Tuesday, October 24th

Time	Talk / Poster title	Speaker
8:00	**Registration**	
9:00	**Welcome** *Prof Mª Isabel Lucena, Department of Pharmacology, School of Medicine, chair of the LOC; Prof. Juan Jose Hinojosa Torralvo, Dean of the Law Faculty, and Prof. Zaida Díaz Cabiale, Research and Innovation Vice Chancellor from UMA*	
9:30	**Opening Lecture** *Chair: Juan Manuel Falcón Pérez [CIC bioGUNE-RTA,CIBERehd, Derio (GEIVEX)]*	
	"EVs and Parasitic Diseases: let´s MOVE-on"	**Hernando del Portillo**
10:15	**Session I: Parasitic EVs** *Chairs: Juan Manuel Falcón Pérez and Susana Santos [i3S - Instituto de Investigação e Inovaçao em Saúde, Universidade do Porto (PNEV)]*	
	O1. "Functional characterization of a hypothetical spleen-dependent P. vivax gene and the role of EVs in spleen-parasite interactions"	Albert Ayllón
	O2. "Proteomic profiling of plasma-derived extracellular vesicles in patients with visceral leishmaniasis: a biomarker discovery. "	Ana Torres
	O3. "Are Extracellular Vesicles from trematodes sufficient to cause fibrosis in liver? Evaluation of the effects of EVs in vitro and in vivo."	Christian Miquel Sánchez-López
	O4. "Multiparametric study to optimize EV production for immunomodulatory potential"	Christophe Wong
11:10	**Coffee break**	
11:45	**Session II: Infection and interspecies communication** *Chairs: Esther Nolte-'t Hoen [Utrecht University (NLSEV)] and Nele De Langhe [Ghent University (BESEV)]*	
	O5. "Intercellular communication strategies of human polyomaviruses: The role of extracellular vesicles in Merkel cell polyomavirus pathogenesis."	Ute Andrea Westerkamp
	O6. "Impact of Klebsiella pneumoniae outer membrane vesicles on antibiotic resistance"	Marie Burt
	O7. "SARS-CoV-2 infection during pregnancy alters profile of placenta-derived extracellular vesicles indicating placental dysregulation"	Isabel Graf
	O8. "RNA profiles of plasma extracellular vesicles in patients with sepsis"	Berit Brusletto
	O9. "Colorectal cancer EVs alter the gut microbiome"	Rawan Maani

O10. "Amniotic fluid stem cells-derived extracellular vesicles reprogram dendritic cell functions in autoimmune neuroinflammation"	Giorgia Manni
O11. "Study of the anti-inflammatory effects of industrial-produced lemon nanovesicles on inflammatory bowel disease"	Vincenza Tinnirello
O12. “Inflammatory modulation through proteolytic active extracellular vesicles and bacteriomimetic vesicles”	Yildiz Daniela
O13. "Tetraspanins as an immunogenic adjuvant in lipid nanovesicles"	Fernando del Burgo

13:30 **Lunch Break**

14:45 **Afternoon lecture**

Chair: Benedetta Bussolati [Department of Molecular Biotechnology and Health Sciences, University of Torino (EVita)]

“Harnessing Nature's nanoSecrets: Microalgal-Derived Extracellular Vesicles as Bio-Based Nanoparticles for next-level Pharmaceutical and Cosmetic applications” — **Antonella Bongiovanni**

15:30 **Session III: Cardiovascular and metabolic diseases**

Chairs: Benedetta Bussolati and
María Pardo Pérez [Instituto de Investigación Sanitaria De Santiago (GEIVEX)]

O14. "PLANT EDIBLE EVs MODULATES THE INFLAMMATORY RESPONSE IN AN ACUTE PANCREATITIS MODEL"	Christian Miquel Sánchez-López
O15. "INTERORGAN CROSSTALK THROUGH EVs AND THEIR IMPLICATION IN OBESITY: FUNCTIONAL ROLE AND CARGO OF BIOMARKERS"	Nerea Lago Baameiro
O16. "Adipocyte-derived extracellular vesicles are endocrine regulators of insulin secretion in mice"	Konxhe Kulaj
O17. "Microvesicles display opposite coagulolytic balance according to their cellular origin and activation status"	Akhil Antony Konkoth

16:30 **Coffee break**

17:15 **Session IV: Fundamental EVs Biology**

Chairs: Christian Neri [INSERM (FSEV)] and
Wolf Holnthoner [Ludwig-Boltzmann-Institute for Traumatology (ASEV)]

O18. "Extracellular lipid vesicles induce Ca2+ signals in target cells"	Alina Milici
O19. "Genome-Wide-CRISPR/Cas9 screening identifies the COMMANDER recycling complex as a key player in EV cargo delivery"	Miguel Palma-Cobo

O20. "Unravelling molecular drivers of extracellular vesicle-mediated cargo transfer"	Maria Laura Tognoli
O21. "A role for integrin beta 1 in extracellular vesicle-mediated functional RNA delivery"	Omnia Elsharkasy
O22. "siRNA screening using a novel rapid radioactive-based assay reveals a role for SNAP29 in exosome release"	Krizia Sagini
O23. "Exploring the Versatile Role of LAMP2A: Insights into Exosomal Cargo Loading and Endosomal Identity in Inter-Cellular Communication"	Joao Vasco Ferreira

18:30 **poster walk I (with wine and cheese)**

P1.1"Surface functionalizations for the preferential capture of extracellular vesicle"	Cristina Potrich et al.
P1.2 "Isolation of astrocytes-derived extracellular vesicles from brain, to decipher their influence on recovery after stroke"	Hind Haj Ahmad et al.
P1.3 "Genetic and metabolic factors affecting EV-secretion in Bacillus cereus"	Tanja Edelbacher et al.
P1.4 "Role of Extracellular Vesicles in Müller glia-neuron crosstalk during neuroinflammation"	Cristiano Lucci et al.
P1.5 "Investigating the behavior of extracellular vesicles after a neuroinflammatory stimulus"	Lien Cools et al.
P1.6 "Adipocyte-derived extracellular vesicles: the quest for a proper isolation protocol"	Lisa Mennens et al.
P1.7 "Transparent reporting and centralizing knowledge in bacterial extracellular vesicle research"	Nele De Langhe et al.
P1.8 "Fecal extracellular vesicles: potential applications in disease diagnosing, prognosis, and monitoring"	Chanaka Premathilaka et al.
P1.9 "Studies of extracellular vesicle heterogeneity at the single vesicle level"	Reet Kurg et al.
P1.10 "Quantitative proteomic analysis of serum-purified exosomes identifies putative antimonial failure-associated biomarkers in immunosupressed mice with visceral leishmaniasis"	Lorena Bernardo et al.
P1.11 "Identification and characterization of plasma-derived extracellular vesicles in burn-septic shock patients"	Martina Schiavello et al.
P1.12 "Evaluation of different fluorescent labelling strategies to track exosome biogenesis, loading, and delivery "	Rebecca Piccarducci et al.
P1.13 "A different perspective on EV surface engineering: the protein corona “variable”"	Miriam Romano et al.
P1.14 ”Multi-platform ligands for Extracellular Vesicles integrated isolation and analysis"	Marina Cretich et al.
P1.15 "B cell receptor engagement boosts the secretion of extracellular vesicles that modulate activation capacity in human B lymphocytes"	Saara Hämälistö et al.

P1.16 "Production and characterization of Saccharomyces cerevisiae extracellular vesicles containing recombinant PQLC2, a lysosomal amino acid membrane transporter. "	Jose Luis Vázquez-Ibar et al.
P1.17 "Detection of tumor-derived extracellular vesicles interactions with immune cells is dependent on EV-labelling methods"	Lorena Martin Jaular et al.
P1.18 "Development of EV tools labelled with fluorescent or luminescent cargos"	Etienne Lourdin-de Filipis et al.
P1.19 "Spatio-temporal analysis of the intercellular transfer of extracellular vesicles between mammary cells"	Simon Marie et al.
P1.20 "Multiparametric characterization of the secretome produced by mesenchymal stromal cells: towards regulatory compliance"	Elise Madec et al.
P1.21 "Workflow optimization for the isolation and characterization of human milk extracellular vesicles"	Jose Luis Moreno Casillas et al.
P1.22 "Analysis of protein and miRNA cargo of human conjunctival EVs"	Laura García-Posadas et al.
P1.23 "Study of host-symbiont communication mediated by extracellular vesicles in Blattella germanica"	David Saiz-Martínez et al.
P1.24 "Effects of Growth Medium and Incubation Time on Fungal Extracellular Vesicles: Insights into Differential Composition and Characteristics""	Lucia Monteoliva et al.
P1.25 "Protection Against Invasive Candidiasis with Vesicle Immunization: Promising Advances towards an Effective Vaccine"	Raquel Martínez López et al.
P1.26 "Optimization of EV isolation and their separation from lipoproteins by size exclusion chromatography"	Joaquín Morales et al.
P1.27 "ELECTROCHEMICAL SENSOR FOR POINT-OF-CARE QUANTIFICATION OF EXTRACELLULAR VESICLES "	Maria del Carmen Blanco Lopez et al.
P1.28 "Comparative analysis of extracellular vesicle-derived protein extraction methodologies for mass spectrometry analysis"	Carmen Ráez Meseguer et al.
P1.29 "COMPARISON OF METHODS FOR ISOLATING EXTRACELLULAR VESICLES FROM HUMAN PLASMA"	Esther Serrano-Pertierra et al.
P1.30 "In vitro binding of seminal extracellular vesicles (sEVs) to porcine sperm increases with co-incubation time."	Ana Parra et al.
P1.31 "Proof of concept of using a membrane-sensing peptide for sEVs affinity-based isolation"	Beatriz Benayas et al.
P1.32 "Characterisation of circulating microvesicle content in horse plasma in the search of biomarkers for the diagnosis of equine metabolic syndrome."	Beatriz Ortiz Guisado et al.
P1.33 "Study of the in vivo biodistribution of hepatocyte-released extracellular vesicles in mice models for metabolic syndrome progression"	Clara Garcia Vallicrosa et al.
P1.34 "RNA-seq in blood circulating EVs shows the presence of bacterial RNAs in multiple sclerosis patients."	David Otaegui et al.

P1.35 "Small extracellular vesicles but not microvesicles from the parasitic liver-fluke Opisthorchis viverrini promote cell proliferation in human cholangiocytes"	Javier Sotillo et al.
P1.36 "Attenuation of SASP through sEV biogenesis interruption"	Juan Antonio Fafian Labora et al.
P1.37 "CHARACTERIZATION OF EVs FROM APOPLASTIC FLUID OF CAROB PULP: ANTI-INFLAMMATORY AND ANTIBACTERIAL ACTIVITY "	Mari Cruz Manzaneque-López et al.
P1.38 "INVOLVEMENT OF EXTRACELLULAR VESICLES SECRETED BY INFECTED CELLS ON THE PERSISTENCE OF THE DISEASE. LONG COVID, DIAGNOSIS AND PROGNOSIS."	Pilar Martin Duque et al.
P1.39 "Characterization of Mesenchymal Stromal Cells derived Extracellular Vesicles from 3D Hollow-fiber Bioreactor culture"	Marta Sanroque Muñoz et al.
P1.40 "Proteomics profile of peritoneal EVs from CD38-deficient lupus mice reflects a defective expression of the immunoproteasome and increased abundance of proteins related with inflammatory-resolving processes."	Mercedes Zubiaur et al.
P1.41 "A method compatible with a clinical setting to isolate extracellular vesicles from cerebrospinal fluid"	Nil Salvat-Rovira et al.
P1.42 "ORGAN-ON-A-CHIP TECHNOLOGY TO STUDY EXTRACELLULAR VESICLES-MEDIATED CRYPTIC INFECTIONS IN MALARIA"	Núria Sima Teruel et al.
P1.43 "Sepharose CL-2B is more efficient than CL-6B for isolating porcine seminal extracellular vesicles in purity by size exclusion chromatography."	Pablo Martínez-Díaz et al.
P1.44 "Regulation of of cell envelope remodeling to mediate EV production in the human pathogen Mycobacterium tuberculosis"	Rafael Prados-Rosales et al.
P1.45 "The profiling of extracellular vesicle subpopulations in human Huntington's disease brains identifies Alix as a novel neuropathology marker"	Rocío Pérez-González et al.
P1.46 "Fida: A novel technique for determination of protein binding on Extracellular Vesicles "	Anna-Kristin Ludwig et al.
P1.47 "Small particles carrying great potential – Extracellular vesicles in Parkinson's disease research"	Fanni Annamaria Boros et al.
P1.48 "Unraveling signaling mechanisms of renal extracellular vesicles in ciliopathies"	Alina Frei et al.
P1.49 "Pro-coagulant extracellular vesicles mediate smoking-induced pulmo-vascular inflammation"	Isabell Burhorst et al.
P1.50 "Bacterial extracellular vesicles repress the vascular protective factor RNase1 in human lung endothelial cells"	Isabell Burhorst et al.
P1.51 "Extracellular vesicles cargo of the proteolytically active ADAM10 or ADAM17 shape the inflammatory response and drive disease severity "	Ahmad Aljohmani et al.
P1.52 "Enhance small RNA NGS analyses with caRNAge - Bias detection, ncRNA isoforms and improved validation chances"	Kirchner Benedikt et al.

