxidative stress occurs due to an imbalance between the intake of pro-oxidant and antioxidant substances within the cell. Among the substances most involved in this imbalance, Reactive Oxygen Species (ROS) play a pivotal role. The preservation of balance oxidative state allows the skin to respond better and more readily to the environment, such as pollutant, insults and slows down the skin aging.

Vitamin C (Vit C) is a powerful hydrophilic antioxidant with several applications in the cosmetic field. Vitamin E (VIt E) is a lipophilic antioxidant and has a primary role in protecting cell membranes against oxidative stress and maintaining the collagen network in the skin. It is an important ingredient in many cosmetic products. Both vitamins can neutralize the ROS and constitute a strong line of defence in retarding free radical induced cellular damage.
The aim of this study is to determine the biological response of both commercial cosmetic formulations containing Vit C or Vit E.
Both creams were tested to determinate cell viability on Human Endothelial Cells (HECV), the items were evaluated at different concentrations from 20 to 0.01 mg/mL.
Protection against ROS damage was performed treating HECV with the test creams followed by 3mM of H_2O_2 to induce oxidative stress. Then the cell viability was to evaluate the capability of ROS protection in this cell model.
In conclusion, both cosmetic formulations shown an antioxidant activity in both the non-cellular and cellular system. In the case of Vit C cream at a concentration of 0,1 mg/mL while the Vit E cream at 10 mg/mL.

P18

Bioreactor-based investigation platform for unravelling bone mechanotransduction mechanisms: synergic pro-osteogenic effect of direct perfusion and pulsed electromagnetic fields on biomimetic bone tissue models

S. Gabetti[1,2], F. Daou[3], B. Masante[1,2,4], E. Zenobi[5], E. Scatena[5], F. Mochi[6], G. Putame[1,2], A. Sanginario[7], C. Del Gaudio[8], L. Rimondini[3], C. Bignardi[1,2], A. Cochis[3], D. Massai[1,2]

[1]Department of Mechanical and Aerospace Engineering and PolitoBIOMed Lab, Politecnico di Torino; [2]Interuniversity Center for the Promotion of the 3Rs Principles in Teaching and Research, Pisa; [3]Laboratory of Biomedical Materials, Center for Translational Research on Autoimmune and Allergic Disease-CAAD, Department of Health Sciences, University of Piemonte Orientale, Novara; [4]Department of Surgical Sciences, University of Torino; [5]E. Amaldi Foundation, Roma; [6]Hypatia Research Consortium, Roma; [7]Department of Electronics and Telecommunications, Politecnico di Torino; [8]Italian Space Agency, Roma, Italy

Presenting author:
S. Gabetti. E-mail: ✉ stefano.gabetti@polito.it

Bone fractures represent a growing socio-economic burden worldwide. Although biophysical stimuli, such as internal mechanical loading and external Pulsed Electromagnetic Field (PEMF), are known to promote bone healing, the triggered mechanotransduction mechanisms remain partially unknown, leading to empirical treatments. In view of reducing or even replacing animal tests, we developed a bioreactor-based platform for culturing and investigating biomimetic Bone Tissue Models (BTMs) under direct perfusion and PEMF stimulation.
The platform, based on 3 Independent Culture Chambers (CCs) connected to a perfusion unit, is combinable with a PEMF stimulator for automated culture of BTMs. For the biomimetic BTMs, polylactic acid scaffolds mimicking the trabecular bone structure were seeded with human mesenchymal stem cells (4 x 10^6/scaf-

fold). BTMs were housed in the CCs and cultured under direct perfusion (flow rate=0.3 mL/min) for 21 days, with or without PEMF stimulation (intensity=1.5 mT, frequency=75 Hz) for 4 h/day (n=3). In parallel, the control group was statically cultured (n=3). Collagen type I (COL1) and II (COL2) expressions were assessed by qPCR and high-throughput RNA sequencing was performed.
The combination of the biophysical stimuli significantly boosted COL1 (6.5-fold) and COL2 (4.3-fold) expression in comparison to the control, showing a synergic pro-osteogenic effect of flow-induced shear stress and PEMF stimulation. Interestingly, the transcriptome analysis revealed the activation of immune response pathways and an increased expression of angiogenesis and osteogenesis upstream regulators.
The proposed investigation platform will enable reaching a thorough understanding of the correlations between applied biophysical stimuli and biological response at the cell- and tissue-scale, with the final aim to define the rationale basis for improved biophysical stimulation treatments.
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P19

Inhibition of the hexosamine biosynthesis pathway affects the epithelial-mesenchymal transition in pancreatic ductal adenocarcinoma

V. Brancato[1,2], B. Zerbato[1], J. Calviello[1], E. Domaneschi[1], F. Chiaradonna[1]

[1]Department of Biotechnology and Biosciences, University of Milano-Bicocca; [2]Center for Genomic Science IIT@SEMM, Italian Institute of Technology, Milan, Italy

Presenting author:
V. Brancato. E-mail: ✉ virginia.brancato@unimib.it

Investigate cancer through Two-Dimensional (2D) cell culture is not enough to recapitulate the complexity underlying the interactions among cell types. Recent technologies improved the micro-physiological systems able to copycat the tissue–tissue interfaces, spatio-temporal chemical gradients and microenvironments. These dynamic bioengineered *in vitro* models could be adopted in pre-clinical studies for drug testing, in line with the 3Rs principles. Pancreatic Ductal Adenocarcinoma (PDAC) is the third leading cause of cancer deaths. Only about 20% of patients are eligible for surgery, so PDAC treatment is based on chemotherapy, mainly Gemcitabine (GEM) alone or in combination. PDAC is resistant to gemcitabine treatment and it is characterized by a strong desmoplastic reaction that impairs the chemotherapeutics diffusion. It is necessary to found new molecules that can overcome GEM chemoresistance. The Hexosamine Biosynthesis Pathway (HBP) is recurrently upregulated in PDAC. The FR054 inhibitor targets the PGM3 enzyme in the HBP, affecting both N and O-glycosylation. Previous studies showed that the growth inhibition induced by GEM alone is significantly enhanced by the combined treatment with FR054. Epithelial-Mesenchymal Transition (EMT) is a process leading to the morphological tumor cells changes, to cell polarity loss and cytoskeleton changes. EMT is a crucial event in the tumor cells invasion and migration. The aim of the research is to investigate how FR054 inhibits HBP and modulate EMT and desmoplastic proteins. Pancreatic cancer cell spheroids and 2D cell lines are challenged with FR054 alone or in combination with GEM. Modulation of genes (RNAseq) and proteins (western blot and immunofluorescence), confirmed the FR054 ability to inhibit the HBP in 2D and spheroids, inducing growth arrest. Furthermore, FR054 modulates the levels of important proteins in EMT both in 2D and in Three-Dimensional (3D) models, attributing to FR054 an effect on important cancer hallmarks: invasion and metastasis.

P20

Development of new eco-sustainable wound dressing based on *Rosa canina L.* extract encapsulated into nanovesicles and loaded on broom fibres

C. Cappadone[1], M. Rossi[1,2], M. Mandrone[3], S. Rossello[1,2], V. Sallustio[2], A. Abruzzo[2], B. Luppi[2], F. Bigucci[2], T. Cerchiara[2]

[1]Pharmaceutical Biochemistry Lab., Department of Pharmacy and Biotechnology, Alma Mater Studiorum, University of Bologna; [2]Drug Delivery Research Lab., Department of Pharmacy and Biotechnology, Alma Mater Studiorum, University of Bologna; [3]Pharmaceutical Botany Lab., Department of Pharmacy and Biotechnology, Alma Mater Studiorum, University of Bologna, Italy

Presenting author:
C. Cappadone. E-mail: ✉ concettina.cappadone@unibo.it

Wound healing represents a significant health and economic burden on society and, despite recent advances in wound care, it remains a challenge. In addition, increasing ecological awareness stimulates the development of new sustainable products. In this context, Spanish broom fiber represents a viable alternative to the use of cotton for the preparation of biocompatible dressings. Compared to cotton, broom cultivation does not require special care, fertilizers, pesticides, and herbicides. Moreover, it can be grown in soils unsuitable for food crops. The aim of this work was the development of an eco-sustainable Spanish Broom dressings impregnated with glycethosomes containing *Rosa canina L.* extract for the treatment of wounds and skin injuries. Glycethosomes are nanovesicles containing 10% of glycerol and 20% of ethanol, prepared using the solvent injection method. The chemical-physical characterization of the formulation obtained showed nanometric size (150-200 nm), good polidispersion index (lower than 0.3), negative ζ-potential (-30 mV) and optimal encapsulation efficiency (~90%). Additionally, glycethosomes showed a good stability for up to 6 months. The biocompatibility of the nanovesicles was tested on human dermal fibroblasts, and no cytotoxic effects were detected with all the used concentrations. Therefore, impregnation of the broom dressings with glycethosomes was carried out. Cells grown in conditioned culture medium showed a reduction in wound closure time by the scratch test. Moreover, a reduction of intracellular ROS levels was detected. In conclusion, due to antioxidant and healing properties, the Spanish broom wound dressings may represent an interesting alternative to traditional cotton dressings.
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P22

Ethical considerations in the investigation of ferulic acid's therapeutic effect on fertility in MSG-induced testicular damage

A. Can[1], M. Acikel Elmas[1], M. Kolgazi[2], S. Arbak[1], Y. Isil Ulman[3]

[1]Acibadem University, School of Medicine, Department of Histology and Embryology, Istanbul; [2]Acibadem University, School of Medicine, Department of Physiology, Istanbul; [3]Acibadem University, School of Medicine, Department of History of Medicine and Ethics, Istanbul, Turkey

Presenting author:
A. Can. E-mail: ✉ anil.can@live.acibadem.edu.tr

This paper deals with ethical aspects of a biomedical study demonstrating effects Of Monosodium Glutamate (MSG) and ferulic acid over male fertility. It benefits from the PREPARE Guidelines all through the research process. Firstly, a thorough literature search was carried out to formulate an original and robust study, and all referential sources were carefully listed in a well documented manner to comply with research integrity, responsibility and accountability while applying for the Ethics Committee. The justification for the use of laboratory animals and suitability of the selected species were based on scientific necessity as well as the ethical principle of Replace. The research methodology was properly planned to prevent unnecessary use of animals. Secondly, the correct number of animals in each group was carefully reckoned. Calculation was carried out by using ANOVA power analysis with 80% power, 0.05 statistical significance and 0.6 effect size that determine to take eight rats in each group as an optimum number to assess research hypothesis in line with the ethical principle of Reduction. Thirdly, Refinement measures were properly considered: the laboratory animals were scrupulously monitored regarding their physical and psychological changes on daily basis; all precautions were taken to minimize pain and distress on animals; discomfort were minimized by keeping rats in a laboratory environment with a temperature of 22±2°C and a standard light/dark (12/12 hours) cycle all through the study. The animals were randomly divided into 5 groups and housed together to minimize stress and prevent social isolation. Pain was alleviated by using anesthesia during euthanasia. In conclusion, this study aligns with research integrity and the 3Rs principles by justifying the use of animals, minimizing the number required, and implementing refinement measures to enhance animal welfare in investigating the curative effect of ferulic acid on fertility damage induced by MSG.

P23

Coaxial bioprinting for *in vitro* tumour models

P. De Stefano, E. Bianchi, L. Libutti, A. Scaletti, G. Dubini

Laboratory of Biological Structure Mechanics, Department of Chemical, Materials and Chemical Engineering "G. Natta", Politecnico di Milano, Italy

Presenting author:
P. De Stefano. E-mail: ✉ paola.destefano@polimi.it

Cancer is the second leading cause of death worldwide. Therapy failure rate is still high, so creating complex and accurate *in vitro* models is mandatory to better understand the complex mechanisms behind drug response and to better build up the preclinical research. Bioprinted cancer models represent a significant improvement by mimicking the complex physiological Three-Dimensional (3D) architecture. The most common basement membrane used *in vitro* as substrate for 3D cell culture is Matrigel™ thanks to peculiar biochemical properties. However, 3D bioprinting of free-standing matrigel™ constructs is still an open point: it is hardly bioprinted pure with standard pneumatic-extrusion systems, so it is commonly combined with other bioinks. Nonetheless, the use of other materials introduces additional variabilities, which limits their usage. Therefore, in our previous work, we have presented a custom-made volumetric-extrusion bioprinter, able to produce free-standing matrigel™ scaffolds with good shape fidelity. However, this strategy might be expensive as it may require high matrigel™ content.
For this reason, we have designed and fabricated a modular three-fluid coaxial nozzle capable of producing a fibre with a core suitable for cell encapsulation, such as diluted matrigel™ (1:1 in PBS) and an outer layer of pre-crosslinked alginate to provide mechanical support. We exploited stereolithography 3D printing technology, using a biocompatible resin, to fabricate a nozzle made of two main parts screwed together and optically accessible. For the optimization of fibre bioprinting, we performed definition of optimal flow rate and printing speed for both internal and external layer. We also optimized cross-linking by coupling pre-crosslinking during the printing process, providing mechanical stability, and post-crosslinking through immersion in calcium chloride solution to achieve long-term stability in aqueous solution strictly necessary for long-term cell culture studies.

P24

3D bioprintied infected skin model as a platform for drug and therapies screening

S. Villata[1,2], R. Cue-Lopez[3,4], D. Baruffaldi[1], C. G. Gaglio[1], P. Bosh[3], F. C. Pirri[1,2], L. Napione[1,2], F. Frascella[1,2], E. Martinez-Campos[3,4]

[1]Dipartimento di Scienza Applicata e Tecnologia, Politecnico di Torino, Italy; [2]PolitoBIOMed Lab, Politecnico di Torino, Italy; [3]Departamento de Química Macromolecular Aplicada, Instituto de Ciencia y Tecnología de Polímeros, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain; [4]Grupo de Síntesis Orgánica y Bioevaluación, Instituto Pluridisciplinar (UCM), Unidad Asociada al Instituto de Ciencia y Tecnología de Polímeros, Instituto de Química Médica (CSIC), Madrid, Spain

Presenting author:
C. G. Gaglio. E-mail: ✉ cesare.gaglio@polito.it

Skin is the largest human organ and it is the barrier between the human body and external environment. Skin-on-a-chip models are for sure fundamental for *in vitro* drug testing and they are replacing conventional Two-Dimensional (2D) cell culture and animal models. Bacteria-induced infections is one of the major clinical challenges for effective skin repair and regeneration after an injury or in some pathological conditions.
Methacrylated Gelatin (GelMA) was synthesized and dissolved in culture medium, adding photo-initiator. Human fibroblasts were

then encapsulated in GelMA and 3D printed. Immediately after printing the architectures were photopolymerized and human keratinocytes were seed on top of them. Air Liquid Interface culture started and new medium was supplied and refreshed every two days. The models were analysed after 14 and 31 days. After 31 days the models were wounded and infected with both gram positive (*S. Aureus*) and gram negative (*E. Coli*) bacteria. The infection lasts 24h. At this point, the infected models were treated with penicillin-streptomycin.
After 31 days the skin was improved than after 14 days: the epidermis was thicker with good cytokeratin, filaggrin and CD29 expression, fibroblast began to produce collagen type I and α-sma, marker of the skin fibroblasts becoming able to participate in wound healing process. After 31 days it was also observed elongation of fibroblasts into the matrix and change in shape of epidermis, that became more compact.
Observing the behaviour of bacteria on the wounded model after the penicillin-streptomycin treatment it was possible to notice that the wound allows the bacteria to grow more but it also permits the antibiotic to better operate, obtaining an enhanced antibacterial effect.
For these reasons, the 3D bioprinted infected skin model could be used in the future as a realistic platform for drugs and therapies screening to have predicting results and to avoid the use of animals in clinical trials.

P25

Glycosignature impact in 3D-bioprinted models of gastrointestinal cancer

F. Cadamuro[1], L. Santos Ferreira[2,3] F. Pinto[2,3,4], C. Albuquerque Reis[2,3,4], F. Nicotra[1], L. Russo[1,5]

[1]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Italy; [2]i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal; [3]ICBAS – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto, Portugal; [4]IPATIMUP – Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Portugal; [5]CÚRAM, SFI Research Centre for Medical Devices, National University of Ireland, Galway, Ireland

Presenting author:
F. Cadamuro. E-mail: ✉ francesca.cadamuro@unimib.it

Three-Dimensional (3D) *in vitro* models are important tools to mimic the tumor human microenvironment to develop new therapies and go further to a more personalized medicine. They guarantee more reliable results than *in vivo* animal model, which do not possess human cells and therefore is not able to recapitulate the human physiology due to differences in molecular, immunological, genetic and cellular responses. Understanding the molecular features associated with cancer progression is important for the identification of new targets and develop advanced therapies. In this context, we aim to develop a 3D *in vitro* model of gastrointestinal cancer, able to mimic not only the physical and mechanical properties of the Extracellular Matrix (ECM) but also to resemble the cell-ECM interactions, in which glycans are involved. Indeed, the ECM glycosignature influences cell behaviour and is associated with tumorigenic processes. We developed 3D bioprinted models functionalised with selected glycans to replicate gastrointestinal cancer ECM. For that, we selected gelatin and hyaluronic acid as starting materials to formulate a hydrogel upon proper crosslinking. Gelatin was functionalized with 3'-sialylgalactose, 6'-sialylgalactose and 2'-fucosylgalactose. We evaluated the effect of the different glycans in the ECM properties and cancer cell behaviour. From the swelling test, SEM and SAXS/WAXS analysis the morphology and physical properties of the hydrogels resulted affected from the difference glycans functionalization. Hydrogels were then tested with human colon and gastric cancer cells (HT29 and MKN45) as models and with patient-derived colon and gastric cancer organoids. Single cell proteomic analysis on the bioprinted HT29 cells in the 3D-printed matrices showed significant differences depending on the different glyco-signature. Moreover, our glycosylated matrices showed the capacity to growth patient derived gastrointestinal organoids and that the behaviour is related to the glycosignature.

P26

Three-dimensional cultures of endothelial cells and fibroblasts in a microfluidic multi-compartmental device as an alternative to animal models

C. Bodio[1], A. Milesi[2], L. G. Pradotto[3,4], P. L. Meroni[1], M. O. Borghi[1,5], E. Raschi[1]

[1]Experimental Laboratory of Immunological and Rheumatologic Researches, IRCCS, Istituto Auxologico Italiano, Milan; [2]Laboratory of Clinical Neurobiology, IRCCS, Istituto Auxologico Italiano, San Giuseppe Hospital, Piancavallo (PN); [3]Division of Neurology e Neurorehabilitation, IRCCS, Istituto Auxologico Italiano, Piancavallo (PN); [4]Department of Neurosciences, University of Turin; [5]Department of Clinical Sciences and Community Health, University of Milan, Italy

Presenting author:
E. Raschi. E-mail: ✉ raschi@auxologico.it

Two-dimensional (2D) *in vitro* cultures have been used since 1900 to study biological processes in life science, thanks to easy handling, high reproducibility and low cost, but with some limitations, above all the loss of tissue/organ architecture. Moreover, the employment of animals has been recently limited by the 3R's principles encouraging the development of alternative models.
Using traditional 2D cultures, we previously demonstrated the pathogenic role of immune complexes from patients affected by Systemic Sclerosis, a chronic autoimmune disease in which Endothelial Cells (EC) and fibroblasts are the main actors.
Our aim was to set-up an innovative Three-Dimensional (3D) multi-compartmental dynamic model to confirm/strengthen the above results.
Human EC and fibroblasts were cultured into proper matrices to reproduce the original sources. The 3D structures were grown up in two interconnected bioreactors thanks to a peristaltic pump which simulates blood flow, to mimic the *in vivo* cross-talk.
We observed that fibroblasts could secrete new Extra Cellular Matrix and EC led to an organized structure with fusion of blood islands and formation of primitive vascular plexus, indicating that both cells were able to colonize and remodel the respective matrix, reproducing the tissue of origin.
These results support our 3D model as an evolution from the classical 2D *in vitro* cultures, offering a reliable tool to shed light into

the molecular mechanisms involved in SSc pathogenesis. Moreover, this model represents an innovative approach offering the possibility to interconnect different cell types in a dynamic environment and to reproduce *in vitro* and *in vivo* tissue/organ architecture. Further applications will consist in investigating the pathogenic mechanisms of several diseases, testing new drugs, employing stem cells to build the organs for transplantation through a model resembling the *in vivo milieu* with a significant focus on the ethical animal handling.