P1.53 "Role of extracellular vesicles in the pathophysiology of Alzheimer's disease; modulation of amyloid β aggregation"	Mohsin Shafiq et al.
P1.54 "Bacterial vesicles block viral replication in macrophages via TLR4-TRIF-axis"	Marie Burt et al.
P1.55 "TFF and SEC may lead to loss of a pro-angiogenic effect in Müller cell-conditioned media"	Melanie Schwämmle et al.
P1.56 "ROLE OF THE PRION PROTEIN IN EXTRACELLULAR VESICLES UPTAKE"	Santra Brenna et al.
P1.57 "The presence of cell-free DNA in plasma-derived small extracellular vesicles"	Kristína Lichá et al.
P1.58 "Metagenomic profiling of fecal-derived bacterial membrane vesicles in non-alcoholic fatty liver disease and drug-induced liver disease"	Cristina Rodriguez Diaz et al.
P1.59 "A novel spectral flow cytometry workflow to assess the RNA topology of submicron particles"	Jillian Bracht et al.
P1.60 "CHARACTERIZATION OF EXTRACELLULAR VESICLES FROM NEURONAL PRIMARY CULTURES"	Mª Teresa Fernández Sánchez et al.
P1.61 "Developing a scalable method for isolating and purifying milk-derived extracellular vesicles"	Alberto Dávalos et al.
P1.62 "The miRNA cargo of jejunal extracellular vesicles depends on the level of insulin resistance in patients with morbid obesity"	Ailec Ho Plágaro et al.
P1.63 "The effects of fecal-microbe-derived extracellular vesicles on intestinal mRNA expression depend on the type of patient"	Flores Martín Reyes et al.
P1.64 "The inflammatory microenvironment generated in acute pancreatitis modify the surface of circulating exosomes increasing their inflammatory activity"	Daniel Closa et al.
P1.65 "Proteomics of Circulating Extracellular Vesicles in Patients with Bacterial sepsis"	Kari Bente Foss Haug et al.
P1.66 "MARCO as a potential human biomarker present in plasma-derived extracellular vesicles from a patient co-infected with Leishmania and HIV "	Nuno Santarem et al.
P1.67 "The liver stage of Plasmodium infection – written in extracellular vesicles?"	Bárbara Teixeira et al.
P1.68 "Functional characterization of hiPSC-derived endothelial cells as an in vitro model for studying EV-mediated tissue repair in myocardial tissue"	Monika Orpel et al.
P1.69 "The impact of graphene-based substrates on proangiogenic properties of human mesenchymal stem cells and their extracellular vesicles - significance for cardiovascular repair"	Sylwia Noga et al.
P1.70 "Manganese overexposure induces the release of manganese-loaded extracellular vesicles in microglia"	M. Rosario Sepulveda et al.
P1.71 "Modifications in the protein corona of exosomes after exposure to the inflammatory microenvironment generated during acute pancreatitis"	Montserrat Carrascal et al.

P1.72 "Transport of RNA from mouse to bacteria via extracellular vesicles: probing specificity and function"	Xiaochen Du et al.
P1.73 "Identification and characterization of Trypanosoma cruzi proteins present in circulating EVs from patients with chronic Chagas Disease"	Berta Barnadas et al.
P1.74 "Proteasome activity is increased in Extracellular Vesicles isolated from the plasma of Fasciola hepatica-infected cattle"	Aranzazu González et al.
P1.75 “Viral GPCR-activated EV release as a novel therapeutic target in brain cancer”	Lotte Di Niro et al.

Wednesday, October 25th

Time	Talk	Speaker
9:00	**Morning lecture** *Chair: Charlotte Lawson [Royal Veterinary College, London (UKEV)]*	
	"Convergence of G protein-coupled receptor (GPCR) and extracellular vesicle biology"	**Martine Smit**
9:45	**Session V: Cancer immunity** *Chairs: Jason Webber [Swansea University (UKEV)] and Basant Kumar Thakur [University Hospital Essen (GSEV)]*	
	O24. "Tumour endothelial cells derived – extracellular vesicles control the local and systemic anti-tumor immune response modulating mTOR/G-CSF pathway"	Alessandro Sarcinella
	O25. "Colorectal cancer-derived extracellular vesicles affect the immunomodulatory properties of hepatocytes supporting liver metastatic colonization"	Elisa Costanzo
	O26. "Using Menstrual Blood-Derived Stromal Cell Secretome for Shifting Tumor-Associated Macrophages towards an Immunoreactive Phenotype"	María Ángeles de Pedro
	O27. "Effect of plasma-derived small EVs from HNSCC patients on NF-κB signaling in macrophages"	Diana Huber
	O28. "Modulation of PDAC immune response through cancer EVs"	Carolina Dias
10:50	**Coffee break**	
11:30	**Session VI: EVs in Cancer progression** *Chairs: Marina Cretich [National Research Council of Italy, Istituto di Scienze e Tecnologie Chimiche (EVita)] and Kendra Maass [DKFZ/KiTZ Heidelberg (GSEV)]*	
	O29. "Extracellular vesicle microRNAs contribute to Notch signaling pathway in T-cell acute lymphoblastic leukemia."	Tommaso Colangelo
	O30. "Chromatinized DNA derived from AML extracellular vesicles alters bone marrow mesenchymal stem cells behavior by inducing non-mutational dysfunction of p53"	Jamal Ghanam
	O31. "YBX1 containing small EVs derived from AML influences osteogenic differentiation of mesenchymal stromal cells in the bone marrow microenvironment"	Venkatesh Kumar Chetty
	O32. "Breast adipose tissue-derived extracellular vesicles from women with obesity stimulate mitochondrial-induced dysregulated tumor cell metabolism"	Alberto Benito-Martin

O33. "Tumoral periprostatic adipose tissue derived exomicroRNAS regulate tumour suppressor RORA gene in prostate cancer cells "	Matilde Rodríguez Chacón
O34. "Decoding the impact of EVs in the tumor microenvironment: Insights into EV-DNA dynamics and implications for disease progression"	Enrique Bastón
O35. "Ferroptosis transmission by small extracellular vesicles in epithelial ovarian cancer cells"	Juan Antonio Fafian Labora
O36. "ENHANCING CURCUMIN'S PHARMACOKINETICS USING EXTRACELLULAR VESICLES AS DRUG DELIVERY SYSTEMS FOR IMPROVED CANCER TREATMENT"	Malika Singh

13:10 **Lunch Break**

14:30 **Afternoon lecture**

Chair: María Yáñez-Mó [CBM-SO/IIS-IP, Universidad Autónoma de Madrid, (GEIVEX)]

"Tetraspanins and extracellular vesicles: together and forever" **Fedor Berditchevski**

15:15 **Session VII: EVs towards the clinic in cancer**

Chairs: Maja Kosanovic [INEP (Srb-EV)] and
Tobias Tertel [Institute for Transfusion Medicine, University Hospital Essen (GSEV)]

O37. "Manipulation of Tumor Extracellular Vesicles biodistribution and uptake in vivo by a versatile cell-surface "EV-Trap" "	Vincenzo Verdi
O38. "Identification of EV-biomarker candidates from peripheral and local tumor plasma in head and neck squamous cell carcinoma using proteome analysis before/after tumor removal surgery"	Dapi Menglin Chiang
O39. "Digital Detection of EGFR Mutations in Lung Cancer Using Tumor-Derived EVs in Blood Plasma "	Yoon-Kyoung Cho
O40. "An Early Brain Cancer Diagnosis Approach Based on Bioinformatics and Machine Learning Analysis of Human Plasma Exosome Transcriptomics"	Tayfun Tatar
O41. "PD-L1 on large extracellular vesicles is a predictive biomarker for therapy response in tissue PD-L1-low and -negative patients with non-small cell lung cancer"	Kerstin Menck
O42. "Saliva-derived small extracellular vesicles as biomarker for head and neck cancer – does saliva provide better diagnostic potential than plasma?"	Linda Hofmann

16:20 **Coffee break**

17:15 Session VIII: EVs in liquid biopsy

Chairs: Alicia Llorente [Oslo University Hospital (NOR-EV)] and
Gloria Alvarez-Llamas [Fundación Jiménez Díaz Hospital (GEIVEX)]

O43. "Plasma Exosomal Non-Coding RNA Profile Associated with Renal Damage Reveals Potential Therapeutic Targets in Lupus Nephritis"	Ana Flores-Chova
O44. "A Selected signature of miRNAs in Urinary Extracellular Vesicles Allows Noninvasive Detection of Graft Fibrosis in Renal Transplant Patients"	Marta Clos-Sansalvador
O45. "Identification of a plasma extracellular vesicle surface signature associated with nephritis in systemic lupus erythematosus"	Olga Martinez-Arroyo
O46. "Altered small RNA-secretome in Huntington´s Disease neurons"	Marina Herrero
O47. "Synovial fluid-derived EVs in Equine Osteoarthritis – Correlation between the proteome and phospholipidome for biomarker identification"	Laura Varela
O48. "Single vesicle imaging flow cytometry approach to unravel the molecular profile of endometriosis-related extracellular vesicles as a source of potential biomarkers"	Karolina Soroczyńska
O49. "Physical association of low density lipoprotein particles and extracellular vesicles unveiled by complementary single particle analysis techniques"	Estefanía Lozano-Andrés

18:30 poster walk (with wine and cheese) SPONSORED by ISEV

P2.1 "Comparative proteomics of canine osteoblast and osteosarcoma cell-derived extracellular vesicles enriched by different methods "	Daniela Cortes et al.
P2.2 "Engineered Extracellular Vesicles for Targeting and Activation of Lymphatic VEGFR-3"	Wolfgang Holnthoner et al.
P2.3 "Differential effect of cytotoxic therapy on primary and metastatic clear cell sarcoma and their EVs"	Djenana Vejzovic et al.
P2.4 "Surface Plasmon Resonance for Sensitive Detection of Chemokine Receptors CXCR4 and CXCR7 on Extracellular Vesicles: Implications for Cancer Diagnosis and Beyond"	Kaat Verleye et al.
P2.5 "Contribution of endothelial progenitor cell derived extracellular vesicles in bone regeneration "	Cyril Bouland et al.
P2.6 "Extracellular vesicles from chronic lymphocytic leukemia cells induce the differentiation of monocytes into tumor-associated macrophages"	Nathan Dubois et al.
P2.7 "Scalable production of extracellular vesicles from dental pulp stem cells cultivated in a hollow-fiber bioreactor"	Paula Pincela Lins et al.
P2.8 "Towards clinical translation of exosome-based mutation analysis using next-generation sequencing"	Rebekka Van Hoof et al.

P2.9 "Colorectal cancer cells during chemotherapy treatment release EVs that induce cancer associated fibroblasts reprogramming"	Giulia Artemi et al.
P2.10 "The Role of JAK-STAT1 in Exosome Biogenesis and miRNA Regulation in A549 Lung Cancer Cell Line"	Dian Salih et al.
P2.11 "Dissecting exosomal-miRNAs as key-players for the early identification of an aggressive subtype of lung adenocarcinoma"	Francesco Mazzarelli et al.
P2.12 "EVs from solid and fluid tissues face each other as multiscale biomarkers for Intensive Care Unit Acquired Weakness "	Lucia Paolini et al.
P2.13 "Proof-of-concept study on the use of tangerine-derived nanovesicles as RNAi delivery vehicles toward mammalian cells "	Nima RabieNezhad Ganji et al.
P2.14 "EXTRACELLULAR VESICLE RELEASE AND CARGO ARE ALTERED BY CAVEOLIN-1-OVEREXPRESSION AND MODULATE TUMOR MICROENVIRONMENT IN A MODEL OF RHABDOMYOSARCOMA"	Rachele Agostini et al.
P2.15 "Study of extracellular vesicles from accessible biological fluids to characterize individual responses to physical activity"	Stephanie Fondi et al.
P2.16 "Comparative Analysis of Cell Surface Markers CD38, CD138, and CD269 on mm plasma cells-Derived Extracellular Vesicles: Plasma cells-derived EVs identification in Liquid Biopsies?"	Sebastien Charles et al.
P2.17 "Upstream and downstream innovations in large-scale manufacturing of mesenchymal stem cells EVs in stirred tank bioreactor "	Thibaut Fourniols et al.
P2.18 "Using Raman spectroscopy for biochemical characterization of extracellular vesicles generated by thyroid tumor cell-fibroblast interplay: preliminary reports"	Ana Carolina Donadio et al.
P2.19 "Finding new EVs associated biomarkers of early post-menopausal osteoporosis in a Minipig model."	Arantza Infante et al.
P2.20 "Development and application of a secretome from mesenchymal stem cells with enhanced osteogenic capacity for the treatment of osteoporosis"	Alberto González González et al.
P2.21 "Orange-derived extracellular vesicles as viable nanocarriers of bioactive cargoes"	Joao Tomé-Carneiro et al.
P2.22 "Milk-derived extracellular vesicles as nanocarriers of dietary phenolic compounds"	María-Carmen López de las Hazas et al.
P2.23 "Isolation of exovesicles in the host containing pathogen DNA"	Mercedes Gomez-Samblas et al.
P2.24 "Characterization of ovarian cancer ascites-derived extracellular vesicles for their usage in precision medicine"	Antonio Jose Serrano-Muñoz et al.
P2.25 "Comparation of platelet-like particles (PLPs) and platelets (PLTs) derived EV"	Carolina Torres et al.
P2.26 "Exploring the impact of donor pool size on the consistency and features of Platelet Lysate-derived Extracellular Vesicles"	Andreu Miquel Amengual Tugores et al.
P2.27 "Functionalization of MSC-EVs with senolytic drugs and aptamers to target the vasculature"	Ainara González-Moro et al.