P27

Development of personalized preclinical models for drug screening in Chronic Lymphocytic Leukemia using 3D bioprinting

M. Cellani[1], R. Pinos[1], F. Barbaglio[1], L. Scarfò[2,3], P. Ghia[2,3], C. Scielzo[1]

[1]Division of Experimental Oncology, Malignant B Cells Biology and 3D Modelling Unit, IRCCS Ospedale San Raffaele Milano; [2]School of Medicine, Università Vita-Salute San Raffaele, Milano; [3]Division of Experimental Oncology, B-Cell Neoplasia Unit and Strategic Research Program on CLL, IRCCS Ospedale San Raffaele, Milano, Italy

Presenting author:
M. Cellani. E-mail: ✉ cellani.marco@hsr.it

Preclinical models employed for haematological cancer research are mainly Two-Dimensional (2D) *in vitro* culture and animal models. However, they present many limitations that can be potentially overcome by Three-Dimensional (3D) cell culture. The aim of the project is to employ 3D bioprinting to generate personalized preclinical models to study the response to therapies in Chronic Lymphocytic Leukemia (CLL), the most common adult leukemia in the western world, that remains incurable. MEC1 CLL cell line and primary cells were bioprinted within different hydrogels supporting cell viability and treated with chemotherapy for 24 and 72h at time zero post printing or after 7 days of 3D culture adaptation. RNAseq analysis on 3D bioprinted primary cells after 7 days of culture show that cells compared to 2D cultured overexpress genes involved in proliferation, survival and homing within lymphoid tissues. Prompted by these results we employed 3D bioprinted CLL cells, to evaluate their preclinical application for drug testing. We firstly observed an increased resistance to the drug in MEC1 cells after 7 days of adaptation in static culture compared to 2D. In addition, dynamic culture settings were found to further influence cell response in 3D. Similarly, we observed that 3D bioprinted primary cells show a higher resistance to chemotherapy if compared to 2D, after 72h treatment. We could achieve comparable response by increasing the dose of drug however, by treating the primary cells after 7 days adaptation in 3D they became resistant at both doses. By rtPCR we demonstrated that genes identified by RNAseq and potentially involved in resistance to therapy are differentially expressed in 3D settings compared to 2D culture. We are implementing the co-printing with other cells types mimicking the lymphoid environment to evaluate the contribution of the microenvironment in response to drugs. These results pave the way for the generation of more complex *in vitro* models to assess the response to target therapies in a personalized manner.

P28

Designing 3D bioprinted meniscal scaffold taking inspiration from Extracellular Matrix (ECM) features

M. Bracchi[1], A. Panunti[1], F. Cadamuro[1], F. Barbugian[1], F. della Torre[2], M. Crippa[2], L. Rigamonti[2], M. Bigoni[2], G. Zatti[2], M. Turati[2], F. Nicotra[1], L. Russo[1,3]

[1]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Italy; [2]Department of Medicine and Surgery, University of Milano-Bicocca, Italy; [3]CÚRAM, SFI Research Centre for Medical Devices, National University of Ireland, Galway, Ireland

Presenting author:
M. Bracchi. E-mail: ✉ m.bracchi11@campus.unimib.it

The menisci are C-shaped cushions found in the knee articulation between the femoral condyles and the tibial plateau which main function is the weight distribution. Their Extracellular Matrix (ECM) is highly hydrated, and mainly composed of collagen, Glycosaminoglycans (GAGs), adhesion glycoproteins and elastin. The meniscus is composed by two portions, the inner and the outer, with different composition, fibres orientation and properties. The meniscal tissue has a low vascularization and low regeneration potential; on the other hand, injuries are very common.
Here in this work, the generation of Three-Dimensional (3D) bioprinted meniscal tissue is proposed. To mimic the ECM morphology of meniscal tissue, ECM has been characterized by Scanning Electron Microscope (SEM) analysis. Furthermore, major ECM components have been characterized and compared. The mechanical properties of the tissue were then analysed with a Finite Element (FE) simulation on models obtained from the segmentation of DICOM files from nuclear magnetic resonance on patients.
A 3D bioprinted meniscal scaffold is proposed and to mimic the inner and outer inner and the outer parts of the meniscus two bioprintable ECM mimetics have been produced and characterized.

P29

InExpose and vivoFlow system: advances in Refinement and Reduction

S. Di Girolamo[1], G. Terribile[2], G. A. Sancini[2,3]

[1]PhD in Experimental Neuroscience, University of Milano-Bicocca, Monza (MB); [2]School of Medicine and Surgery, University of Milano-Bicocca, Monza (MB); [3]Nanomedicine Center, Neuroscience Center, University of Milano-Bicocca, Monza (MB), Italy

Presenting author:
S. Di Girolamo. E-mail: ✉ s.digirolamo5@campus.unimib.it

In last decades, scientific research is doing its utmost to implement and achieve the ethical principles of the 3Rs. In this respect, our state-of-the-art platform inExpose system (SCIreQ; Emka Technologies, Sterling, USA) is an example. The latter is a compact exposure system of precision inhalation in nose-only mode, whose computer-controlled nebulizers allow automated generation of aerosols with a precise concentration in the order of seconds. Moreover, our modular platform is combined with vivoFlow system (SCIreQ, Emka Technologies), that allows a whole-body mode inhalation and a contemporary recording of rodents plethysmo-

graphic values. Our combined platform offers the great opportunity to performing small-scale studies, evaluating small animal numbers in precise controlled conditions. Thus, this system allows us to study nanoparticles impacts on murine models along repeatable and non-invasive exposures. Moreover, we have the possibility not only of minimising any animal distress but also of monitoring the animal welfare during the entire exposure procedures assuring with a better reliability and repeatability of scientific results. In conclusion, this innovative nanotechnological platform is open to the contribution of research groups united by the aim of identifying the toxicological profile of inhaled nanoparticles and validating the development and refinement of experimental models to study the efficacy and safety of the inhalation administration route of new drugs.

P30

3D glioblastoma *in vitro* models to identify the impact of ECM in tumor progression

F. Barbugian[1], E. Calciano[2], L. Russo[1,3], F. Nicotra[1]

[1]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Italy; [2]Department of Molecular Medicine, University of Pavia, Italy; [3]CURAM SFI Research Centre for Medical Devices, National University of Ireland, Galway, Ireland

Presenting author:
F. Barbugian. E-mail: ✉ f.barbugian@campus.unimib.it

The Extracellular Matrix (ECM) is a dynamical microenvironment where aberrant balance of proteins, glycoproteins, Glycosaminoglycans (GAGs) and Hyaluronic Acid (HA) favor tumor progression and invasiveness.
The generation of tailorable, *in vitro* systems able to replicate physical and biochemical features of ECMs will contribute to spreading light on the role of the cell microenvironment features determining the pathological event and will allow to develop animal-free drug testing protocols.
In this work, we aim to understand and test the effect of biochemical and physical behavior in Glioblastoma Multiforme (GBM) microenvironment and display how *in vitro* systems candidate as forerunners for future biomedical studies. To this end, HA was crosslinked with different ECM proteins, exploiting linkers with different lengths and branching. The selected formulations were first tested with three cell lines to obtain an *in vitro* 3D bioprinted GBM model suitable for high-performance, predictive screening. Next, their dynamical properties were investigated by applying a flow rate. Finally, since cells respond to a variety of *stimuli*, we have also synthesized and functionalized a glycoconjugate hydrogel to understand the effect of glucose on GBM proliferation and invasion. The outcome of these three studies was the preparation of neurospheres inside the hydrogel, which we ultimately tested for different drugs for cross-validation.

P31

The prolonged effects of Russian chrysotile on an *in vitro* 3D human lung epithelial tissue

V. Almonti[1,2], S. Mirata[2,3], S. Vernazza[2,3], S. Tirendi[2,3], A. F. Gualtieri[4], M. Passalacqua[2,3], S. Penco[2,3], J. Markus[5], S. Letasiova[5], S. Scarfi[1,2], A. M. Bassi[2,3]

[1]Department of Earth, Environment and Life Sciences, University of Genova, Italy; [2]Inter-University Center for the Promotion of the 3Rs Principles in Teaching & Research (Centro3R), Pisa, Italy; [3]Department Experimental Medicine, University of Genova, Italy; [4]Department of Chemical and Geological Sciences, University of Modena and Reggio Emilia, Modena, Italy; [5]Mattek *In Vitro* Life Science Laboratories, Bratislava, Slovak Republic

Presenting author:
V. Almonti. E-mail: ✉ vanessaalmonti@gmail.com

Asbestos fibres, including the amphiboles and the serpentine Chrysotile (CHR), are classified as carcinogens by the International Agency for Research on Cancer (IARC). Today CHR "safe" mining takes place only in few countries like Russia, China, Africa, and South America, although, to mitigate the health risks associated with CHR, various international organizations, including the World Health Organization (WHO), have advocated for a complete ban on asbestos.
This study investigated the effects of a Russian Chrysotile (CHR) from the Yasny mine, on a Three-Dimensional (3D) human lung organotypic *in vitro* model: the EpiAirway™ (MatTek Corp, MA, USA). This model was used to investigate the 12-days effects of CHR, physically separated in two fractions of different fibre lengths (<5 and >5 µm) on viability, barrier integrity, and inflammation. Results were compared to the effects of Crocidolite (CRO) fibres considered as a carcinogenic positive control. The results showed that tissue viability was significantly reduced for both CHR fractions and CRO at 24 h, although at 12 days the viability returned to the values of the untreated control in all samples, indicating a significant resilience of the tissue in the long term, also confirmed by the morphological analyses and the Transepithelial Electrical Resistance (TEER) measurements. Gene expression analyses at 24 h revealed an increase in IL-1β, IL-6 and IL-8 pro-inflammatory cytokines, while ELISA tests showed a significant release of IL-1β, TNFα and IL-8 at 24 and 48h. Conversely, at 12-days of fibre exposure, only CRO and the CHR largest fraction induced an increase of IL-1β, and IL-8 gene expression compared to the untreated control. This preliminary study gives new insights into the role of fibre length in the toxicity of minerals and suggest the possibility to use the physiologically more relevant 3D lung tissue models to study the long-term effects of mineral fibres with respect to the traditional Two-Dimensional (2D) lung cellular models.

P32

Novel three-dimensional *in vitro* models of endometriosis

C. Volpini[1,2,3], N. Bloise[1,2,3], B. B. Mendes[4], J. Oliveira[4], J. Conde[4], L. Visai[1,2,3]

[1]Molecular Medicine Department (DMM), Centre for Health Technologies (CHT), UdR INSTM, University of Pavia, Italy; [2]Medicina Clinica-Specialistica, UOR5 Laboratorio di Nanotecnologie, ICS Maugeri, IRCCS, Pavia, Italy; [3]Interuniversity Center for the Promotion of the 3Rs Principles in Teaching and research (Centro 3R), University of Pavia Unit, Italy; [4]ToxOmics, NOVA Medical School, Faculdade de Ciências Médicas, NMS|FCM, Universidade Nova de Lisboa; Lisboa, Portugal

Presenting author:
C. Volpini. E-mail: ✉ cristina.volpini01@universitadipavia.it

Due to the increasing understanding of the molecular pathways involved in endometriosis, numerous advanced Three-Dimensional (3D) *in vitro* models have been developed. The objective of this study was to establish a 3D *in vitro* model for endometriosis to evaluate the active targeting of CD44 overexpressing endometriotic cells using gold nanoparticles conjugated with anti-CD44 antibodies (Au@antiCD44).
To achieve this, Platelet Lysate (PLT) was diluted with an Alginate (ALG) solution, resulting in a PLT-ALG composite that functioned as an embedding structure for the 3D model. ALG alone was used as the control. Two distinct endometriotic cell lines were utilized: Z12 cells, which overexpress CD44, and Thesc cells, which exhibit low CD44 expression. Viability tests using trypan blue and spatial distribution analyses employing Confocal Microscopy (CLSM) were conducted. Biocompatibility assessments with trypan blue and receptor recognition analyses using CLSM and Flow Cytometry (FACS) were performed to evaluate the interaction of Au@antiCD44 with the aforementioned cell lines. The qualitative analysis using CLSM and the quantitative assessment with trypan blue dye demonstrated that cells exhibited superior growth within the PLT-ALG hydrogel over time, extending up to 8 days, compared to cells cultured in ALG alone. Viability assays conducted after incubation with the nanovector confirmed the biocompatibility of the conjugated nanoparticles with both cell lines. CLSM and FACS data revealed that the nanoconjugate exhibited enhanced recognition of the CD44 receptor on Z12 cells (which overexpress CD44) compared to Thesc cells. In conclusion, further investigations employing 3D *in vitro* models are necessary to evaluate the toxicity and efficacy of this Photothermal Therapy (PTT) nanoplatform against endometriotic cells overexpressing CD44.

P33

In vitro functional characterization of iNeurons Parkinson phenotype

A . Andolfi[1], D. Di Lisa[1], G. Uras[2,3], S. Grasselli[1], S. Lucas Del Pozo[3], S. Martinoia[1], A. H. V. Schapira[3], L. Pastorino[1]

[1]Department of Informatics, Bioengineering, Robotics and System Engineering, University of Genoa, Italy; [2]Department of Biomedical Sciences, University of Sassari, Italy; [3]Department of Movement and Clinical Neurosciences, Institute of Neurology, University College London, Royal Free Hospital, London, UK

Presenting author:
A. Andolfi. E-mail: ✉ andrea.andolfi@edu.unige.it

Parkinson's Disease (PD) is the second most common neurodegenerative disorder, following Alzheimer's disease, and is characterized by a lack of dopamine in the basal ganglia. The current focus of PD symptomatic therapy revolves around techniques that replace dopamine. Among the various genetic risk factors for PD, the GBA gene is the most significant in terms of numerical importance, with mutations found in 5-15% of PD patients.
Induced Pluripotent Stem Cells (iPSCs), which are generated through cellular reprogramming techniques, have had a transformative impact on stem cell research. They provide researchers with access to a diverse range of human cell lines and enable the creation of both diseased and healthy *in vitro* models, thereby reducing animal testing.
Using an *in vitro* model of human iPSC-derived neurons, researchers have demonstrated how differences in neuronal morphology, connectivity, and electrical activity can be indicative of Parkinson's disease.
Microelectrode Arrays (MEAs) have emerged as a valuable non-invasive method for recording extracellular neural activity and are considered the leading platform for studying brain function, dysfunction, neurotoxicity, and drug screening in the context of brain-on-a-chip technology.
This study aimed to examine the spontaneous electrical activity of dopaminergic neurons, both healthy and pathological, using MEAs. Specifically, the focus was on investigating the impact of the L444P GBA mutation on their functional activity. Using iPSCs obtained from healthy individuals and PD patients, wild-type dopaminergic neurons and those with the L444P GBA mutation were generated. These two cell phenotypes and primary astrocytes were co-cultured on MEA devices. Through preliminary functional characterization, notable differences in electrophysiological behaviors were observed between the two phenotypes, particularly in burst duration.

P34

NAP: advanced cellular models for studying individual sleep dynamics

S. Cerchio[1], P. Ruther[2], J. C. Schwamborn[3], U. Olcese[4], A. Aarts[5], U. Faraguna[6], A. Ahluwalia[1], C. Magliaro[1]

[1]Research Center E. Piaggio, University of Pisa, Italy; [2]Department of Microsystems Engineering (IMTEK), University of Freiburg, Germany; [3]OrganoTherapeutics SARL, Luxembourg; [4]Cognitive and Systems Neuroscience, Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands; [5]ATLAS Neuroengineering BV, Leuven, Belgium; [6]SleepActa SRL, Montacchiello (PI), Italy

Presenting author:
S. Cerchio. E-mail: ✉ sonia.cerchio@ing.unipi.it

Human sleep is a complex physiological process necessary for organisms' energy levels restoration and for the correct functioning of the brain. Insufficient sleep is an under-reported epidemic with sleep disturbances being common early signs of neurodegeneration. Clinical research is currently challenging the assumption that human sleep is a one-fits-all phenomenon: breaking new grounds into sleep research is needed. In this scenario, the NAP research consortium proposes here a new science-toward-technology paradigm enabling the study of human individual sleep and the identification of the effects of sleep deprivation and sleep-related disorders at the microscale. In order to reach this aim, next generation brain models will be generated, the cyborganoids: biohybrid constructs obtained from induced pluripotent stem cells of both sexed healthy and Parkinsonian patients. Those constructs will be integrated with microelectrodes for tracking neural dynamics and fluidics for ensuring adequate nutrient supply. Cyborganoids will be characterized in terms of mass, structure and metabolism, as an intimate association exists between these parameters and sleep behaviours. Normal sleep-wake cycles and sleep deprivation will be simulated through new experimental procedures in the cyborganoids, while human sleep patterns and mass will be tracked through wearables. Allometric scaling relating the mass of an organism to its sleep-wake cycles will be exploited to compare *in vitro* and *in vivo* data and define the cyborganoids ability to model human cycles. Structural and functional investigations on cyborganoids from healthy and PD patients, subjected to normal sleep habits and

sleep loss, will return individual-relevant information, as sleep-related anomalies, that cannot be probed by means of the currently available technologies.
Overall, the NAP project (Grant Agreement 101099310) will powerfully impact both the fields of basic research and personalized medicine.

P35

Generation of retinal organoids from Human Induced Pluripotent Stem Cells (hiPSCs)

S. Tirendi[1,2], A. M. Bassi[1,2], S. Mirata[1,2], V. Almonti[2,3], S. Scarfì[2,3], S. Vernazza[1,2]

[1]Department of Experimental Medicine (DIMES), University of Genoa; [2]Inter-University Center for the Promotion of the 3Rs Principles in Teaching & Research (Centro 3R), Pisa; [3]Department of Earth, Environment and Life Sciences (DISTAV), University of Genoa, Italy

Presenting author:
S. Tirendi. E-mail: ✉ tirendisara@gmail.com

Glaucoma is a neurodegenerative disease characterized by vision loss, due to Retinal Ganglion Cell (RGC) death. The RGCs are the first retinal neurons generated during development from multipotent Retinal Progenitor Cells (RPCs), followed by other retinal cells type, *i.e.* bipolar, horizontal and amacrine cells, Muller glial cells, and cone/rod photoreceptors. Since the precise mechanism underlying neurodegeneration in glaucoma is not fully understood, Retinal Organoids (ROs) derived from Human Pluripotent Stem Cells (h-IPSCs) could be a valuable tool to investigate the early steps involved in the glaucomatous cascade. ROs, also known as "mini-retinas," follow the *in vivo* steps of retinogenesis by providing an *in vivo* human model to study the development and behavior of RGCs, hence the harmful effects of this neurodegenerative disease. Herein, the h-IPSCs were differentiated towards ROs, according to Amélie Slembrouck-Brec *et al.* protocol. The retinogenesis was evaluated over time by analyzing the expression of several markers, such as NOGGIN, DKK1, LHX2, PAX6, and BRN3a. Notably, the gene expression of BRN3a, a specific RGC marker, increased from day 28 to day 56 of the differentiation protocol. In this regard, the real-time PCR proves that separation with MACS sorting for CD90/THY-1 at day 56 was successful. Although these preliminary results have led to a satisfactory result, further investigations will be necessary to evaluate, through more specific markers, how many of the CD90-positive cells are also RGCs.