P2.28 "Effect of tumor-derived extracellular vesicles on the differentiation and maturation of monocytes and dendritic cells"	Almudena Rocha Mulero et al.
P2.29 "HEK293T-derived Extracellular Vesicles overexpressing miR219a-5p: a good model for further studies in neurodegenerative diseases"	Jone Karmele Arizaga Echebarria et al.
P2.30 "Delivery of shRNA minicircles by extracelullar vesicles to halt Parkinson's disease neurodegeneration"	María Izco Gaviria et al.
P2.31 "Plant exosome-like nanoparticles: building up novel animal-free therapeutic agents for drug delivery"	Miriam Morales Rodríguez De Lope et al.
P2.32 "Tetraspanin pattern characterization of extracellular vesicles in multiple sclerosis disease by ExoView R200+ platform"	Rocío del Carmen Bravo Miana et al.
P2.33 "Implication of secreted CEMIP protein in dermal fibroblast behavior "	Maria Larrinaga et al.
P2.34 "Evaluation of microneedle arrays as potential delivery systems for bacterial extracellular vesicles"	Alba Cortés Carbonell et al.
P2.35 "Anti-inflammatory effect of extracellular vesicles from synovial fluid on osteoarthritic synoviocytes"	Alvaro Compañ-Bertomeu et al.
P2.36 "IMPLEMENTATION OF SCAFFOLDS EMBEDDED WITH EXOSOMES AS PLATFORMS TO ATTRACT METASTATIC CELLS "	Belén Azanza Hernández et al.
P2.37 "Omics analysis of prostate cancer-derived extracellular vesicles to unravel their role in tumour generation and proliferation"	Belén Pastor Navarro et al.
P2.38 "Use of natural nanotherapies for liver fibrosis based on curcumin encapsulated in milk sEVs"	Beatriz Salinas et al.
P2.39 "Extracellular Vesicles-miRNAs signature in metastatic breast cancer diagnosis"	Coral González-Martínez et al.
P2.40 "Designing a nucleic acid-loaded theragnostic hybrid nanosystem based on extracellular vesicles-coated gold nanoparticles"	Cristina Fornaguera et al.
P2.41 "Combination of cation-exchange chromatography and filtration for lipoprotein depletion and plasma RNA detection"	Estela Sánchez-Herrero et al.
P2.42 "Production and isolation of F7 peptide tagged extracellular vesicles loaded with α-galactosidase A to improve cell internalization and in vitro efficacy "	Marc Moltó et al.
P2.43 "Cancer Stem Cell secreted Extracellular Vesicles lead tumor plasticity regulation and stroma activation in TNBC models" "	Joaquin Seras-Franzoso et al.
P2.44 "Plasma-derived extracellular vesicles from ovarian cancer patients and their impact on healthy ovarian epithelial cells"	Lidia Lorenzo Catoira et al.
P2.45 "IL-8/CXCR1 axis induces release of small extracellular vesicles from A2780 ovarian cancer cells with a pro-angiogenic profile"	Manuel Varas-Godoy et al.
P2.46 "EV-encapsulated Pt nanoparticles as radiosensitizers for antitumoral therapy"	Miguel Encinas-Gimenez et al.

P2.47 "EXOGAG, the new method for the isolation of Extracellular Vesicles and Glycoproteins, unmasks biomarkers and new molecular mechanism in kidney disease."	María Pereira Hernández et al.
P2.48 "STUDY AND VALIDATION OF THE METABOLIC SIGNATURE OF EXTRACELLULAR VESICLES AS A PROGNOSTIC AND PREDICTIVE MARKER IN PANCREATIC CANCER"	Pilar Espiau Romera et al.
P2.49 "Unveiling the Potential of Tumour-Derived Exosomes in Ewing Sarcoma: Implications for Dynamic Cell-Cell Communication and Tumour Spreading"	Raquel Melero Fernández de Mera et al.
P2.50 "Exploring a multiplexed bead-based flow cytometry assay designed for profiling of CNS-derived extracellular vesicles"	Alexandra Brahmer et al.
P2.51 "Challenges of scalable production and effective purification of mesenchymal stromal/stem cell derived extracellular vesicles for clinical application"	Tanja Kutzner et al.
P2.52 "EVs as Biomarkers for Monitoring Response to Radiotherapy "	Aoife Gahlawat et al.
P2.53 "Exploration of novel EV-associated transcriptional biomarker candidates for early chemotherapy response prediction in cancer cells"	Christian Grätz et al.
P2.54 "Small Extracellular Vesicles as Biomarker for Myocardial Infarction with Non-Obstructive Coronary Arteries (MINOCA)"	Christien Beez et al.
P2.55 "Forming of a Protein Corona on Extracellular Vesicles increases Uptake into Immune Cells"	Laura Dietz et al.
P2.56 "A comparative analysis of circulating tumor DNA in extracellular vesicles and circulating cell-free DNA in non-small cell lung cancer"	Katharina Maria Richter et al.
P2.57 "EV miRNA and protein composition of primary glioblastoma cells changes in 3D organoid models"	Marlene Reithmair et al.
P2.58 "Bacterial membrane vesicles as potential biomarkers for bacteremia diagnostics"	Mia Yu et al.
P2.59 "Cre-loxP reporter tumor model to study EV uptake and its effects"	Viviane Ponath et al.
P2.60 "A Comparative Engineering Study Distinguishing Breast Cancer Exosomes from Normal Exosomes using their Property of Thermal Stability "	Samer Al-Hakami et al.
P2.61 "Characterizing extracellular vesicles derived from tumor-associated macrophages in the ovarian cancer microenvironment"	María Gómez-Serrano et al.
P2.62 "EphrinB3 acts as a by-pass driver in mutated epidermal growth receptor-driven Non-small cell lung cancer treatments and is loaded in extracellular vesicles."	Albano Cáceres-Verschae et al.
P2.63 “Sweet EV’: Tracing different glucose carbon isotopic labeling patterns in EVs versus source breast cancer cells"	Thi Tran Ngoc Minh et al.
P2.64 "Analysis of plasma-isolated extracellular vesicles – a way to predict treatment response in immune checkpoint inhibitor treated metastatic non-small cell lung cancer patients?"	Petra Hååg et al.

P2.65 "Exploring the role of Rab3 in exosome release from prostate cancer cells"	Silvana Romero et al.
P2.66 "THE USE OF EV MEMBRANE PROTEINS AS TARGETS FOR CAPTURING URINARY EVs IN NOVEL THERMAL LATERAL FLOW IMMUNOSENSORS"	Beatriz Martín Gracia et al.
P2.67 "Isolation and Analysis of Extracellular Vesicles for the Evaluation of Microsatellite Instability in Endometrial Cancer"	Carlos Honrado et al.
P2.68 "Enhancing Brain Organoid Growth through Electrical Stimulation-Induced Amplification of Extracellular Vesicles"	Sahba Mobini et al.
P2.69 "The proteome of extracellular vesicles in DLBCL plasma patients is associated to the immune response and signaling transduction"	Ana Sofia Carvalho et al.
P2.70 "Lipidomic analysis of hypoxic small extracellular vesicles from head and neck squamous cell carcinoma cells revealed upregulation of glycerophospholipids"	Alicja Głuszko et al.
P2.71 "Immortalized and primary adipose tissue- derived mesenchymal stem/ stromal cells as a source of extracellular vesicles for tissue repair – comparative study"	Patrycja Dudek et al.
P2.72 "Actin rearrangement reduces CD20 and ICAM1 antigens on tumor-derived EVs and improves lymphoma immunotherapy."	Beata Pyrzynska et al.
P2.73 "Inactivation of EWSR1-FLI1 oncogene characteristic of Ewing sarcoma using Therapeutic Extracellular Vesicles (TEVs)."	Raquel Melero Fernandez de Mera et al.
P2.74 "Effects of Trophoblast Extracellular Vesicles on D-Galactose Induced Premature Aging of Keratinocytes"	Mirjana Nacka-Aleksić et al.
P2.75 "Characterisation of extracellular vesicles from mesenchymal stem/stromal cells as new delivery system for functionalized gold nanoparticles"	Maja Kosanović et al.
P2.76 "Developing an Extracellular Vesicle RNA Test for Early Detection of Patients at Risk of Aggressive Prostate Cancer"	Jason Webber et al.
P2.77 "Using extracellular vesicle glycoproteins for early detection of aggressive prostate cancer"	Demi Pritchard et al.
P2.78 "Novel EV quantification methods and their use in ageing biomarker discovery"	Benjamin Raven et al.
P2.79 "Transcriptomic analysis of astrocyte origin enriched plasma EVs in Alzheimer’s Disease"	Fatma Busra Isik et al.
P2.80 "Developing a device for the isolation of ovarian cancer exosomes using nanoporous filteration "	Sadeka Nujhat et al.
P2.81 "Identification of Pro-resolutive ω3 Oxylipins in Human Milk Extracellular Vesicles for Potential Therapeutical Application in Inflammatory Bowel Diseases "	Marta Gómez Ferrer et al.
P2.82 "The interplay between metalloprotease cleavage and extracellular vesicle secretion of immune ligands affects tumour recognition"	Silvia López Borrego et al.

Thursday. October 26th

Time	Talk	Speaker
9:00	**Session IX: Therapeutic opportunities of EVs** *Chairs: Alireza Fazeli [Estonian University of Life Sciences (BSEVs)] and Ewa Zuba-Surma [Jagiellonian University in Krakow (PSEV)]*	
	O50. "Serum Extracellular Vesicle miRNA Profiling to determine Extracorporeal Photopheresis Response in Graft versus Host Disease"	Kimberly Schell
	O51. "Uptake of extracellular vesicles by the liver and the effect of ApoB-rich protein corona on their biodistribution"	Krisztina Németh
	O52. "Immunomodulatory effects of placenta MSC-derived EVs in a novel 3D, immune-competent in vitro human small airway model of cystic fibrosis"	Malgorzata Czystowska-Kuzmicz
	O53. "Vectorised radiotherapy in oncology using milk small extracellular vesicles"	Beatriz Salinas
9:50	**Sponsored talks**	
	“Clinical Scale Production of Extracellular Vesicles in a 3-D Hollow Fiber Bioreactor"	John J.S. Cadwell [**Fibercell systems**]
	“Efficient isolation of biologically active EVs”	Dr. Gonçalo Regalo [**FUJIFILM Wako**]
	“EV Therapeutics and EV Diagnostics: A qEV PurePath Approach”	Dr. Stephane Mazlan [**IZON**]
10:50	**Coffee Break**	
11:30	**Session X: Manufacturing EVs** *Chairs: Antonella Bongiovanni [National Research Council of Italy (EVita)] and Saara Laitinen [R&D Manager (FISEV)]*	
	O54. "Extracellular vesicles from human iPS cells as new tool enhancing biological functions of cord blood-derived hematopoietic stem and progenitor cells"	Elżbieta Karnas
	O55. "Clonal Immortalized Mesenchymal Stromal Cells: an ideal cell source for a reproducible and standardized production of therapeutic extracellular vesicles."	Yanis Mouloud
	O56. "Manufacturing of Wharton’s Jelly Mesenchymal Stromal Cells-derived Extracellular Vesicles for Clinical Application"	Yvan Courageux
	O57. "Standardized cell factories for production of biologically active, clinical-grade MSC-EVs"	Marieke T. Roefs
	O58. "Incorporating Extracellular Vesicles from dendritic cells in fibrinogen and magnesium scaffolds to promote bone regeneration"	Maria Cardona-Timoner

12:30 Sponsored talks

"Nanosight and Evs: introducing the new NanoSight Pro"	Dr. Francisco J. López Jimenez [**IESMAT**]
"Colocalization of Biomarkers with the New Particle Metrix ZetaView© x30 Family"	Dr. Christina Klasen [**Solmeglas/Particlemetrix**]
"Quantitative analysis of single EV and their subpopulations with super-resolution solutions"	Mrs Dassine ZOUAOUI [**Abbelight**]

13:15 Lunch break

14:30 Session XI: Engineering EVs

Chairs: Pieter Vader [University Medical Center Utrecht (NLSEV)] and Michele Guescini [University of Urbino Carlo Bo (EVita)]

O59. "Optimization of EV-protein tagging strategies"	Diego Baranda Martínez-Abascal
O60. "Enhancing Immunomodulatory Potential of Immortalized Mesenchymal Stromal Cell-Derived Extracellular Vesicles through Overexpression CD39, key regulator of the purinergic adenosine signaling pathway"	Mohmed Elbeltagy
O61. "Mesenchymal stroma cell-derived extracellular vesicles show altered functionality after knock out of genes related to immunomodulatory capacity using CRISPR/Cas9"	Tobias Tertel
O62. "Therapeutic Extracellular Vesicles (TEVs) to deliver Cas9-sgRNA ribonucleoproteins for the treatment of recessive dystrophic epidermolysis bullosa."	María Iranzo Martínez
O63. "Improving Exosome Production and Immunogenicity for More Effective CAR T Cell Therapy"	Paula Heredia

15:30 Sponsored talks

"Sensitive and Quantitative Detection of EV Subpopulations Using Dedicated Nano-Flow Cytometry"	Ben Peacock [**Nanofcm**]
"FLEXible Flow solutions for Extracellular Vesicle Research"	Matthew B. Goff [**Beckman Coulter**]
"Identifying and characterizing EVs need vision and colour- Cytek have both! "	Michal Maj [**Cytek**]

16:30 Coffee break

17:15 **Session XII: Advances in EV methodology**

Chairs: Marija Holcar [Faculty of Medicine, University of Ljubljana (SIN-EV)] and Vendula Hlaváčková Pospíchalová [Faculty of Science, Masaryk University (CzeSEV)]

O64. "Efficient non-enzymatic isolation of brain-derived extracellular vesicles"	Andreu Matamoros-Anglès
O65. "Membrane Sensing Peptides: reversible affinity isolation of Extracellular Vesicles from minimally pre-treated biological fluids"	Roberto Frigerio
O66. "Affinity capture nanodiagnostic devices for EV biomarker detection"	Teresa Valero
O67. "Utilizing Lectins for Glycan Analysis on Extracellular Vesicles (EVs)"	Maria-Anthi Kakavoulia
O68. "Selective detection of biological nanoparticles labeled with gold nanoparticles in a heterogeneous sample"	Fredrik Eklund
O69. "Cell surface area as normalization factor to study prostate cancer EV targeting to specific cell types"	Marije Kuipers
O70. "The role of extracellular vesicles in COVID-19 infection disease progression"	Claudia Maria Radu

18:45 **GEIVEX and GSEV Annual assemblies**

21:00 **Simposium dinner @ Baños del Carmen**

Friday, October 27th

Time	Talk	Speaker
9:30	**Closing lecture** *Chair: Bernd Giebel [Institute for Transfusion Medicine, University Hospital Essen, Universtity of Dusburg- Essen (GSEV)]*	
	"Escaping the Endosome: Overcoming the Final Barrier for Effective Drug Delivery"	**Benjamin Winkeljann**
10:15	**Coffee Break**	
10:45	**Session XIII: MOVE fellows** *Chairs: Kerstin Menck [University of Muenster (GSEV)] and María Yáñez-Mó [CBM-SO/IIS-IP, Universidad Autónoma de Madrid (GEIVEX)]*	
	O71. "Evaluation of the role of CD133-Extracellular vesicles secreted by TNBC cells in promoting cancer aggressiveness"	Mireia Gomez-Duro
	O72. "Protein corona of EVs is essential for skin cell self organization and aids wound healing"	Martin Wolf
	O73. "Proteomic comparison between non-purified CSF and CSF-derived EVs from patients with Alzheimer's, Parkinson's and Lewy body dementia "	Yael Hirschberg
	O74. "Red Blood Cells-derived Extracellular Vesicles as targetable drug delivery vehicles"	Maria Chiara Ciferri
	O75. "Development of a protocol for the isolation and characterization of extracellular vesicles from Manila clam hemolymph"	Valentina Moccia
	O76. "Characterization of mRNA loading onto mammal extracellular vesicles by STORM Imaging"	Monica Guarro
	O77. "LC-MS/MS-based proteome profiling of CSF-derived extracellular vesicles in Alzheimer's disease"	Natalia Valle-Tamayo
	O78. "Functional and Potency Assays for Profiling Bioreactor Derived Mesenchymal Stromal Cell Extracellular Vesicles in Chronic Kidney Disease"	Sergio Garcia Garcia
	O79. "Determining the Function of Matrix Bound and Secreted Vesicles in Mineralisation"	Genevieve Anghileri
13:00	**Awards and closing remarks**	

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INTERNATIONAL SOCIETY for EXTRACELLULAR VESICLES

Oral Communications

Functional characterization of a hypothetical spleen-dependent P. vivax gene and the role of EVs in spleen-parasite interactions

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Plasmodium vivax can be considered an asymptomatic chronic infection as up-to 90% of cases are only detected by PCR. Noticeably, the spleen has recently emerged as a cryptic erythrocytic niche in natural infections where the largest parasite biomass is hidden. Moreover, we have identified parasite genes whose expression is dependent on an intact spleen. In addition, circulating extracellular vesicles (EVs) from natural infections contain parasite proteins acting as intercellular communicators that facilitate cytoadherence of P. vivax-infected reticulocytes to human spleen fibroblasts (hSF).