P36

Genetic algorithms for the identification and design of physiologically relevant 3D constructs

F. Fontana[1], P. Mancini[1,2,3], E. Botte[1,2,3], C. Magliaro[1,2,3], A. Ahluwalia[1,2,3]

[1]Research Centre 'E. Piaggio', University of Pisa; [2]Department of Information Engineering, University of Pisa; [3]Interuniversity Centre for the Promotion of 3R Principles in Teaching and Research (Centro 3R), Pisa, Italy

Presenting author:
F. Fontana. E-mail: ✉ f.fontana13@studenti.unipi.it

Physiological relevance is defined as the ability of a model to replicate the *in vivo* counterpart. It consists in identifying universally-respected biophysical rules to use as design criteria *in vitro*. For instance, two constraints for designing physiologically relevant *in vitro* constructs are the optimisation of cell viability - and thus of the oxygen (O_2) utilisation - and the minimization of the surface energy. In addition, organisms are able to adapt to environmental perturbations. Here Genetic Algorithms (GAs) are used to optimize the combination of morphological parameters enabling the construct to respect such constraints. The algorithm starts by creating a random pool of individuals: cell-laden ellipsoidal constructs with randomly positioned surface protrusions. The morphological genes (*i.e.*, protrusions' number and construct volume) are randomly defined in Matlab and used as input for the Comsol (COMSOL Inc., Stockholm, Sweden) model studying resource consumption for two O_2 boundary conditions. To assess the individuals' ability to adapt and balance cell viability and surface energy a Fitness Function is used evaluating the so-called biophysical genes that are: the difference in the fraction of volume with CO_2 <0.04 [mol/m^3] for two boundary conditions (delta), and the sphericity (psi), a surface energy proxy. The highest score individuals are the Elite Children directly populating the Next Generation while the others are the Crossover (combining two individuals' morphological genes) and Mutation (applied to one individuals' morphological genes). The convergence is achieved reaching the maximum number of generations. The optimal construct has delta⟶0 and psi⟶1, suggesting the feasibility of this approach. Having an *in silico* protocol automatically predicting the optimal morphology of Three-Dimensional (3D) constructs (based on the evaluation of biophysical parameters) enables the development of high-fidelity and cost-effective lab on a laptop which could augment or even substitute costly *in vitro* models.

P37

Osteosarcoma: from 2D to 3D model to study natural-like chalcones with antitumor activity

M. Rossi[1], C. Cappadone[1], M. Malgorzata Rydzyk[1], C. Pellegrino[1], F. Rossi[1], G. Farruggia[1], A. Bisi[2], S. Gobbi[2], E. Malucelli[1], P. Blasi[1]

[1]Department of Pharmacy and Biotechnology, Alma Mater Studiorum, University of Bologna; [2]Department of Pharmacy and Biotechnology, Alma Mater Studiorum, University of Bologna, Italy

Presenting author:
M. Rossi. E-mail: ✉ martina.rossi12@unibo.it

Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents. To date, OS treatment focuses on surgical therapy, combined with adjuvant chemotherapy. However, the prognosis remains very poor, especially when surgery is not possible, or drug resistance sets in. Therefore, research for alternative therapeutic approaches is of paramount importance both in terms of *in vitro* tumor models and new therapeutics. Here we focused on the development of Three-Dimensional (3D) *in vitro* models, able to mimic the tumor microenvironment to test Licochalcone A (Lic A), a promising natural compound against OS, and other chalcones selected from a wide in-house library. First, molecules were tested on MG63 and 143B osteosarcoma cell lines in Two-Dimensional (2D) culture models. The cytotoxic and cytostatic effects were analyzed through an automated live-imaging platform (Livecyte®). Livecyte®, through quantitative phase imaging, allows to screen large sets of compounds selecting the most promising. The seven selected compounds with significant antiproliferative effect (IC50<20 μM) were

tested on Multicellular Tumor Spheroids (MCTSs) as 3D *in vitro* models. MCTSs were obtained by liquid overlay and, after treatment, the IC50 was estimated through the AlamarBlue™ assay while propidium iodide was used to assess cell viability by confocal microscopy. The invasion assay was used to study the compounds ability to inhibit cancer cells spreading. When tested in 3D, only two chalcones proved to be effective even if the concentration had to be increased of about one order of magnitude if compared to 2D. At the higher concentrations needed for 3D treatment some compounds were not enough soluble to be tested. Interestingly, Lic A and the two selected compounds showed also the ability to inhibit cell spreading. In conclusion, the application of 3D models allows to optimize the preclinical studies reducing cost, time and animal sacrifice.

P38

Set up of a dry eye model using 3D reconstructed human corneal tissues: a new prospective for medical device testing

G. Bray, G. Galgani, V. Citi, V. Calderone

Dipartimento di Farmacia, Università di Pisa, Italy

Presenting author:
G. Bray. E-mail: ✉ g.bray@studenti.unipi.it

Dry Eye Disease (DED) is an ophthalmological disorder due to complex etiopathogenetic factors. It is usually treated with eye drops, containing medical devices or drugs. Various models have been created for studying the pathogenesis of DED, using three-dimensional (3D) tissue cultures. 3D models are a novel field of research that can mimic *in vitro* the structure and complexity of the physiological environment. They can predict an *in vivo*-like response and lead to a reduction in animal use for research purposes.
The aim of this project was the set-up of an innovative DED *in vitro* model, using 3D reconstructed human corneal tissues. The model was then used to evaluate different formulations useful in the prevention and treatment of DED.
To recreate the dry-eye model, tissues were maintained in dry condition (DC: 40°C, 40% Rh, HYP-DRY) for different periods, with the addition of sorbitol 0.6M to the culture medium, for assessing the protective effect of sodium hyaluronate 0.2% + tamarind seed polysaccharide 0.2% (HA+TSP), trehalose + sodium hyaluronate (TR), hydroxypropyl guar (HG) and dexamethasone 1 mg/mL (DEX).
At the end of the procedures, tissue viability was evaluated by MTT assay and the release of IL-1β was quantified by ELISA method.
DC for 24h caused a massive tissue damage (viability: 10%): treatments demonstrated that HA+TSP significantly increases tissue viability compared to untreated tissues. However, any treatment reduced IL-1β production.
DC for 16h caused a significant tissue damage (viability: 40%); treatments did not increase tissue viability, however a significant reduction in IL-1β release has been recorded both for HA+TSP and DEX.

P39

Down-scaling metabolic dynamics of living communities *in vitro*

E. Botte, P. Mancini, C. Magliaro, A. Ahluwalia

Research Centre "E. Piaggio", University of Pisa; Department of Information Engineering, University of Pisa; Inter-University Centre for the Promotion of the 3Rs Principles in Teaching and Research (Centro 3R), Pisa, Italy

Presenting author:
E. Botte. E-mail: ✉ ermes.botte@ing.unipi.it

Coexistence has been extensively documented to shape individual behaviour across scales. Properly rescaled, these concepts are likely to hold in cellular systems and should be considered in the design of human-relevant models of tissues and organs. In turn, creating a population of coexisting cell constructs might furnish insights into the metabolic behaviour of biological communities, providing tractable down-scaled models of ecosystems.
To date, no attempts in investigating to what extent inter-individual interactions affect metabolic dynamics at the cellular level have been reported. In this perspective, here we present a study designed to explore how oxygen (O_2) metabolism of cell aggregates depends on their coexistence. An ecosystem (*i.e.*, a multi-construct model where the crosstalk among cell aggregates sharing a common environment drives metabolic dynamics) was first developed *in silico*, combining finite element and statistical analysis to evaluate the influence of community abundance and their spatial distribution on O_2 uptake in simple geometries and ideal conditions. Then, multi-spheroid systems were adapted to be reproduced *in vitro* for experimental validation.
Computational predictions suggest metabolic cooperation of cell-laden spheroids, as they adapt their O_2 consumption when tightly packed. Similar behaviour was observed *in vitro*, matching *in silico* results, and hence indicating the feasibility of a predictive and translational framework to replicate key ensemble behaviours which drive resource management in living systems.
Further analyses are ongoing, incorporating heterogeneities and environmental disturbances in virtual and *in vitro* ecosystems. Albeit preliminary, the outcome of this work represents the first step towards understanding how multiple cell constructs can interact *in vitro* in terms of resource consumption, with potential applications as alternative methods in diverse fields, such as biomedical sciences and ecology.

P40

hiPSCs-derived neurons as a model to study Developmental Neurotoxicity (DNT): focus on *in vitro* neurotransmitter release

S. Amato[1], C. Cervetto[1,2], F. Pistollato[3], S. Baldassarri[4], F. Zara[4], A. Bal-Price[3], G. Maura[1], M. Marcoli[1,2]

[1]Department of Pharmacy (DIFAR), Section of Pharmacology and Toxicology, University of Genoa; [2]Interuniversity Center for the Promotion of the 3Rs Principles in Teaching and Research (Centro 3R), Pisa; [3]European Commission, Joint Research Centre (JRC), Ispra (VA); [4]Unit of Medical Genetics, IRCCS Istituto Giannina Gaslini, Genova, Italy

Presenting author:
S. Amato. E-mail: ✉ sarah.amato@edu.unige.it

Human Induced Pluripotent Stem Cells (hIPSCs) and their neuronal/glia derivatives are considered a valid resource to set up an *in vitro* model to study Developmental Neurotoxicity (DNT). The official guidelines propose an *in vitro* battery to assess DNT through the evaluation of specific neuronal endpoints, such as cell proliferation

and apoptosis, differentiation into neurons and glia, neuronal migration, synaptogenesis, and neuron network formation; however, none of these tests includes the possibility to assess the neurotoxicant effects on neurotransmitters release and clearance, creating a gap in the biological applicability of such a battery. Hence, we implemented a high-performance liquid chromatography-based method to measure glutamate and aspartate release in a previously characterized model of hIPSC-derived neurons at different differentiation stages. The analytical method involves precolumn derivatization with o-phalaldehyde, followed by separation on a C18 reverse-phase chromatography column and fluorimetric detection. We determined the ability of the hIPSC-derived neurons to release glutamate and aspartate in basal conditions or upon depolarization, as well as after exposure to known neurotoxicants, alone or in three different chemical mixtures. Concurrently, the effect of such molecules was determined through non-conventional electrophysiology on multi electrode arrays, to evaluate any alteration of the neuron-glia network collective behavior. Our results suggest that hIPSC-derived neurons are able to release glutamate in a vesicular manner, and the treatment with different chemicals induces alterations at this level. Our methodology provides essential information about hIPSC-derived cultures in the context of DNT, contributing to clarify the mechanisms underlying neurotoxicants-induced damage. Moreover, given its sensitivity, the analysis of neurotransmitters release should be considered a reliable *in vitro* test to define functional neuronal endpoints.