The aim of this study is to functionally characterize P. vivax spleen-dependent genes and to determine the role of EVs in spleen-parasite interactions.

Due to the lack of in vitro culture, we are using CRISPR-Cas9 to knock-in P. vivax genes into P. falciparum. We have generated a stable clonal transgenic line expressing a hypothetical spleen-dependent gene, as determined by genomic integration, RT-PCR and western blot and confocal microscopy determined its surface localization. Moreover, functional assays demonstrated that EVs from P. vivax patients, purified either by size exclusion chromatography or direct immunocapture, facilitated the binding of this transgenic line to hSF. Last, we performed RT-qPCR against known important adhesins during malaria infection and observed how PvEVs stimulation led to upregulation of investigated adhesins. Finally, single-cell RNAseq of stimulated hSFs confirmed the upregulation of certain adhesins.

In conclusion, we have implemented CRISPR-Cas9 to knock-in P. vivax genes into P. falciparum. Such approach allowed the characterization of a hypothetical spleen-dependent coding gene, which we named P. vivax spleen-dependent protein 1 (PvSDP1). Binding of PvSPD1-infected RBC to hSF was facilitated by EVs obtained from plasma of infected patients exclusively. Similar approaches are being pursued to study other spleen-dependent genes and we are further investigating the role of EVs for formation of cryptic infections, a major challenge towards elimination of this human parasite.

Proteomic profiling of plasma-derived extracellular vesicles in patients with visceral leishmaniasis: a biomarker discovery.

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Extracellular vesicles (EVs) in plasma have gained significant interest for their diverse functions in physiology and pathology, particularly in infectious diseases like leishmaniasis. Limited understanding of host-pathogen EV interactions during infection motivates research in this area, especially with the re-emergence of visceral leishmaniasis in the Mediterranean basin. The intricate cargo of EVs, with their unique molecular pattern, holds potential as biomarkers for diagnostics and prognostics. Currently, there are no validated biomarkers to assess clinical efficacy of immunocompetent individuals with visceral leishmaniasis, emphasising the need for further exploration of EV protein cargo in this context.

Plasma-derived EVs from clinical samples of active and cured patients at 1, 3 and 6 months were isolated by combining the methods of size-exclusion chromatography and ultracentrifugation. The size, concentration, and morphological integrity of EVs were assessed using nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and BCA assay. These methods confirmed successful isolation of exosomes within the expected size range (100-200 nm) with the appropriate morphology. Furthermore, western blot analysis was performed to verify differential expression of both EV protein markers CD63, CD81 and TSG101.

Tandem mass tags (TMT)-labelled coupled with liquid chromatography mass spectrometry (LC-MS/MS) was used to identify differently enriched proteins in the content of the patient's EVs. In total, 480 proteins were quantified, and a high variability of protein expression was observed between groups of patients. According to a primary bioinformatic analysis of total protein levels in active and cured patients, without distinguishing between curing times, 57 proteins were downregulated, and 66 proteins were upregulated.

The quantitative proteomics study identified possible biomarkers that differentiate between active and cured states of the disease. The identified biomarkers not only offer insights into the patient's immune status but also shed light on the underlying mechanisms associated with an effective response to treatment.

Are Extracellular Vesicles from trematodes sufficient to cause fibrosis in liver? Evaluation of the effects of EVs in vitro and in vivo.

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The trematodes Fasciola hepatica and Dicrocoelium dendriticum can co-exist in the liver and bile ducts of their mammalian hosts, including humans. Most pathological effects of these parasites are associated with liver fibrosis resulting from the migration of the flukes, their excretory/secretory products (ESP) and local inflammation. The manipulation of the host response primarily occurs through the release of ESP at the host-parasite interface, including extracellular vesicles (EVs). These EVs may have effects on different immune cells, as well as liver cells.

Objective: Analyze the effects of EVs released by F. hepatica (FhEVs) and D. dendriticum (DdEVs) in vitro, on hepatic stellate cells (HSC) and hepatocytes, and in vivo, in mouse livers.

Methods: Parasites obtained from local abattoirs were cultured for 4h. EVs were purified by differential centrifugation and Size Exclusion Chromatography (SEC), and characterized by NTA, TEM and immunogold labeling. LX-2 and HepG2 cells were treated with EVs from both parasites and transwell assays were performed at different times. Cells were analyzed through Label-free quantitative proteomics and cell responses were monitored by MTT, RT-qPCR, ELISA and flow cytometry. For in vivo assays, EVs were administered subcutaneously to C57BL6 mice, and the, expression of liver markers was assessed by RT-qPCR and Syrius red staining.

Results: Cultured hepatic cells exhibited different responses to DdEVs and FhEVs in terms of cell proliferation and wound healing assays, and enhanced HSC activation by different pathways. FhEVs stimulate extracellular matrix proteins (ECM) release and regulatory cytokines, while DdEVs influence the expression of distinct ECM proteins and NF-κβ. Although mice livers did not display evident fibrosis upon short-term exposure to EVs, significant alterations in mRNAs levels of fibrogenic proteins were observed, which became more pronounced with prolonged exposure.

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Multiparametric study to optimize EV production for immunomodulatory potential

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Extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) have immunomodulatory potential and demonstrated similar therapeutic effect as cell therapies. However, many factors have an influence on EVs therapeutical activity, such as the cellular source, the conditions of production, the state of the producing cell or the EV isolation process. This project targets the optimization of the whole EV manufacturing process to establish a link between process and immunomodulation properties.

To do this, we use an innovative method of sequential EV production based on shear stress, and compare it against classical starvation process. Moreover, we compared adipocyte-derived and umbilical cord-derived MSCs, and three isolation processes, ultracentrifugation (UC), tangential flow-filtration (TFF) and size exclusion chromatography (SEC). Global EV characterization was realized by size and concentration measures, and EV identity. To assess the immunomodulation activity, we use in vitro potency assays with THP-1 monocytes and macrophages or peripherical blood mononuclear cells (PBMCs) assessing the expression of NF-κB and IRF pathways, combined with pro-inflammatory cytokines dosage, cargo analysis through multiplexed protein dosages and miRNA-sequencing.

Results show a dose-dependent and process-dependent immunomodulatory effect of EVs in our in vitro potency assays. Notably, sequential EV production allows for massive EV production while maintaining their cargo and their immunomodulatory effect. EV isolation by UC and SEC decreased immunomodulatory potential and the concentration of key anti-inflammatory proteins such as TGF-β, whereas isolation by TFF maintained EV potency. In-depth protein cargo dosage showed difference between EVs from different cell sources. Further work will be focused on clarifying the impact of each parameters on the immunomodulatory effect.

The work highlights the impact of process on active biomolecules located inside or outside EVs and on immunomodulatory potential, and specifically outlines the importance of selective EV manufacturing process for therapeutical applications.

Intercellular communication strategies of human polyomaviruses: The role of extracellular vesicles in Merkel cell polyomavirus pathogenesis.

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Extracellular vesicles (EVs) have gained interest as intercellular communicators in various diseases, including cancer. In cancer progression, EVs play a significant role in interactions within the tumor microenvironment (TME). However, the functions of EVs in the pathogenesis of Merkel cell carcinoma (MCC) and concerning modifications of the TME remain elusive. Individual publications mention miRNA-375 as EV cargo, which is captured via EVs in fibroblasts and possibly activates the TME. Our group showed that in MCC tumors which are virally induced by MCPyV, the viral oncoprotein sT is crucial for tumor progression by altering the TME. Here, we conduct a comprehensive description and analysis of EV classes e.g., their protein and nucleic acid cargos from patient-derived virus-positive MCC cell lines expressing or not expressing sT.

We employed high-throughput sequencing of RNA cargos, imaging flow cytometry (IFCM) and proteomics to characterize EVs from MCPyV-positive MCC cells to analyze their role in MCPyV pathogenesis.

For this, EVs from MCC wt cells and MCC cells in which sT was downregulated by shRNA expression were isolated via ultracentrifugation. Nanosight, IFCM and Cryo-EM were used for phenotypical analysis. Content and composition of the EVs were determined by RNA-Seq and mass spectrometry.

HTS of RNAs together with proteome analysis suggest that EV cargos do not simply reflect the parental cell composition. Notably, most highly expressed proteins, transcripts and miRNAs from the parental cells were not detected in EVs. Interestingly, fragmented and intron-containing mRNAs were highly abundant in MCC-derived EVs. Proteome analysis shows an enrichment of histones, particularly in cells expressing sT protein. Pathway analysis of miRNA cargos revealed an enrichment of EV-miRNAs with predicted functions in TME regulation.

Based on this comprehensive characterization of MCC EVs, current experiments address the qualitative differences of EVs in MCC progression, especially in angiogenesis induction and activation of TME resident macrophages.

Impact of Klebsiella pneumoniae outer membrane vesicles on antibiotic resistance

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Klebsiella pneumoniae (Kp) is a pathogenic bacterium that can cause pneumonia. The emergence of hypervirulent and multidrug-resistant strains has made Kp one of the antibiotic-resistant pathogens prioritized by the WHO. It is essential to understand their mechanisms of pathogenicity. Our study investigates the role of outer membrane vesicles (OMVs), which are nano-sized structures consisting of a lipid bilayer, in the interplay between antibiotics and bacteria. Specifically, we analyze the impact of stress conditions on OMV release and their effect on antibiotic resistance in Kp.

Our results indicate that Kp releases OMVs of varying size and amount under different stress conditions. We show that exposure to polymyxin B (PB) leads to significant changes in the vesicle composition but is not associated with an upregulation of PB resistance genes. Lipidomics analyses reveal that PB alters the

composition of OMVs but not of the source bacteria. Importantly, we demonstrate that OMVs protect Kp from PB in a dose-dependent manner and reduce antibiotic stress response confirmed by proteomics. We show that OMVs also confer protection against PB in E.coli, Salmonella enterica, and Pseudomonas aeruginosa. We generated artificial vesicles that validate this. OMVs were also protective against the polymyxin colistin, but not against the carbapenem meropenem or the aminoglycoside gentamicin. Finally, we show that OMVs protect Kp against PB in ex vivo and in vivo infection models.

In summary, our study highlights the critical role of OMVs in promoting antibiotic resistance in Kp and other antibiotic-resistance priority pathogens. Our data suggest that OMVs might promote bacterial replication and subsequent spreading in the host when the infection is treated with polymyxins.

SARS-CoV-2 infection during pregnancy alters profile of placenta-derived extracellular vesicles indicating placental dysregulation

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Introduction: During pregnancy the placenta ensures the adequate supply of oxygen, nutrients and immunological factors to the fetus. Stressors, such as infections or maternal obesity, can lead to impairment of placental transfer function and subsequently risk the healthy development of the unborn child. However, we are still lacking possibilities for the detection of placental dysregulation during ongoing pregnancy - which to date is primary based on ultrasound assessments. In this context, placenta-derived extracellular vesicles (EV) might reflect the placental function and bear potential as prognostic and diagnostic markers. We aim to investigate the profile of placenta-derived EVs in healthy pregnancies and unearth alterations upon SARS-CoV-2 infection.

Methods: EVs were isolated via cushion ultracentrifugation from serum samples of third trimester healthy (n=40) and SARS-CoV-2 infected (n=25) pregnant women. Nanoparticle-Tracking Analysis and Transmission Electron Microscopy were performed to validate the isolation. Imaging Flow Cytometry was applied to analyze the surface protein profiles using antibodies against CD81, CD63 and CD9 as well as PLAP (placental-alkaline phosphatase) and CD34 as placental-endothelial markers. Subsequently, placenta-derived EVs were enriched and proteomic analysis was performed by Liquid-chromatography coupled Tandem-Mass-Spectrometry. Lastly, clinical parameters were integrated for correlations.

Results: In healthy pregnancies, levels of placenta-derived EV are influenced by the number of previous pregnancies, but are neither correlated with the placental volume nor the blood flow to the placenta. Infection with SARS-CoV-2 decreased overall EV levels, but increased the frequency of placenta-derived EVs. Additionally, the levels of CD81+ EVs were increased upon infection and positively correlate with the placenta-derived EV. Proteomic analysis revealed a downregulation, especially of RNA-associated genes and upregulation of genes associated with the immune response after SARS-CoV-2 infection, which parallels reduced placental transfer rates.

Conclusion: Placental EV profiles mirror alterations in the placental functionality and serve as markers for placental stress and dysregulation after SARS-CoV-2 infection.

RNA profiles of plasma extracellular vesicles in patients with sepsis

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Background: Sepsis is an organ dysfunction caused by a dysregulated host response to an infection. A complex pathophysiology of different pathogens reveal a need of diagnostic markers to distinguish between sepsis types which appear clinically alike. Extracellular vesicles (EV)s are suggested to play a role in sepsis and might be a potential source of biomarkers which rapidly could determine characteristics of a pathogen and be of benefit in sepsis management. The aim was to characterize and compare the RNA content in EVs from patients with meningococcal sepsis, meningococcal meningitis and pneumococcal sepsis.

Methods: Plasma EVs in samples taken on submission to hospital, were isolated from patients with meningococcal sepsis (n=6), meningococcal meningitis (n=6), pneumococcal sepsis (n=6) and healthy controls (n=3) by size-exclusion chromatography. EVs were quantified and further characterized by nanoparticle-tracking analysis, flow cytometry, western blotting and transmission electron microscopy. EV-RNA was isolated using ExoRNeasy Midi Kit and further analyzed for RNA subtypes by Affymetrix microarray HTA 2.0. Data was analyzed with Partek Genomics Suite and Ingenuity Pathway Analysis (IPA).

Results: The microarray analysis detected 14909 transcripts where the most abundant were mRNA, lncRNA, long intergenic non-coding RNA (lincRNA), Havana Chromosome and piRNA. Compared to the control group, 5927 (meningococcal sepsis), 390 (meningococcal meningitis) and 2341 (pneumococcal sepsis) transcripts were detected with significantly different levels. From these IPA predicted, from the mRNA levels, variable effects on biofunctions and canonical pathways showing overall distinct upregulation of immune and inflammatory related functions in the meningococcal groups while downregulation in the pneumococcal group.

Conclusion: EVs and EV RNA were successfully isolated from patient plasma. Significant differences in biofunctions and canonical pathways were observed in patients with meningococcal sepsis, meningococcal meningitis or pneumococcal sepsis. These new observations can potentially contribute to discovery of novel biomarkers to distinguish between sepsis types.

Colorectal cancer EVs alter the gut microbiome

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Background: the human gut microbiota plays a vital role in the regulation of various physiological processes, and alterations in the composition and function of the system (dysbiosis) are associated with the pathogenesis of colorectal cancer (CRC) 1. Although the causative link between CRC and microbiota is widely investigated, the underlying microbiota-gut interactions are not well understood yet. It is evident that CRC-derived extracellular vesicles (EVs) have an impact on various oncogenesis processes 2, however, their impact on the surrounding microbiota is not clear. Therefore, we hypothesise that EVs could have an impact on the microbiota and contribute to dysbiosis. Methods: two CRC cell lines (SW480, SW620) were cultured in CELLine AD 1000 bioreactor flasks, and blood was collected from CRC patients and healthy individuals. EVs were isolated from the culture media and blood plasma by size-exclusion chromatography and characterised by nanoparticle flow cytometry (NanoFCM), western blotting, ELISA, and transmission electron microscopy (TEM). The impact of the EVs on the bacterial (MG1655 E. coli (Laboratory strain) and 11G5 E. coli (CRC-associated strain)) phenotypic characteristics (growth curve, biofilm formation) was assessed. Flow cytometry, confocal microscopy, and TEM were performed to assess the interactions between EVs and E. coli strains. Results: NanoFCM analysis showed a high yield of EVs with characteristic size profiles, and EVs markers detection confirmed the presence of EVs. TEM analysis indicated an interaction between the EVs and E. coli with clear surface binding, and EV treatment had an impact on bacterial phenotypic characteristics; an increase in E. coli growth and a decrease in the ability of the bacteria to form biofilm were shown. Overall, EVs appeared to be capable of mediating CRC-microbiome interactions.

Amniotic fluid stem cells-derived extracellular vesicles reprogram dendritic cell functions in autoimmune neuroinflammation

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Introduction
Dendritic cells (DCs) are potent antigen-presenting cells that control adaptive immunity and balance effector and regulatory components of the immune response. Amniotic fluid stem cells-derived extracellular vesicles (HAFSC-EVs) are characterized by important immunoregulatory properties. In this study we focus on the potential of HAFSC-EVs to promote tolerogenic effects on specific subsets of DCs in preclinical model of multiple sclerosis (MS).
Methods
HAFSC-EVs were isolated by ultracentrifugation and characterized by scanning electron microscopy, nanoparticle tracking analysis and western blot for size, number and the expression of specific markers. Lipidomic, proteomic and miRNA analysis were used to completely characterize EVs. Murine DCs and T cells were isolated from bone marrow and spleen. Human peripheral blood mononuclear cells were isolated from peripheral blood by Ficoll-Paque. Confocal microscopy and cytofluorimetric analysis were used to evaluate EV uptake to cells. Experimental autoimmune encephalomyelitis (EAE) model was induced in C57BL/6 female mice immunized with MOG35–55 peptide.
Results
We found that HAFSC-EVs are preferentially internalized by murine and human conventional dendritic cell type 2 (cDC2), but not by other cDCs and their uptake on cDC2 is mediated by the cell surface receptor ITGβ1. Analysis of protein and miRNA cargo revealed the enrichment of several immunoregulatory pathways in HAFSC-EVs. Immunogenic cDC2 conditioned with HAFSC-EVs acquired strong tolerogenic functions. HAFSC-EVs are able to suppress pro-inflammatory cytokines IL-6, IL-12 and TNF-a by cDC2 activated with LPS. Transfer of cDC2 conditioned with HAFSC-EVs in vivo resulted in suppression of autoimmune responses and significant improvement in the clinical score of EAE.
Summary/Conclusion
These data demonstrate that HAFSC-EVs contribute to reprogram inflammatory cDC2 to tolerogenic functions, leading to the control of autoimmune responses.

Study of the anti-inflammatory effects of industrial-produced lemon nanovesicles on inflammatory bowel disease

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Inflammatory bowel diseases (IBD), whose most prominent clinical manifestations are Crohn's disease and ulcerative colitis, are phlogistic disorders of the gastrointestinal tract, for which there is still no definitive cure. Conventional therapies are often not useful in the treatment of IBD and are associated with adverse side effects. Considering the inefficiency of treatment and the incidence of IBD, which is dramatically increasing globally, the goal today is to prevent the disease. In this context, the study and use of plant-derived extracellular vesicles received great attention among the scientific community, and it is thought that they have the potential to be a good preventive approach to many inflammatory diseases.

Here, we aim to highlight the biological properties of nanovesicles from Citrus limon isolated on industrial scale (iLNVs). In particular, to evaluate whether treatment with iLNVs can counteract the symptomatology of IBD, we tested the iLNVs in vitro on human THP1-M0 macrophages stimulated with LPS, and in vivo on a model of DNBS-induced colitis in rats.

Our results show that pre-treatment with iLNVs, significantly reduces the expression levels of pro-inflammatory cytokines and increases the levels of anti-inflammatory ones on THP1-M0 cells. In addition, in vivo findings show that, compared with control cases, pretreatment with iLNVs results in lower macroscopic inflammatory scores associated with severe colitis. In addition, histological analysis reveals that iLNVs restrict disruption of colon architecture, which is typical in animals affected by colitis.

Considering the promising results, further studies will be carried out to evaluate the molecular basis underlying the observed effects.

Inflammatory modulation through proteolytic active extracellular vesicles and bacteriomimetic vesicles

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Inflammatory modulation through proteolytic active extracellular vesicles and bacteriomimetic vesicles

Extracellular proteolysis and extracellular vesicles (EVs) are fundamental processes in short- and long-distance communication in the body. Key players, releasing the soluble domains of membrane-bound ligands and receptors such as TNF and TNFR through so-called shedding, belong to the family of `a disintegrin and metalloproteinases` (ADAMs). These events have been thought to occur mostly at the same membrane compartment (“in cis”). However, we could show that ADAMs released on exomes are proteolytic active and capable of proteolysis in trans. Cargos and interacting molecules, such as matrix metalloproteinases (MMPs), may further account for their tissue-destructive capacity as seen in patient samples of periodontal disease and Porphyromonas gingivalis infection, respectively. Furthermore, these exosomes are transferred to the circulation upon pulmonary infection (bacterial and Sars-Cov2, respectively) and release by leukocytes. This long-range communication may account for systemic side-effects as seen during the Covid19 pandemic.
EVs are not only released by eukaryotic cells, but also by probiotic bacteria such as Lactobacillus. Although their beneficial effects in tissue regeneration have been shown in various studies, their therapeutic use as living materials is especially limited in immunocompromised patients. Therefore, we developed a hydrogel based on membrane vesicles (MVs) produced by Lactobacilli to circumvent these shortcomings. In in vitro models, we observed an increase in cell migration and anti-inflammatory effects of the MV-loaded hydrogels dependent on the bacterial culture condition correlating with the proteomic repertoire. In vivo, MV-loaded hydrogels were able to improve wound healing and to reduce scar formation.
Our results create a solid basis for the future targeting and application of eukaryotic and probiotic-derived vesicles in the treatment of inflammatory dispositions and stimulates the initiation of further preclinical trials.

Tetraspanins as an immunogenic adjuvant in lipid nanovesicles

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Climate change and loss of biodiversity are two of the most important consequences of human activities that are contributing to accelerate the appearance of new infectious agents. Vaccines can elicit a prophylactic or therapeutic immune response and are the best way to prevent and treat infectious diseases. Numerous vaccines have been approved and are currently in use worldwide, but there are still numerous pathogens against which no protective vaccine has been developed. For example, to date there is no effective vaccine against diseases caused by protozoa in humans.

We have developed a new vaccine platform that combines liposome technology with the properties of natural exosomes: a lipid-base nanovesicle whose surface is decorated with recombinant tetraspanins and that carries recombinant immunogenic proteins from any pathogen against which we want to induce protection.

Our proof of concept was carried out with recombinant proteins KMP-11, from Leishmania, and Spike, from SARs-CoV-2 virus. Nanovesicles were injected intraperitoneally in mice and showed to produce a strong IgG response as detected in the sera of immunized mice. This response was quantitatively higher than that produced by soluble pathogen recombinant proteins or nanovesicles carrying the pathogen protein without the tetraspanin. This adjuvant effect suggests that our patented vaccine platform could be easily adapted to prevent and combat new infectious diseases.

PLANT EDIBLE EVs MODULATES THE INFLAMMATORY RESPONSE IN AN ACUTE PANCREATITIS MODEL

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Acute pancreatitis (AP) is an acute inflammatory process of the pancreatic gland that may lead to local and systemic complications. Cytokines and oxidative stress play a role in the early pathophysiological events of the disease [1]. Previous studies have shown the anti-inflammatory and antioxidant properties of extracellular vesicles isolated from pomegranate juice (PgEVs) [2]. The aim of this work was to assess the role of PgEVs in the modulation of the inflammatory response and oxidative stress in AP.

AP was induced by 3 intraperitoneal injections of L-arginine (3 g/kg bw, administered at hourly intervals). Animals were sacrificed at 72 h after last injection. A group of mice with AP was treated with PgEVs administered as subcutaneous injections 2 hours before the first L-arginine injection.

The histopathological study showed that the inflammatory process and tissue edema were reduced with the PgEVs treatment. The increase in pancreatic lipase activity was lower with PgEVs administration. Furthermore, increased phospho p65 and the expression of the pro-inflammatory cytokine Il6 were reduced in mice treated with PgEVs after AP induction. Redox pairs, such as oxidized glutathione/reduced glutathione, and homocystine/homocysteine were diminished by the PgEVs treatment

Our results show the anti-inflammatory and antioxidants properties of the PgEVs in acute pancreatitis, by avoiding the activation of p-p65/IL-6 pathway and recovering the redox thiol status.

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2. doi: 10.1039/d2fo01806c.

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INTERORGAN CROSSTALK THROUGH EVs AND THEIR IMPLICATION IN OBESITY: FUNCTIONAL ROLE AND CARGO OF BIOMARKERS

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Obesity is a leading global multi-disease that continues to increase exponentially; therefore, it is urgent to characterize new molecular mechanisms involved, as well as to identify early biomarkers of risk for comorbidities, which will also allow the classification of patients into different phenotypes to establish personalized therapy. We believe that EVs secreted by metabolic organs and their comprising cells such as obese hypertrophied adipocytes, hepatocytes under fatty liver disease, inflamed adipose tissue (AT) resident-macrophages, inactive brown adipose tissue, and sedentary muscle, are establishing a dynamic dialog among each other, and all together, with the CNS by crossing the blood-brain barrier, exacerbating the deleterious events linked to this pathology. Moreover, all these obesity-EVs may also reflect treatment interventions such as exercise, diet, and bariatric surgery, being able to participate on reverting deleterious alterations. Once we have established several functional roles of obese AT shed EVs (ADIPOsomes) promoting hypertrophy and insulin resistance of healthy adipocytes, as well as inflammation of macrophages, we show here the crosstalk of obese adiposomes with healthy hepatocytes. Moreover, we illustrate the characterization of lipid hypertrophied hepatocytes secreted EVs (NAFLDsomes) and their functional effect upon the interaction with healthy hepatocyte cells. Finally, we describe the proteome of brown adipose cells EVs (BATosomes) upon thermogenesis activation, a process of great interest as it beneficially dissipates energy through uncoupled respiration and heat production, becoming great promise for the treatment of obesity. The capacity of thermogenic active BAT-EVs to promote thermogenesis in non-activated brown adipocytes is also explored. As conclusion, we show here the functional crosstalk among altered metabolic tissues through EVs, identifying also potential biomarkers of disease with application to the clinical practice.

Adipocyte-derived extracellular vesicles are endocrine regulators of insulin secretion in mice

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Background and Aims: In the early pre-diabetic stages of type 2 diabetes, β-cells respond to insulin resistance by increasing insulin secretion to decrease the elevated plasma glucose levels. Various insulinotropic signaling mediators like glucose, fatty acids and metabolic hormones inform the β-cells about this metabolic demand. Here, we add white adipocyte-derived extracellular vesicles (AdEVs) to this list of stimulants for insulin secretion.