P41

Optimization of tumor spheroids for the study of molecular mechanisms in chemotherapy resistance

G. Paties Montagner[1,2], L. Cacopardo[2,3,4], S. Piaggi[1], A. Ahluwalia[2,3,4], A. Corti[1]

[1]Department of Translational Research NTMS, University of Pisa Medical School; [2]Research Center "E. Piaggio", University of Pisa; [3]Department of information Engineering, University of Pisa; [4]Centro 3R, Pisa, Italy

Presenting author:
G. Paties Montagner. E-mail: ✉ g.patiesmontagner@studenti.unipi.it

Drug-resistance is one of the major disadvantages of chemotherapy leading to failure of most cancer drugs. Although the phenomenon is well known, its molecular mechanisms remain unclear. *In vitro* studies using Two-Dimensional (2D) cell cultures and immortalized cell lines inadequately represent the human pathophysiological condition. Three-Dimensional (3D) models have been thus proposed to fill the gap between *in vitro* chemotherapeutic studies and the human *in vivo* condition. In this study, 3D bioprinting was used to fabricate tumour spheroids using HeLa cells. Integration of HeLa cells into a gelatine and alginate matrix to create tumoroids has not been reported in the literature. Optimisation studies were focused on the definition of material combination and printing parameters to support the growth of these cells. Thanks to a custom bioprinter, alginate drops with controlled shape were generated using needles with different diameters (750 and 1500 µm) and collected in 0.1M calcium chloride bath. after 15 minutes, the spheroids were incubated with different concentration of Microbic Transglutaminase (mTG) for 24h at 37°C.
Viability was assessed using the Alamar Blue and Live and Dead assays, and optimal biomaterial combination was determined as 1% w/v alginate and 250U/g mTG-10% w/v gelatine. Moreover, cell viability at 24h was higher for the bigger needle with respect to the smaller one, probably because of lower shear stress during extrusion. However, after 5 days, viability was higher in the smaller spheroids. This is likely related to better nutrient diffusion over time.
To conclude, in this study, optimal bioprinting conditions for the generation of tumour spheroids based on HeLa cells were defined. Furter studies will focus on understanding 3D cell culture performance under chemotherapy treatment with respect to 2D cultures. This will contribute to improve the relevance of *in vitro* tools for the study of molecular mechanisms underpinning chemotherapy resistance, in line with the 3R principles.

P42

Glioblastoma giant polyploid stem cells: from genomic profiles to *in vitro* study of new therapeutic strategies

M. Giambra[1,2], A. Sirtori[1], S. Redaelli[1], S. Tabano[3,4], A. Montesi[1], V. Berni[1], A. Curto[1], M. Ghizzi[1], A. Di Cristofori[1,2,5], C. Giussani[1,5], A. Bentivegna[1]

[1]School of Medicine and Surgery, University of Milano-Bicocca, Monza (MB); [2]PhD program in Neuroscience, University of Milano-Bicocca, Monza (MB); [3]Division of Pathology, Research Laboratory Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan; [4]Medical Genetics, Department of Pathophysiology and Transplantation, Università degli Studi di Milano; [5]Division of Neurosurgery, Azienda Socio Sanitaria Territoriale - Monza (MB), Ospedale San Gerardo, Italy

Presenting author:
M. Giambra. E-mail: ✉ m.giambra1@campus.unimib.it

Despite the aggressiveness of the treatment, Glioblastoma (GBM) remains the deadliest malignant primary brain tumor with patients' median survival of 15 months. *In vitro* and *in vivo* evidence support the existence of subpopulations of GBM cells expressing stemness-related markers, the so-called Glioma Stem Cells (GSCs). Among them, the Polyploid Giant Cancer Cells (PGCCs) differ from diploid tumor cells in morphology, size, chromosomal abnormalities, tumorigenic capacity, radioresistance and chemoresistance. The origin of PGCCs is associated with the abnormal expression of several cell cycle-related proteins, such as Aurora Kinases (AURKs). In addition, recent studies evidenced an abnormal expression of AURKs in different types of tumors, including GBM, making AURKs a new possible therapeutic target. The aim of this work is to investigate a new therapeutic strategy inhibiting the AURKs through danusertib, given alone or in combination with temozolomide, the first-line therapy in GBM. The cytotoxic effects were evaluated on four patient-derived GSC 2D-cultures, two of them defined as PGCC for their particular features, after the molecular and cytogenetic characterizations. The well-known temozolomide resistance of the GSCs was demonstrated to be independent from the TP53 mutational status and the methylation status of the MGMT promoter. Furthermore, after the treatment, an unexpected cytostatic effect was observed in the GSCs. Interestingly, only the PGCCs were sensitive to danusertib alone treatment, showing alterations in the nuclear morphology and increasing their chromosome content. A possible synergistic effect of the combined treatment was observed in some cell cultures as well, even if the mechanism underlying this synergy and the association with GSC ploidy status is still unclear. In conclusion, the efficacy of

danusertib on the PGCCs was confirmed and the potential benefit of its combination with temozolomide was proposed for the first time to our knowledge.

P43

Investigating zebrafish (D. rerio) development and behavior to assess the hazard of antimicrobial CuO nanoparticles

B. Negrini[1,2], P. Floris[1], P. Bonfanti[1], A. Colombo[1], C. Bragato[1], M. Saibene[1], P. Mantecca[1]

[1]POLARIS Research Center, Department of Earth and Environmental Sciences, University of Milano-Bicocca; [2]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Italy

Presenting author:
B. Negrini. E-mail: ✉ b.negrini1@campus.unimib.it

Nanoparticles (NPs) and nano-enabled products emerged as novel antimicrobial agents with proven efficacy against Antimicrobial Resistant (AMR) bacteria, which can be found in water bodies associated to fish farming. CuO NPs have been extensively used as bactericidal agent, yet their potential toxicity to cells and organisms is recognized. This work aims at evaluating the nanosafety of CuO-based nanomaterials in exploitation scenarios by using zebrafish (D. rerio), a promising model organism for high-throughput developmental and behavioral screening. Sonochemically Synthesised Water-Based Cuo (wCuO) NPs were studied, in comparison with sonochemical Zn-doped CuO (ZnCuO) NPs to investigate any differences in response based on the NPs physico-chemical structures and identify CuO-induced adverse outcomes. NPs suspensions were characterized by TEM, DLS and ICP-OES. The aquatic toxicity potential was assessed by the Fish Embryo acute Toxicity test (OECD n. 236). Zebrafish embryos were exposed to NPs at increasing concentrations (0.1, 1, 10, 100 mg/L) for 96 hours and screened every 24 hours for lethal and sub-lethal endpoints, to calculate LC50 and EC50. No significant lethal effects were found. The morphometric analyses revealed significant differences in all of the NPs-treated embryos' parameters with respect to controls. A complete lack of hatching was already evident at the lower concentrations for wCuO, while this effect decreased in ZnCuO-treated embryos. To investigate the mechanism preventing the hatching, the spontaneous tail coiling was analysed in pre-hatching embryos by using the DanioScope Software, but no significative differences were found compared to control. Collectively, these results suggest that the modulation of the NPs physico-chemical structure (*i.e.*, metal doping) may contribute to their safety profile and that the mechanism responsible for the hatching delay induced by this class of NPs needs to be investigated at further levels (*e.g.*, molecular).

P44

Stromalized microtissue as new model to study histone methylation profile involved in PCa aggressiveness

G. Gangarossa[1], M. Iozzo[1], L. Ippolito[1], E. Pardella[1], G. Sgrignani[1], E. Pranzini[1], G. Comito[1], G. Sandrini[2], R. Galli[3], G. Mugnaini[3], M. Bonini[3], C. Capatano[2], E. Giannoni[1], P. Chiarugi[1]

[1]Department of Experimental and Clinical Biomedical Sciences, University of Florence, Italy; [2]Institute of Oncology Research (IOR), Università della Svizzera italiana (USI), Bellinzona, Switzerland; [3]CSGI & Department of Chemistry "Ugo Schiff", University of Florence, Sesto Fiorentino (FI), Italy

Presenting author:
G. Gangarossa. E-mail: ✉ giulia.gangarossa@unifi.it

It has been demonstrated that lactate secreted by Cancer-Associated Fibroblasts (CAFs) in the prostate Tumor Microenvironment (TME) plays an essential role in tumor cells' metabolic reprogramming that culminates in the enhancement of their pro-aggressive features. In this context, the availability of specific metabolites can regulate an epigenetic-based gene expression through their effects on modifications of DNA and histones. Focusing on the role of lactate in histone modifications, our preliminary data show: i) an increase of histone methylation H3K4me3 promoted by lactate in 22rv1 cells (also in 3D cultures) impaired by OICR-9429, a specific inhibitor of MLL/WDR5 HMT; ii) a reduction, following OICR-9429 treatment, in lactate-induced 22rv1 migration ability; iii) a positive correlation between H3K4me3 level and tumor grading and progression, in both aggressive human PCa tumor microarrays and primary tumors derived from lactate-treated mice. Although our findings are *in vivo* confirmation of methylation level, we strongly need to enlarge the number of experiments, possibly fulfing the 3R principles. Therefore, we aim to set-up three-dimensional (3D) stromalized prostate microtissues, closely recapitulating key PCa microenvironment traits, to evaluate the effect of the targeting of lactate transporter and of the H3K4 methylation machinery in stromal and tumor compartments. In preliminary experiments we co-cultured PCa cells together with CAFs on biodegradable gelatin porous scaffold to monitor the crosstalk between the two cell populations and the endogenous extracellular matrix deposition but our tentatives were unsuccessful until now. We are testing different porosity and size spheres performing co-culture of stroma/tumour cells at different time setting to obtain microtissues as alternative models to animal use. Future analysis (*e.g.*, IHC staining, etc) will allow us to study the role of stromal lactate in epigenetic regulation, specifically in methylation directly on microtissue.