Methods: AdEVs were isolated from lean and obese mice, and fully characterized for their yields, structural morphology and protein cargo. Their systemic biodistribution, cellular uptake and signaling function were characterized by fluorescent tracking or SILAC labelling followed by (phospho-)proteomic analyses. Last, their functional impact on glucose-stimulated insulin secretion (GSIS) and glucose tolerance was tested in vitro in MIN6 cells and isolated murine islets and in vivo in mice. The presence of relevant AdEV cargo was compared in human AdEVs and EVs isolated from the human stromal vascular fraction.

Results: AdEVs from obese mice significantly enhanced GSIS in cells and mice. Stimulation of GSIS was attributed to the AdEV-mediated and β-cell-directed transfer of distinct proteins that perturbed protein and phosphosite dynamics and increased total protein abundances that feed into insulinotropic pathways linked with the metabolic amplification of GSIS and insulin granule docking. Ultimately, AdEV exposure promoted first phase insulin secretion and lowered glucose excursions in normoglycemic mice after a glucose bolus.

Conclusion: In the early stages of prediabetes, AdEVs deliver insulinotropic protein cargo to β-cells, thereby priming them toward a hypersensitive state to ultimately maintain normal glucose levels by increasing insulin secretion.

Microvesicles display opposite coagulolytic balance according to their cellular origin and activation status

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Background
Microvesicles (MVs) display not only procoagulant but also profibrinolytic properties. Therefore, their effects on hemostasis may result from an equilibrium between these two activities that can be termed as “coagulolytic balance”. However, the variations of this MV coagulolytic balance have not been investigated in different subpopulations of MVs based on their cellular origin and the activation status of their parental cells.
Purpose
Investigate the coagulolytic balance and functional consequences of the main circulating subsets of MVs.
Methods
MVs from neutrophil, monocyte, platelet, and endothelial cell origin were generated either in vitro from both unstimulated and stimulated purified human primary cells or directly isolated from the human plasma of healthy donors or septic patients by immunomagnetic separation. MV-dependent procoagulant and profibrinolytic activities were assessed by a panel of functional assays such as Factor Xa, Thrombin, and Plasmin generation assays. MV-induced clot structure was investigated by scanning electron microscopy and fibrinography.
Results
Purified MVs from stimulated monocyte and endothelial cells showed high procoagulant activity as evidenced

by tissue factor-dependent FXa (22±2.64 and 17.33±1.33 mUDO/min, p=0.0018 and p=0.02 respectively) and thrombin generation (106.7±13.5 and 55±13.79 thrombin peak nM, p=0.0007 and p=0.36, respectively). Clots induced by the procoagulant MVs were denser and displayed more clot stability and resistance to fibrinolysis as demonstrated by longer lysis time (p=0.0004 and p=0.04) as shown by the scanning electron microscopy and fibrinography. Additionally, monocyte and endothelial MVs isolated directly from plasma from patients with sepsis also showed higher FXa generation. In contrast, only MVs of neutrophil origin displayed a profibrinolytic profile exhibiting significantly high plasmin generation capacity both in vitro (p=0.0001) and in sepsis plasma (p=0.0002).

Conclusion

We showed the different circulating MV subsets display opposite coagulolytic balances according to their cellular origin and activation status which may impact hemostatic imbalance in thrombotic conditions like sepsis.

Extracellular lipid vesicles induce Ca2+ signals in target cells

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Due to their involvement in a wide range of pathologies and the highly specific transported cargo, extracellular vesicles (EVs) gained tremendous attention in the field of intercellular signaling. Most EV studies focus on the long-term effects on target cells, that occur after successful endocytosis. However, the acute events preceding the internalization of the vesicles, i.e. the initial interactions with the plasma membrane, are likely to be sensed by specialized receptors on the cell surface. As a low-cost, easy production, and customizable alternative for EVs, synthetic lipid vesicles (SLVs) are good candidates for the study of fundamental interactions between EVs and recipient cells. In this study, we hypothesized that extracellular lipid vesicles activate mechano-sensory channels upon their interaction with the plasma membrane of recipient cells. To test this, we determined the effects of natural EVs and SLVs on TRPA1, a Transient Receptor Potential cation channel known to be activated by mechanical perturbations in the plasma membrane. We used Fura-2-based fluorescence imaging to assess possible changes in intracellular Ca2+ concentrations triggered by lipid vesicles. We found that acute (

Genome-Wide-CRISPR/Cas9 screening identifies the COMMANDER recycling complex as a key player in EV cargo delivery

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The outstanding potential of Extracellular Vesicles (EVs) in medicine, deserves a detailed study of the molecular aspects regulating their incorporation into target cells.
We have performed a Genome Wide CRISPR screen by flow cytometry sorting, to identify possible molecular candidates that may regulate EV uptake.
We have used a GWC cell library in K562 cells, which includes 10 sgRNAs for each gene of the whole human genome, plus negative controls, summing up a total of 250,000 individual sgRNAs. Each cell has inserted in its genome a single sgRNA and, thus, has a single gene deletion. Total EVs were isolated from SKMEL147 human melanoma cell line by ultracentrifugation at 100,000g. EVs were characterized by NTA, Electron Microscopy, Western blot and confocal microscopy, and stained with Alexa633-C5-Maleimide, a fluorescent compound able to covalently bind to sulfhydryl residues present in surface proteins. For each assay 500x106 cells and 3.6x1012 of stained EVs were used in order to have a 2000x coverage of the library.
After two hours of EV incubation with cells, the cell culture was washed and analysed with a flow cytometer for cellular sorting. 5% high and low A633 fluorescence populations were sorted.
Total genomic DNA was obtained, and sgRNAs sequences amplified, adding Illumina adaptors as overhangs by PCR. NGS Illumina sequencing was made to quantify the enrichment of the sgRNAs within the sorted populations compared to the unsorted control. According to the results of the assay several members of the COMMANDER complex seem to be of importance for EV uptake, appearing as significant hits in our assay. Quantitative EV uptake assays previously developed in the laboratory, based on Luciferase or in Maleimide, were used to validate the hits found in the screen in KO cell lines for the different components of the COMMANDER complex.

Unravelling molecular drivers of extracellular vesicle-mediated cargo transfer

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Extracellular vesicles (EVs) are thought to mediate intercellular communication by transferring bioactive cargo from donor to acceptor cells. The EV content delivery process within acceptor cells, however, is still poorly characterized and the molecular players at the donor EV/acceptor cell interface have not been elucidated.
Here, we set out to identify critical surface proteins on EVs that mediate functional cargo transfer in recipient cells and understand how these proteins affect different steps of the delivery process. First, we mapped the surfaceome of isolated EVs by coupling high purification steps (size exclusion chromatography followed by density gradient ultracentrifugation) and mass spectrometry. We identified approximately 200 proteins that decorate the membrane of MDA-MB-231 and hTERT-MSC-derived EVs. Next, we performed a siRNA-based screen targeting the aforementioned genes to evaluate their involvement in EV-mediated (RNA) cargo transfer in a co-culture system. We are now validating the screen hits, via chemical or antibody-based inhibition, and evaluating their involvement in the EV-content delivery process with in-house designed assays. Among the surface proteins identified via mass spectrometry, tetraspanins CD63 and CD9 have been previously implicated in EV-mediated cargo transfer. However, using two different reporter assays (CROSS-FIRE and Nanoluciferase assay) we found that both CD63 and CD9 are dispensable for EV-mediated cargo transfer, both on the donor EV's and on the acceptor cell's side. Furthermore, additional data similarly supports dispensability for CD81 in this process.
In conclusion, we have identified EV surface proteins and, through a RNA interference-based screen, we discovered both potential novel regulators, currently under validation, and non-regulators of the EV cargo transfer process. This study has the potential to answer outstanding questions in EV biology on the mechanisms of EV-mediated content delivery, as well as advance the drug delivery field by providing native cargo delivery enhancers for testing in therapeutic formulations.

A role for integrin beta 1 in extracellular vesicle-mediated functional RNA delivery

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Introduction
While uptake mechanisms for extracellular vesicles (EVs) have been widely explored, the underlying molecular mechanisms through which EVs are able to provide functional RNA transfer to recipient cells are not understood. Here, we demonstrate a role for integrin beta 1 (ITGB1) on recipient cells in EV-mediated uptake and RNA delivery.

Methods
To study RNA transfer we used a fluorescent stoplight reporter system, which is activated by Cas9 upon functional sgRNA delivery (De Jong et al., Nat Commun. 2020). HEK293T reporter cells were transfected with ITGB1 siRNA or non-coding siRNA as a control. EVs from MDA-MB-231 cells expressing a targeting sgRNA were isolated by tangential flow filtration followed by size exclusion chromatography and were then added to reporter cells. Functional RNA transfer was quantified by flow cytometry. For uptake experiments, EVs were labelled using MemGlow640. Additionally, intracellular EV trafficking was tracked in live cells using a Nanoimager-S (ONI). A role for different potential ITGB1 binding partners on EVs was screened using blocking antibodies or peptides.

Results
ITGB1 mRNA knockdown efficiency in recipient cells was ~ 85%. In cells transfected with ITGB1 siRNA, functional RNA delivery was strongly reduced. This was observed both in a co culture setting and by adding isolated EVs. Complementarily, knockdown of ITGB1 or ITGB1-blockage using neutralising antibodies resulted in a significant decrease in EV uptake in recipient cells. Moreover, using live imaging, we observed localisation of MDA-MB-231 EVs with ITGB1 and their trafficking together inside recipient cells. Initial screening for ITGB1 binding partners showed that blocking fibronectin resulted in a decrease in RNA transfer.

Conclusions
MDA-MB-231-EV uptake and EV-mediated functional RNA transfer into HEK293T recipient cells is mediated by ITGB1. In-depth understanding of the molecular mechanisms underlying EV-mediated RNA transfer could contribute to achieving more efficacious EV-mediated RNA delivery and improved RNA therapeutics.

siRNA screening using a novel rapid radioactive-based assay reveals a role for SNAP29 in exosome release

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Extracellular vesicles (EVs) have been implicated in many pathophysiological conditions. Therefore, strategies that can affect EV release are of biological interest and may also have important clinical implications. Nevertheless, the molecular mechanisms responsible for EV secretion are not completely understood.
To investigate the molecular machinery required for the release of small EVs (

Exploring the Versatile Role of LAMP2A: Insights into Exosomal Cargo Loading and Endosomal Identity in Inter-Cellular Communication

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Exosomes, a type of extracellular vesicle derived from endosomes, play a critical role in intercellular communication by transferring lipids, RNAs, and proteins between cells, tissues, and organs. Our recent study focuses on elucidating the mechanism of exosomal LAMP2A loading of cargo (e-LLoC). We demonstrated that proteins containing a KFERQ pentapeptide are selectively loaded into a specific subset of exosomes, in a process that depends on the membrane protein LAMP2A. The e-LLoC sorting system primarily operates in early endosomes, facilitating the loading of proteins into intraluminal vesicles at the endosome limiting membrane. Surprisingly, this loading process does not rely on components of the ESCRT machinery like TSG101 and VPS4B but rather involves ESCRT-independent machinery such as CD63, PDCD6IP/ALIX, SDCBP/syntenin-1, RAB31, and the lipid ceramide. Our data also reveals an asymmetric distribution of endosomal machinery, where LAMP2A-enriched endosomes preferentially contain non-ESCRT machinery, while LAMP2A-depleted endosomes exhibit a preference for ESCRT machinery. Mass spectrometry analysis of exosomes, early endosomes, and late endosomes further supports the potential involvement of LAMP2A in endosomal organization. We found that LAMP2A knockout may lead to alterations in endosomal contents, affecting factors such as plasma membrane fusion, cortical actin association, RAB27A and PIP(4,5) mediated membrane tethering, as well as enhanced RAB7 association and endolysosomal maturation. However, the precise role of LAMP2A in influencing these aspects of endosomal identity remains unknown. Overall, our findings suggest that LAMP2A plays a broader role in the endocytic pathway, beyond the targeting of proteins into exosomes, potentially providing a distinct identity to a specific subset of endosomal compartments.

Tumour endothelial cells derived – extracellular vesicles control the local and systemic anti-tumor immune response modulating mTOR/G-CSF pathway

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Circulating tumour-derived extracellular vesicles are suspected to contribute to metastatic spreading. Here we investigated the impact of circulating extracellular vesicles derived from tumour-endothelial cells (TEVs) in the expansion of distant metastatic dissemination. Therefore, we focus on the role of immune cells in controlling this process using the murine 4T1 triple negative breast cancer (TNBC) syngeneic model. Indeed, 4T1 cells were intravenously injected and then, mice were exposed to circulating TEVs from day 7. The lung, spleen, and bone marrow (BM) were recovered and analysed by FACS and immunohistochemistry analyses which revealed a significant enrichment of Ly6G+/F4/80+/CD11b+ cells and Ly6G+/F4/80-/CD11b+ in the lung and in the spleen, while Ly6G+/F4/80-/CD11b+ in the BM indicating the occurrence of a systemic and local immune suppression. Thus, increased expression of PD-L1, PD-1, and iNOS in the tumour mass were further supported by the TEV immune suppressive properties. Furthermore, an increase of CD11b+ cells, PD-L1+ myeloid and cancer cells, upregulation of immune checkpoint like LAG3, CTLA4 and PD-1 in T-cells, the release of ROS and NOS, and the impaired T-cell-mediated cytotoxic effect in co-culture of TEVs preconditioned PBMCs and cancer cells has been demonstrated by in vitro experiments. Noteworthy, in vivo experiments confirmed the increase of granulocyte-colony stimulating factor (G-CSF) level, and its involvement in reshaping the immune response. Mechanistically, mTOR has been found to be enriched in TEVs supporting G-CSF release. In conclusion, we provided evidence that circulating TEVs enriched in mTOR supported G-CSF release thereby granting tumour immune suppression and metastasis outgrowth.

Colorectal cancer-derived extracellular vesicles affect the immunomodulatory properties of hepatocytes supporting liver metastatic colonization

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Colorectal cancer-derived liver metastases (CRC-LM) represent the leading cause of CRC-related deaths. Increasing evidence shows that CRC-derived extracellular vesicles (CRC-SEVs) promote in the liver the formation of a favorable microenvironment for metastatic cells colonization, the so-called pre-metastatic niche (PMN), by affecting the activity of non-parenchymal hepatic cells such as Kupffer cells and stellate cells, while no data is available about the involvement of hepatocytes (Heps), despite being the most conspicuous and functional hepatic cellular component. Our recent study for the first time showed that CRC-SEVs can prompt Heps to undergo epithelial to mesenchymal transition, making them active in promoting fibrosis, known to be a key feature of the PMN. In the light of this consideration, we are currently investigating the ability of CRC-SEVs to lead Heps to promote inflammation and immunosuppression, other two events that together with fibrosis define the hepatic PMN properties. We found that CRC-SEVs elicited in Heps NF-κB activation and IL-6 induction, and modulated the expression of PD-L1. Interestingly, according to data present in literature, this last observation was associated (i) with the increase of GAS6 release and consequent TAM receptor-mediated TGFβ1 expression, and (ii) with the upregulation PD-L2 and VISTA, proteins involved in the regulation of immune tolerance mechanisms. Finally, we found that the conditioned medium of CRC-SEVs-treated hepatocytes led to macrophage M2 polarization, as demonstrated by the significant induction of the expression of IL10 and TNFα and TGFβ1.
Taken together these results, beyond revealing CRC-SEVs ability to induce Heps to actively shape hepatic PMN by affecting their immunomodulatory properties, could provide a new perspective to design more effective diagnostic and therapeutic strategies to the early treatment of liver metastatic disease.

Using Menstrual Blood-Derived Stromal Cell Secretome for Shifting Tumor-Associated Macrophages towards an Immunoreactive Phenotype

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Tumor-associated macrophages (TAMs) play a key role in the tumor microenvironment, influencing cancer progression and therapeutic responses. However, their functional heterogeneity and plasticity make them attractive targets for therapeutic interventions. In recent years, considerable attention has been directed toward the immunomodulatory capacity of menstrual blood-derived stromal cells (MenSCs). In this context, the secretome derived from MenSCs (S-MenSCs) has been highlighted as a promising therapeutic approach. Therefore, the aim of this study was to evaluate the therapeutic potential of S-MenSCs on TAMs, and

subsequently determine whether this effect can be attributed to the extracellular vesicles (EVs) present in it. In our methodological approach, MenSCs from five healthy premenopausal women were cultured in DMEM medium supplemented with 1% penicillin/streptavidin and 1% Insulin-Transferrin-Selenium (ITS). After 48 hours, the conditioned medium was collected and concentrated by ultrafiltration using a 3 kDa cut-off, to obtain the total secretome. The EVs from these secretomes were isolated by ultracentrifugation. On the other hand, human monocytes were isolated from the peripheral blood of healthy donors (n=4) and TAMs were generated in vitro by incubation during seven days in the presence of ascites fluid from ovarian cancer patients. Afterward, TAMs were treated with total S-MenSCs (100 µg/mL) or isolated EVs (comparable number of particles) for three more days in ascites media. Phenotype change was determined by the analysis of representative surface markers by flow cytometry (i.e., CD80, CD86, HLA-II, CD163, CD206, and CD16). Interestingly, our results revealed a TAM phenotype shifted towards a M1-like after treatment with S-MenSCs but no changes could be attributed to isolated EVs. In conclusion, the administration of S-MenSCs could be considered a new therapeutic option to modulate TAMs towards a more pro-inflammatory and immunoreactive phenotype. The functional implications of this shift within the tumor environment will be assessed in future studies.

Effect of plasma-derived small EVs from HNSCC patients on NF-κB signaling in macrophages

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Introduction: As one of the most immunosuppressive cancers, head and neck squamous cell carcinomas (HNSCC) show an increased NF-κB activation with downstream production of immunosuppressive and tumor-promoting factors. Plasma-derived small EVs (sEVs) from HNSCC patients contain molecules, which can contribute to the immunosuppressive tumor microenvironment (TME). Here, we investigate the influence of plasma-derived sEVs of HNSCC patients on macrophages, also with regard to HPV-infection.
Methods: sEVs were isolated from plasma of HNSCC patients and healthy donors by size-exclusion chromatography. Monocytes from buffy coats were used to generate primary macrophage cultures, which were incubated with plasma-derived sEVs to investigate their effects on the proteome, analyzed by mass spectrometry. To examine the difference of HPV-induced HNSCC, RNA sequencing was performed and NF-κB nuclear translocation was determined using sEVs from HPV-positive and -negative HNSCC patients.

Results: SEVs increased NF-κB activation in macrophages, which was reversible by addition of NF-κB inhibitors Bay, CAPE and curcumin. Incubation with sEVs changed the proteome time-dependent and HPV-status of sEV donors affected the RNA profile of treated macrophages. Several NF-κB-related genes were shown to be differentially expressed after incubation with HPV-positive or HPV-negative sEVs, respectively, indicating an HPV-dependent modulation of NF-κB pathways by HNSCC sEVs.

Discussion: Plasma-derived sEVs from HNSCC patients can alter immunosuppressive properties of macrophages. The reversion of NF-κB activation by several inhibitors may be useful for future clinical therapeutic strategies on modulation of tumor-associated macrophages through targeting sEVs in the TME. However, HPV-status of the patients has to be considered.

Modulation of PDAC immune response through cancer EVs

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Pancreatic Ductal Adenocarcinoma (PDAC) is a devastating disease with a mortality rate of 94%. Treatment options remain limited and with minimal benefit to the overall survival of the patients. Thus, unraveling novel strategies to improve disease outcome is of the utmost priority. A promising strategy would be to sensitize these tumors to immunotherapy. We have developed PDAC genetic engineered mouse models (GEMMs) capable of tracing the flow of PDAC extracellular vesicles (EVs) and demonstrated that impairing their secretion by inactivating Rab27a in cancer cells surprisingly led to an earlier tumor onset and decreased survival. Moreover, these tumors could recruit MRP8+ inflammatory myeloid cells, which activated inflammatory cancer associated fibroblasts to produce IL-6, leading to the differentiation of CD4+ cells into a Th17 pro-tumorigenic immune response. Thus, through an impaired secretion of cancer exosomes, we have identified a subset of PDAC tumors that are driven by these proinflammatory events. We believe that targeting MRP8 in this subset of PDAC tumors could constitute a novel therapeutic approach that modulates the immune response and improves the survival of PDAC patients.

Extracellular vesicle microRNAs contribute to Notch signaling pathway in T-cell acute lymphoblastic leukemia.

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T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive T-cell malignancy characterized by genotypically-defined and phenotypically divergent cell populations, governed by adaptive landscapes. Clonal expansions are associated to genetic and epigenetic events, and modulation of external stimuli that affect the hierarchical structure of subclones and support the dynamics of leukemic subsets. Recently, small extracellular vesicles (sEV) such as exosomes were also shown to play a role in leukemia. Here, by coupling miRNome, bulk and single cell transcriptome profiling, we found that T-ALL-secreted sEV contain NOTCH1-dependent microRNAs (EV-miRs), which control oncogenic pathways acting as autocrine stimuli and ultimately promoting the expansion/survival of highly proliferative cell subsets of human T-cell leukemias. Of interest, we found that NOTCH1-dependent EV-miRs mostly comprised members of miR-17-92a cluster and paralogues, which rescued in vitro the proliferation of T-ALL cells blocked by γ-secretase inhibitors (GSI) an regulated a network of genes characterizing patients with relapsed/refractory early T-cell progenitor (ETP) ALLs. All these findings suggest that NOTCH1 dependent EV-miRs may sustain the growth/survival of immunophenotypically defined cell populations, altering the cell heterogeneity and the dynamics of T-cell leukemias in response to conventional therapies.

Chromatinized DNA derived from AML extracellular vesicles alters bone marrow mesenchymal stem cells behavior by inducing non-mutational dysfunction of p53

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Many secrets related to small extracellular vesicles DNA (EV-DNA) nature, loading mechanism, localization, and post-shedding function(s) remain unrevealed. Exploring the DNA fraction of sEVs would help the rapid expansion of the field of EV biology. We have identified a chromatin-like structure we termed EV-chromatin as a novel component of sEVs. We found that EV-chromatin derived from myeloid leukemia blasts (AML-B) modifies the proliferation behavior of bone marrow mesenchymal stem cells (BM-MSCs).

Small EVs were isolated by size exclusion chromatography and characterized according to MISEV2018 guidelines. To study EV-DNA/protein association, sheared EV-chromatin was precipitated using anti-dsDNA antibody and the DNA-associated proteins were identified by mass spec. EV-chromatin was physically characterized by AFM and cryo-EM. For functional studies, EV-DNA and EV-chromatin were separately formulated in artificial polymersomes.

EV-chromatin released by AML-B blasts represents a mixture of DNA and proteins, such as histones and S100 proteins (S100A4, A8, A9, and A16). EV-chromatin from AML-B alters the proliferation of BM-MSCs. Our data suggest that EV-chromatin downregulates the p53-mediated transcription of p21, which was accompanied by a significant increase in MDM2 expression levels. Conversely, treatment of BM-MSC with either Siremadlin (MDM2 inhibitor) or siRNA against MDM2 rescued the transcriptional activity of p53. S100A4 and S100B, the primary proteins of EV-chromatin, can bind to the tetramerization domain of p53 and prevent its nuclear translocation. The EV-chromatin-mediated degradation of p53 could culminate in the synergic activity of S100 proteins that restricts p53 nuclear translocation and MDM2-mediated degradation.

EVs have recently emerged as a major player that promotes leukemogenesis in the bone marrow niche. Here, we have shown that EV-chromatin from AML-B induces non-mutational dysfunction of p53 in BM-MSCs. However, additional clinical investigations are needed to further elucidate how EV-chromatin modulate the molecular pathway mediated by the p53-MDM2 axis in the BM niche.

YBX1 containing small EVs derived from AML influences osteogenic differentiation of mesenchymal stromal cells in the bone marrow microenvironment

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Among various hematopoietic and non-hematopoietic components in the bone marrow microenvironment (BMM), mesenchymal stromal cells (BM-MSCs) are one of the most important critical factors that are known to play a key role in acute myeloid leukemia (AML) progression and contribute to treatment failure or success. Although recent studies demonstrated that small extracellular vesicles (sEVs) released by AML cells transform BMM into a leukemia-permissive microenvironment, it is not completely clear which biological cargo from AML-derived sEVs has a functional role in AML BMM. Therefore, our current study aims to address this phenomenon.

sEVs were isolated from cell-conditioned media of an AML cell line (MV4-11) using a combination of tangential flow filtration (TFF), size-exclusion chromatography (SEC), and ultrafiltration (UF). In parallel, sEVs were isolated from the plasma of AML patients and healthy controls using SEC and UF. sEVs were characterized according to MISEV 2018 guidelines using NTA, TEM, western blot, and flow cytometry.

Our results demonstrated that MV4-11 sEVs increased the proliferation and viability of BM-MSCs. Conversely, key proteins such as CxCl2, Scf, Angpt1, and Col1A1 that are important for the normal hematopoietic function of BM-MSCs were downregulated. Additionally, we revealed that MV4-11 sEVs influenced the osteogenic differentiation of BM-MSCs but not adipogenic or chondrogenic differentiation. Through LC-MS proteomics, we found that many proteins, including YBX1, which has a functional role in numerous cellular processes, were upregulated in both untreated MV4-11 sEVs and BM-MSCs treated with MV4-11 sEVs. Supporting this fact, we observed that YBX1 is enormously upregulated in sEVs derived from AML patients compared to healthy controls. Interestingly, downregulation of YBX1 in MV4-11 cells using YBX1-siRNA and a pharmacological YBX1 inhibitor (SU056) significantly rescued the osteogenic differentiation of BM-MSCs. Altogether, it is clear that YBX1 containing AML sEVs disrupts the normal hematopoiesis in BMM by influencing the osteogenic differentiation of BM-MSCs.

Breast adipose tissue-derived extracellular vesicles from women with obesity stimulate mitochondrial-induced dysregulated tumor cell metabolism

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Breast adipose tissue is an important contributor to the obesity-breast cancer link. Dysregulated cell metabolism is now an accepted hallmark of cancer. Extracellular vesicles (EVs) are nanosized particles containing selective cargo, such as miRNAs, that act locally or circulate to distant sites to modulate target cell functions. Here, we found that long-term education of human breast cancer cells (MCF7, T47D) with EVs from breast adipose tissue of women who are overweight or obese (O-EVs) leads to sustained increased proliferative potential. RNA-Seq of O-EV-educated cells demonstrates increased expression of genes, such as ATP synthase and NADH: ubiquinone oxidoreductase, involved in oxidative phosphorylation. O-EVs increase respiratory complex protein expression, mitochondrial density, and mitochondrial respiration in tumor cells. Mitochondrial complex I inhibitor, metformin, reverses O-EV-induced cell proliferation. Several miRNAs, miR-155-5p, miR-10a-3p, and miR-30a-3p, which promote mitochondrial respiration and proliferation, are enriched in O-EVs relative to EVs from lean women. O-EV-induced proliferation and mitochondrial activity are associated with stimulation of the Akt/mTOR/P70S6K pathway, and are reversed upon silencing of P70S6K. This study reveals a new facet of the obesity-breast cancer link with human breast adipose tissue-derived EVs causing the metabolic reprogramming of ER+ breast cancer cells

Tumoral periprostatic adipose tissue derived exomicroRNAS regulate tumour suppressor RORA gene in prostate cancer cells

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Background: From the first steps of prostate cancer (PCa) initiation, tumours are in contact with the most-proximal adipose tissue called periprostatic adipose tissue (PPAT). The secretion of extracellular vesicles by PPAT may provide new evidence for the understanding of the interactions between adipocytes and tumours. Extracellular vesicles, in particular exosomes, are important carriers of non-coding RNA such miRNAS (exomiRNAS) that are crucial for cellular communication. Analysing the exomiRNA-PPAT content can be of importance for the understanding of its progression and aggressiveness.

Methods: A total of 24 samples of human PPAT and paired perivesical adipose tissue (PVAT) were used. Exosomes were characterized by western blot and transmission electron microscopy (TEM) and uptake by prostate cancer cells was verified by confocal microscopy. Explants were cultured overnight, exosomes isolated, and exomiRNA content expression profile was analysed. Pathway and functional enrichment analysis on putative miRNA targets was performed. In vitro functional studies were evaluated by using PCa cells lines, exomiRNA inhibitors and gene silencers.