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Hybrid spheroids as a model of osteosarcoma

M. Malgorzata Rydzyk[1], M. Pannella[2], C. Cappadone[1], G. Farruggia[1], E. Malucelli[1], C. Pellegrino[1], F. Rossi[1], E. Lucarelli[2], T. Ibrahim[2], S. Iotti[1]

[1]Pharmaceutical Biochemistry Lab, Department of Pharmacy and Biotechnology, Alma Mater Studiorum, University of Bologna; [2]Osteoncology, Bone and Soft Tissue Sarcomas and Innovative Therapies Unit, IRCCS Istituto Ortopedico Rizzoli, Bologna, Italy

Presenting author:
M. Malgorzata Rydzyk. E-mail: ✉ martyna.rydzyk2@unibo.it

Osteosarcoma (OS) is the most common primary malignant bone tumour. The 5-year survival rate of patients treated with current therapy is 70% when the disease is localized and less than 20% when metastatic. Therefore, new drugs, such as tyrosine

inhibitors, must be tested to improve patient's outcome. So far, most of the in-vitro pre-clinical studies have been conducted on a monolayer of OS cells. These models are limited because do not consider the Tumour Microenvironment (TME) in terms of cell composition and Three-Dimensional (3D) structure.
The aim of this work is to develop 3D OS models including different Tumor-Associated Cells (TAC) such as mesenchymal stromal cells, fibroblasts and endothelial cells co-cultured with cancer cells. Mimicking OS TME, we will obtain robust results representing the *in vivo* tumor behaviour.
We built spheroids containing OS cells (MG63 and Saos-2) alone or co-cultured with TAC. Spheroids were made by self-assembly in 96-well ultra-low attachment. Spheroids growth was monitored for 7 days by PrestoBlue assay. Spheroids were fixed and embedded in OCT for further histological characterisation.
Results showed that MG63 monocultures developed a compact spheroid with a regular shape, while Saos-2 cells were unable to form a compact spheroid. When mesenchymal stromal cells, fibroblast and endothelial cells were added to cancer cells, the spheroids became more compact and viable, as stromal cells can furnish a physiological scaffold to tumor tissue. Interestingly, spheroids containing fibroblasts were more viable compared to spheroids containing mesenchymal stromal cells. In sum, these results demonstrate that tumor-associated cells in hybrid spheroids have a role on OS cell behaviour and features. Therefore, they can enable a more accurate model for high-throughput screening of anticancer drugs leading to a reduction in animals used in preclinical studies. Further confirmatory studies will be conducted using patient's derived primary cells.

P46

Evaluation of different approaches to produce breast cancer spheroids for *in vitro* drug testing

N. Bloise[1,2,3], S. Strada[1,2,3], E. Restivo[1,3], V. Sottile[1], L. Visai[1,2,3]

[1]Department of Molecular Medicine, Centre for Health Technologies (CHT), INSTM UdR of Pavia, University of Pavia; [2]Medicina Clinica-Specialistica, UOR5 Laboratorio di Nanotecnologie, ICS Maugeri, IRCCS, Pavia; [3]Interuniversity Center for the Promotion of the 3Rs Principles in Teaching and Research (Centro 3R), University of Pavia Unit, Italy

Presenting author:
N. Bloise. E-mail: ✉ nora.bloise@unipv.it

Three-dimensional (3D) tumor spheroids are powerful *in vitro* models for preclinical screening of anticancer drugs. In the literature, different spheroid production approaches capable of generating uniform spheroids for high-throughput analysis and screening of sensitivity to chemotherapeutic agents have been well described. Although spheroids are valuable *in vitro* tools for preclinical screening of chemosensitivity, not all spheroid generation techniques are equivalent. Therefore, a systematic assessment of spheroid generation methodologies will be useful in evaluating the most appropriate method for spheroid-based functional and target-specific analyses. The purpose of this study was to systematically compare different methods of breast cancer spheroid generation in terms of morphology, growth and chemosensitivity. Breast cancer cell lines (MCF-7 and SK-BR-3) were used to form spheroids by the indicated methods: ultra-low-attachment 96-well U-Plate (PrimeSurface®), ultra-low-attachment 96-well V-Plate (PrimeSurface®), agarose-based culture, agarose-based culture droplets and agarose-based culture mini-droplets. Image-based morphological analysis of spheroids was evaluated to compare spheroid protocols. Preliminary results showed that all methods supported spheroid formation, but differences emerged in terms of time required for spheroid formation, morphology, size, and cell compaction. Overall, we observed that the generation of spheroids on ultra-low attachment V-Plates was simple and fast, generating more compact spheroids in a shorter time than the other methods. Experiments are currently underway to evaluate the sensitivity to chemotherapeutic agents of spheroids derived from the different methods. Indeed, the goal is to assess whether the technique used has a significant impact not only on cell phenotype but also on drug toxicity to identify an appropriate approach of spheroid generation to improve the predictive power of *in vitro* drug screening assays.

P47

Advanced cell culture models for the investigation of the cross talk between triple negative breast cancer cells and activated fibroblasts

A. Colombo, D. Prosperi, M. Colombo, M. Innocenti, L. Fiandra

Department of Biotechnology and Biosciences, University of Milano-Bicocca, Italy

Presenting author:
A. Colombo. E-mail: ✉ a.colombo197@campus.unimib.it

The study of the Tumor Microenvironment (TME) becomes increasingly important in the development for new anti-cancer therapies. Within TME, Cancer Associated Fibroblasts (CAFs) have been identified as critical regulators of the malignant phenotype in several aggressive and desmoplastic tumors. The interaction between tumor cells and activated fibroblasts determines in these tumors: rapid growth, metastatic phenotype, and resistance to chemotherapy. Growth factors, produced by cancer cells, have been shown to be able to activate fibroblasts to CAFs; on the other hand, soluble factors, also including cytokines, released by CAFs are involved in the, proliferation, invasiveness and chemoresistance of cancer cells.
We validated the usage of advanced cell culture models as tools to study the interplay between mammary tumor cells and fibroblasts, taking mouse triple-negative tumor cells and fibroblasts as a case study. We developed two different Three-Dimensional (3D) models, representative of a direct and indirect contact between the two cell types: the co-colture on Transwell and the spheroid, the first one allows to study only the paracrine signals, and the second one allows to investigate both paracrine and cell-contact-based signaling.
With these co-culture systems we found that the fibroblasts underwent activation induced by the TGF-β and the PDGF produced by the tumor cells, which increased their proliferation and IL-6 secretion. The IL-6 secreted by activated fibroblasts enhanced tumor-cell proliferation and chemoresistance. The epithelial-mesenchymal transition was also observed in tumor cells exposed to activated fibroblasts, although IL-6-indipendent.
Therefore, in this study it has been confirmed that the interaction between CAFs and cancer cells into TME is extremely complex and includes several soluble factors. The study of this complexity can be important for the development of new therapies, aimed to target the different pathways involved in this cross-talk.

P48

Experimentally validated *in silico* methodology for proteome-wide identification of (off-)target proteins and enhanced rational design of animal testing: the case of finasteride

A. Di Domizio

SPILLOproject, Italy

Presenting author:
A. Di Domizio. E-mail: ✉ alessandro.didomizio@spilloproject.com

Despite significant investments of time and resources in drug R&D, including animal testing and studies involving healthy volunteers, Adverse Drug Reactions (ADRs) often remain primarily known at a clinical level. The understanding of the underlying biomolecular mechanisms responsible for ADRs, which would enable the design of safer drugs or better utilization of existing ones, frequently proves elusive, even for medications that have been on the market for many years.

A representative example of this is finasteride, a 5-alpha-reductase enzyme inhibitor that has been on the market for decades and is widely used for the treatment of benign prostatic hyperplasia and androgenetic alopecia. However, it can give rise to sexual dysfunctions and psychological and physical disturbances.

To provide a biomolecular interpretation of the adverse effects of such drug, the SPILLO-PBSS (www.spilloproject.com) software has been utilized. This unique tool is able to perform an unbiased analysis of the human structural proteome and to identify drug binding sites, even when they are seemingly inaccessible due to distortion or complete closure (cryptic binding sites).

An off-target protein, previously unidentified or unreported, capable of providing a biomolecular interpretation for the aforementioned ADRs, was discovered by SPILLO-PBSS and experimentally confirmed, leading to its publication in a peer-reviewed scientific journal.

Importantly, by precisely targeting the relevant protein, a biomolecular explanation for the problem could be provided while minimizing animal testing.

Furthermore, leveraging the additional structural information provided by the software it was possible to conduct a Multilevel Cross-Organism Transferability Analysis (MCOTA) between H. sapiens and other organisms, to assess the predictive reliability of various animal models beforehand. This allowed for the design of even more targeted tests, maximizing adherence to the guiding principles of the 3Rs.

P49

Device for 3D cell culture and extensive screening on organoids

E. Bianchi[1], A. O. Botrugno[2], P. De Stefano[1], G. Giovannoni[2], C. Felici[2], A. F. Pellegata[1], P. Monica[1], G. Tonon[2], G. Dubini[1]

[1]Politecnico di Milano - Laboratory of Biological Structure Mechanics (LaBS), Department of Chemistry, Materials and Chemical Engineering 'Giulio Natta', Milano; [2]Functional Genomics of Cancer Unit, IRCCS San Raffaele, Milano, Italy

Presenting author:
E. Bianchi. E-mail: ✉ elena1.bianchi@polimi.it

Organoids are *in vitro*-cultured self-assembling Three-Dimensional (3D) structures derived from stem cells. They are able to recapitulate *in vitro* the structure and functions of the organs of origin. Organoids are then highly promising models for studying development processes, disease modelling and pharmacological research. As an alternative to Two-Dimensional (2D) cell cultures, Patient-Derived Organoids (PDOs) represent an affordable and more realistic model for developing personalized medicine. We designed a platform to culture organoids for drug screening purposes. This platform can host and recover hundreds of independent Matrigel® domes, containing patient-derived organoids, in a 384 multiwell format. Validation of the platform has been performed by exploiting PDOs of colon cancer metastatic to the liver. Organoids growth and proliferation in the platform have been confirmed by extensive testing, as their counterpart cultured in standard conditions. Our platform allows reducing the reagent and the experimental time to possibly fit to the clinical application: an ambitious aim is to use this type of screening to obtain clinically relevant information on drug sensitivity with the approach of precision medicine. The possibility to perform extensive screenings on reduced amount of PDO samples is then a feature that provides an indisputable advantage for pre-clinical research.

The present work is part of the Accelerator Award "Single-cell cancer evolution in the clinics" (A26819, 2018), co-funded by Cancer Research UK and AIRC and within MUSA – Multilayered Urban Sustainability Action, funded by EU, under the National Recovery and Resilience Plan (NRRP).

P50

Antitumor activity of the hydroalcoholic extract of *Artemisia annua L.* in human osteosarcoma: from 2D to 3D models to study antitumor phytocomplexes

C. Pellegrino[1], G. Isani[2], G. Andreani[2], M. Mandrone[3], G. Farruggia[1], M. Rossi[1], F. Rossi[1], E. Malucelli[1], C. Cappadone[1]

[1]Pharmaceutical Biochemistry Lab, Department of Pharmacy and Biotecnology, Alma Mater Studiorum University of Bologna; [2]Department of Veterinary Medical Sciences, Alma Mater Studiorum University of Bologna, Ozzano dell'Emilia (BO); [3]Pharmaceutical Botany Lab, Department of Pharmacy and Biotecnology, Alma Mater Studiorum University of Bologna, Italy

Presenting author:
C. Pellegrino. E-mail: ✉ cristina.pellegrino4@unibo.it

Artemisia annua L. has been used for more than 2000 years for the treatment of various diseases, although today its fame is mainly linked to its anti-malarial activity. Its active principle artemisinin was discovered by the Chinese chemist Tu Youyou. Several studies have shown that artemisinin-related compounds have also cytotoxic effects against cancer cells. Interestingly, the phytoextract is active on the canine osteosarcoma cell cultures, inducing cell cycle arrest and cell death. Three-Dimensional (3D) models represent a useful compromise between the simplicity of Two-Dimensional (2D) monolayers and the complexity of *in vivo* preclinical models, mimicking the physiological complexity of tissues and organs. These models allow to assay *in vitro* a wide range of potential drugs before the preclinical animal testing or

the subsequent clinical phase, avoiding ethical implications and costs associated with the use of animal models. The aim of this study is to test the antiproliferative activity of a hydroalcoholic extract of *A. annua L.* on human osteosarcoma cell cultures. First, we used 2D cultures based on five cell lines (Saos, HOS, U2OS, MG63, 143B), and then spheroids of MG63 and 143B cells, obtained by the Scaffold Free Liquid Overlay technique. Cell proliferation has been tested by Livecyte platform, exploiting Ptychographic Quantitative Phase Imaging (QPI) technology. Moreover, cell death was evaluated using the Alamar Blue assay, and cell cycle progression using flow cytometry. Our results showed that the hydroalcoholic extract of A. annua blocks cell proliferation in 2D and 3D cultures. Furthermore, the extract decreases cell displacement and induces cell death events. Thanks to QPI analysis the typical ballooning feature associated with ferroptosis has been observed. In conclusion, the extract may be considered for further investigation in the context of new anti-cancer drugs for the treatment of osteosarcoma, which is currently an untreatable type of cancer, being without a specific therapy.

P52

Unveiling stress-free biomarkers in several mouse models via digital ventilated cages (DVC®) technologies

G. Rosati[1], S. Gaburro[1,2]

[1]Tecniplast S.p.A., Buguggiate (VA), Italy; [2]North American 3Rs collaborative

Presenting author:
G. Rosati. E-mail: ✉ giorgio.rosati@tecniplast.it

Adopting home cage monitoring systems allows researchers to discover novel findings due to the 24/7 and, in most cases, stress-free environment.
Recently, clear definitions between "benchtop technologies," where animals can spend only one to a few days in an environment, and "real home cage technologies," where animals spend 99% of their lifetime, were published by our North American 3R working group.
Among real-home cage technologies, Digital Ventilated Cages (DVC®) are the only scalable solution that demonstrated the following: 1. Possibility of monitoring 1-1000+ cages simultaneously; 2. Using the animals without amending the animal license because effectively, the animals are staying undisturbed in their home cage while being monitored; 3. Employing environmental enrichment, which typically disturbs any video assessment, is possible because of our micro electromagnetic field technology.
In this talk, we will present three examples: 1. Using Polyuria as a surrogate marker of sustained hyperglycemia (diabetes) in type 1 and 2 diabetes; 2. Identify unseen fighting events in the home cage in group-housed conditions; 3. Unveil early signs of welfare issues in a Covid19 mouse model before any occurrence of clinical signs or body weight loss.
Overall, the DVC® technology is allowing system is allowing scientists to perform 24/7 monitoring in a scalable manner, increasing the welfare aspect while better characterizing the models.

P53

Functionalized liposomes for automated fluorine-18 surface radiolabelling and *in vivo* PET imaging

P. Rainone[1,5], M. Kravicz[1], M. N. Iannone[2], S. Stucchi[1,2], E. Vino[2], E. A. Turolla[1,2], A. Antoniou[3], A. Amenta[4], S. Valtorta[5-7], S. Pellegrino[3], D. Passarella[4], P. Seneci[4], S. Todde[1,2], F. Re[1], R. M. Moresco[1,2,5,6]

[1]School of Medicine and Surgery, University of Milano-Bicocca, Vedano al Lambro (MB); [2]Tecnomed Foundation, University of Milano-Bicocca; [3]Pharmaceutical Sciences Department, University of Milan; [4]Chemistry Department, University of Milan; [5]Nuclear Medicine Department, San Raffaele Scientific Institute IRCCS, Milan; [6]Institute of Molecular Bioimaging and Physiology (IBFM), National Research Council (CNR), Segrate (MI); [7]National Biodiversity Future Center (NBFC), Palermo, Italy

Presenting author:
R. M. Moresco. E-mail: ✉ rosa.moresco@unimib.it

Positron Emission Tomography (PET) is a molecular imaging technique offering a variety of radiolabeled compounds targeting different biological structures or processes. It offers non-invasive measurement of a radiotracer concentration in tissue and is clearly translational, since the same technology can be applied in animal models, as well as in research and clinical use in humans. Preclinical PET imaging plays an important role in the investigation of therapeutic targets to help treatment and diagnosis on several mouse models and allows to monitor a biological process or a therapy response longitudinally in the same mice and in a non-invasive modality, reducing the number of animals and improving the animal welfare according to principle of the 3Rs. Liposomes have great potential as PET imaging agents due to their radiolabeling and drug delivery capability. In this study, fluorine-18 labelled liposomes ([18F]-Lip) were functionalized with a peptide from the receptor-binding domain of the apolipoprotein E (mApoE), useful to promote the blood-brain barrier (BBB) crossing, and with an MMP-sensitive lipopeptide (SG) for an MMP-triggered drug release as potential theranostic agent. High radiochemical purity and suitable radiochemical yields (RCP>99%, non-decay corrected RCY: 5,2±1,3%, n=5) were obtained with [18F]-Lip with a hydrodynamic size smaller than 200 nm, low-medium dispersity, and negative ζ-potential. Brain and systemic biodistribution of functionalized nanoparticle [18F]-Lip(mApoE+SG), and/or nonfunctionalized [18F]-Lip, has been evaluated *in vivo* in an orthotopic mouse model of glioma by PET/CT imaging up to three hours post-injection (n=4). Tumor uptake of functionalized [18F]-Lip(mApoE+SG) was slightly lower than that of [18F]-Lip, but a significant higher level of tumor-to-normal brain parenchyma ratio was determined for the former. This data suggests that peptide functionalization in[18F]-Lip(mApoE+SG) might prevent their uptake in healthy brain tissue.

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3R-SMART: Information and training platform for methods to replace and supplement animal experiments

M. K. Valussi[1], C. Nordmann[2], F. Gumz[2], B. Hiebl[2], N. Linklater[1]

[1]Philipps-University Marburg, Department of Biology/Animal

Physiology, Marburg; [2]Institute for Animal Hygiene, Animal Welfare and Farm Animal Behaviour, Hannover, Germany

Presenting author:
M. Valussi. E-mail: ✉ Melissa.Valussi@biologie.uni-marburg.de

The Directive 2010/63/EU firmly strengthens the adoption of the 3R principle (replacement – reduction – refinement) for the use of animals for scientific and educational purposes.
Against this background, the BMBF-funded project 3R-SMART (https://www.3r-smart.de) was designed as an information and training platform on alternative and supplementary methods to animal experiments.
In addition to showcasing specific examples of alternatives to animal testing, 3R-SMART also covers legal and ethical aspects of working with laboratory animals. Video or text-based content provides either a brief overview or more in-depth information.
The website not only offers instructive content about alternative methods but also news and updates, a calendar for upcoming events and a forum that offers the opportunity to exchange ideas in the field of 3R.
Furthermore, 3R-SMART supports the 3R research activities of various stakeholders by enabling them to present their latest 3R findings on 3R-SMART in order to increase the reach of their research results.
In this way, 3R-SMART is constantly being expanded and developed. Interactive maps of the 3R Centres in Germany and Europe enable interested parties to obtain an overview of the 3R Centres and to find out more about the activities and focal points of the individual 3R Centres.
In order to disseminate and transfer knowledge about the 3Rs, 3R-SMART will make Open Educational Resources (OER) available and also is planning to offer 3R seminars and other learning opportunities. In this context, work is being done in cooperation with LAS interactive (https://las-interactive.de) on a combined continuing education portal (3R-Campus) on laboratory animal science and alternatives to animal experimentation (fee-based) for continuing professional development.

P55

Unraveling ligand binding to HIF-2α: computational approaches in drug design and their contribution to promoting 3Rs principles

L. Callea, S. Motta, L. Bonati

Department of Earth and Environmental Sciences, Italy

Presenting author:
L. Callea. E-mail: ✉ lara.callea@unimib.it

In silico methods are becoming increasingly effective in complementing experiments and providing atomistic descriptions of ligand binding. In particular, in the context of drug design, computational methods have played a crucial role in reducing the number of animals required for studies. These approaches provide reliable predictions of molecular behavior and drug interactions, enabling the evaluation of efficacy and safety *in silico* before conducting animal experiments. In this work, computational methods based on Molecular Dynamics (MD) simulations were employed to study ligand binding to the PAS-B domain of hypoxia-inducible factor 2α (HIF-2α), a pharmaceutical target for cancer therapy. Modeling the binding pathways of ligands to HIF-2α represents a challenging task due to the buried nature of the binding cavity. Through Steered Molecular Dynamics (SMD) and Metadynamics (MetaD) simulations, the ligand binding process, entry pathways, and binding affinities were elucidated. Specifically, SMD simulations were used to identify the preferred unbinding pathway among alternative ones and to help in the construction of the reference pathway for the next step. On the other hand, MetaD was used to provide a more rigorous characterization of the different states and to calculate the binding free energy values. The correct binding affinity scale was determined based on available experimental data, and minima in the Free Energy Surface (FES) were identified, clearly depicting the bound states and intermediate states characteristic of each ligand. These findings can contribute to the development of successful drug design projects and facilitate the evaluation of structure-activity relationships, the prediction of molecular interactions with the target, and the assessment of potential toxicity.

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