Results: Western blot and transmission electron microscopy revealed characteristics of exosomes in PPAT samples. Exovesicles were found be up taken and found in the cytoplasm of PCa cells. Nine exomiRNAs were found to be differentially express between PPAT and PVAT samples. The RORA gene (a RAR Related Orphan Receptor A) was identified as a common target of the putative exomiRNA-regulated pathways. Functional in vitro analysis revealed that RORA gene was regulated by PPAT-derived exomiRNAS and it was found to be implicated in cell proliferation and inflammation.

Conclusion: Tumoral periprostatic adipose tissue is linked with PCa aggressiveness and could be envisaged for new therapeutic strategies.

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Decoding the impact of EVs in the tumor microenvironment: Insights into EV-DNA dynamics and implications for disease progression

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Extracellular vesicles (EVs) have emerged as crucial players in intercellular communication within the tumor microenvironment. Tumor cell-released EVs have the ability to impact in the surrounding microenvironment by transferring bioactive molecules such as proteins, lipids and nucleic acids to neighboring cells in both local and distant sites. Among these bioactive molecules, EV-associated DNA (EV-DNA) has gained substantial attention in recent years as a potential biomarker. Moreover, the presence of DNA within EVs suggests its functional involvement in cell-cell communication and its potential contribution to disease progression. Nevertheless, our understanding of the origin of EV-DNA and its impact on recipient cells remains limited. In this project, we have analyzed the presence of DNA and DNA damage response (DDR) molecules in EVs in melanomas well as the potential extrinsic effect on recipient cells. We have observed that intrinsic DNA damage is associated with a higher secretion of DNA and γ-H2AX in small extracellular vesicles (sEVs). In order to understand the mechanism involved in secretion of DDR-related proteins, we performed proteomic analysis of EVs derived from melanocytes and melanoma cells. Our data support the presence of proteins associated with DNA replication and DNA damage processes in tumor-derived EVs. Interestingly, we have observed that sEV-associated markers (CD9, CD63) co-localized with damaged DNA in areas surrounding micronuclei enucleation, suggesting a potential origin of DDR-related molecules found in EVs. Additionally, our findings demonstrate that melanoma-derived EV-DNA can be transferred into melanocytes, promoting increased DDR and formation of tumors. Our data provide compelling evidence for a novel pathway to mitigate intrinsic DDR within EVs and the

potential extrinsic effects. However, further investigations are necessary to elucidate the in vivo relevance of these findings and their implications for tumor evolution.

Ferroptosis transmission by small extracellular vesicles in epithelial ovarian cancer cells

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Epithelial ovarian cancer is the deadliest gynaecological cancer due to its challenging early diagnosis caused by nonspecific symptoms during the initial stages of the disease. Despite available treatments that show effectiveness in early stages, many patients eventually relapse as cancer cells develop resistance. Hence, there is a need to explore novel approaches to fight resistant cancer cells. About a decade ago, a form of non-apoptotic cell death tiggered by high levels of free iron was discovered.Ovarian cancer cells (OVCA) exhibit alterations in iron metabolism, a feature that can be exploited to target cells resistant to current therapies.Cancer cells release small extracellular vesicles (sEV) that allow communication between themselves and their environment. By releasing signalling molecules to the tumor microenvironment, ferroptotic cancer

cells can either suppress or promote tumor progression through inflammatory pathways.The aim of this study is to investigate whether ferroptotic cancer cells can propagate this process in a paracrine manner. To achieve this, we treated OVCA with ferroptotic modulators and then, confirmed the ferroptosis phenotype assessing several ferroptosis parameters. Then we evaluated the capacity of ferroptotic OVCA-derived sEV to propagate this process and validated the ferroptosis phenotype. Furthermore, to assess whether these ferroptotic cells induced with EV from ferroptosis modulator-treated cells are still able to propagate this process, we collected their sEV and treated OVCA with them.

Our results show that EV derived from ferroptotic cells are capable of triggering ferroptosis in non-ferroptotic OVCA. Furthermore, the sEV released by these new ferroptotic OVCA can propagate this type of cell death. In fact, we observed an increase in sEV production containing high levels of Fe^{2+} during secondary ferroptosis paracrine transmission.

This study establishes a starting point for further investigation into the molecular mechanisms regulating this process, thus potentially being employed as an alternative therapy in cancer treatment in the future.

ENHANCING CURCUMIN'S PHARMACOKINETICS USING EXTRACELLULAR VESICLES AS DRUG DELIVERY SYSTEMS FOR IMPROVED CANCER TREATMENT

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Curcumin, a polyphenol found in turmeric root, has anti-inflammatory and anti-oncogenic properties affecting NF-κB and JAK/STAT signalling pathways. However, poor solubility limits its clinical application. Extracellular vesicles (EVs) can act as advanced drug delivery systems to improve curcumin pharmacokinetics and pharmacological activity. However, optimal EV loading strategies remain undefined.

EVs were isolated from human MDA-MB-231 (aggressive) and T47D (non-aggressive) breast cancer (BC) cell lines using ultracentrifugation (UC). EVs were characterised by nano-flow cytometry (NanoFCM), bicinchoninic protein assay, and western blotting. Both EV types were passively incubated with 1mg/mL of curcumin for 30 minutes at room temperature. EV-curcumin was recovered from unbound curcumin using UC (x120,000xg) and quantified spectrophotometrically at 420nm to evaluate percentage EV-curcumin loading. EV-curcumin solubility (1.1x1011 particles/mL) was assessed in DPBS and compared with curcumin-only controls. The stability of EV-curcumin was compared with curcumin-only controls in DPBS over 150 minutes. Significant differences in CD9 (6.9% vs. 3.1%), CD63 (13% vs 6.37%) markers were recorded for MDA-MB-231 and T47D EVs respectively. Minor variations in particle number (1.71x1010 vs 3.89x1011), mean size (76.18nm vs 80.32nm) and zeta potential (-10.45mV vs -12.43mV) were observed. EV-curcumin percentage loading increased by three-fold and two-fold with increasing concentrations of MDA-MB-231 and T47D EVs, respectively. Similarly, free curcumin's solubility improved by four-fold and five-fold and an average 1.6- and 1.8-fold increase in curcumin stability over 150 minutes was observed for the same. Currently, the therapeutic effects of EV-curcumin vs free curcumin are being compared at concentrations 25, 50 and 100ug/mL using RT-PCR for pro-inflammatory genes (IL-1β, IL-6, TNF-α) and oncogenic genes (p21, p53).

Differences in MDA-MB-231 and T47D EV characteristics were minimal. EV-curcumin's therapeutic potential is apparent through improved curcumin solubility and a slower decline in its stability over time compared to free curcumin. We will next determine EV-curcumin's therapeutic potential in cell culture systems.

Manipulation of Tumor Extracellular Vesicles biodistribution and uptake in vivo by a versatile cell-surface "EV-Trap"

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Tumor-derived EVs are recognized as key mediators playing pivotal roles in pre-metastatic niches formation, in tumor migration and proliferation, in immune-escape and in chemoresistance transfer. Yet, the field is still facing major gaps in our understanding of the role of EVs in vivo. Several approaches have been recently developed to live-track the dynamics and fate of tumor-derived EVs at a single-particle scale in vivo, notably in the zebrafish embryo. However, tools to selectively guide and manipulate tumor EVs dissemination and targeting in vivo are still lacking in the field, complicating efforts to clarify their relative contributions to intercellular communication in pathological development. We reasoned how manipulating tumor EVs biodistribution in vivo by forcing their spatial rerouting towards a chosen cellular target might result in a better comprehension of their biological roles, for instance in pre-metastatic niches formation. Here we describe a novel approach, inspired by Chimeric Antigen Recognition (CAR) strategy, to selectively capture and locally accumulate circulating tumor EVs towards engrafted trapping cells, or endogenous trapping tissues of choice. Focusing on Prostate cancer (PCa)-derived EVs, we combined the pHluorin-genetic tagging of tumor EVs with the expression on target cells of the EV-Trap, a chimeric surface receptor exposing various nanobodies against EVs' surface antigens (synthetic or tumor associated). We assessed the capacity of this EV-Trap to divert EVs from their natural targets and to accumulate PCa-derived EVs at the location of our choice. We demonstrate how the simultaneous presence of PCa-derived CD63-pHluorin+, CD9-pHluorin+ or PSMA+ EVs in a system presenting GFP-Trap or PSMA-Trap accessible cell surfaces results in a significant accumulation of PCa-derived pHluorin+ and PSMA+ EVs towards the EV-Trap sites, both in vitro and in vivo in the zebrafish embryo. This approach will shed new lights on the role of EVs in various physiopathological processes, as on pre-metastatic niches formation.

Identification of EV-biomarker candidates from peripheral and local tumor plasma in head and neck squamous cell carcinoma using proteome analysis before/after tumor removal surgery

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Head and neck squamous cell carcinoma (HNSCC) can severely impact patients' quality of life, especially in advanced stages, causing symptoms such as lumps, pain in the neck or throat, mouth ulcers, hoarseness, loss of voice, and difficulty in swallowing. Limited treatment options exist for patients with second cancers, and younger individuals are increasingly affected by this disease. Although surgery is the primary method for removing HNSCC from the oral cavity, it is crucial to determine the presence of tumor-related antigens in the local area or circulation. Therefore, researchers are exploring the potential use of liquid biopsy with HNSCC proteins as prognostic biomarkers.

Since extracellular vesicles (EVs) contain most abundant cellular information in liquid biopsy, this study aims to investigate the protein content of EVs before and after operation in HNSCC patients using in-house galectin-based glycan recognition particles (EXÖBead) isolation, bead-based flow cytometry (BFC), and high-resolution liquid chromatography mass spectrometry-based proteomics (LC-MS/MS).

Patients' platelet-poor plasma was collected from peripheral venous blood before (BFPP) and one hour after tumor removal surgery (AFPP). Local tumor venous plasma was also collected before (BFTV) and after tumor removal surgery (AFTV). Preliminary BFC results show that EpCAM+ PanEV+, PD-L1+ PanEV+, PanCK+ PanEV+ of EVs-EXÖBead complex were significantly higher in BFPP and BFTV groups but not in AFPP and AFTV. Particle sizes were comparable in all 4 groups. Proteomics result showed that Integrin β-3 (ITGB3/CD61) was significantly higher in BFPP groups compared to AFPP. BFC results also showed that PanEV+ CD45- EpCAM+ CD61+ of EVs-EXÖBead complex was significantly higher in BFPP compared to AFPP.

These results suggest that tumor removal surgery removed majority of tumor-related EVs in peripheral and local area. Furthermore, proteomics and BFC results suggest that ITGB3/CD61 on plasma EVs may be a prognostic marker in HNSCC.

Digital Detection of EGFR Mutations in Lung Cancer Using Tumor-Derived EVs in Blood Plasma

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This study introduces a novel method for the digital detection of tumor-derived extracellular vesicles (tEVs) using a charge-induced fusion approach. By encapsulating molecular beacons within liposomes and compartmentalizing vesicles in picolitre-sized droplets, the platform enables the detection of tEVs in blood plasma samples from lung cancer patients. The system eliminates the need for labor-intensive sample preprocessing, prevents vesicle loss, and simplifies the detection process. Liposomes with tunable surface charges were created using zwitterionic and cationic lipids through microfluidic hydrodynamic focusing. Fusion between liposomes and EVs was confirmed through various analyses, including transmission electron microscopy, size measurements, and a lipid-mixing assay. The platform successfully detected tEVs from the H1975 adenocarcinoma cell line by targeting microRNA 21 (miR-21) using molecular beacon-encapsulated liposomes. Moreover, it accurately detected EGFR L858R and T790M mutations in exosomal RNA from lung cancer patients' blood plasma samples. Fluorescence microscopy confirmed that only droplets containing tEVs exhibited fluorescence, proportional to the target concentration. The platform also showed 100% accuracy in detecting EGFR mutations compared to mutation analysis from tumor tissue. Overall, this study presents a simple and efficient approach for digitally detecting tumor-associated mutations using a droplet-based charge-induced fusion of liposomes and EVs. By preventing unwanted aggregations and enhancing detection sensitivity, this platform offers a sensitive and streamlined method for cancer detection, eliminating the need for extensive sample preprocessing.

An Early Brain Cancer Diagnosis Approach Based on Bioinformatics and Machine Learning Analysis of Human Plasma Exosome Transcriptomics

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Glioblastoma is a high-incidence and high-mortality rate brain cancer type. The gold-standard tissue biopsy suffers from failure in early detection and destructiveness. Exosomes, with their high abundance in biofluids and wide variability of cancer biomarker cargo protected by their lipid bilayer, are promising liquid biopsy candidates.

In this study, we aimed to evaluate the diagnostic potential of exosomal miRNA content for glioblastoma using two Gene Expression Omnibus (GEO) datasets. The first dataset, GSE112462, includes 10 glioblastoma, 9 astrocytoma, 9 oligodendroglioma, and 8 control samples whereas the second dataset, GSE122488, consists of 22 glioblastoma cases versus 16 healthy controls.

From the first dataset, we detected 30 differentially expressed miRNAs between glioblastoma and control samples. KEGG pathway analysis using these miRNAs as well as differentially expressed genes between WHO grade II and grade IV glioblastoma patients from Chinese Glioma Genome Atlas (CGGA) and GliomasDb datasets demonstrated that TGFβ, PI3K-Akt, and P53 pathways were common among two approaches.

The second GEO dataset provided 145 differentially expressed miRNAs, not including any of the top 10 differentially expressed miRNAs from the first dataset. This suggested that biological variation present in such a heterogeneous tumor as glioblastoma may deteriorate the diagnostic value of individual miRNAs as biomarkers.

We hypothesized that a machine learning approach analysing differentially expressed miRNA set can overperform individual miRNAs in terms of diagnosis success. For this purpose, a binary SVM approach was built. By using differentially expressed miRNAs as feature sets, we were able to perfectly distinguish glioblastoma and control samples in both datasets.

Our findings suggest that exosomal miRNA profiling-based liquid biopsy might provide a valuable alternative to tissue biopsy to detect glioma at an early stage non-invasively, and bioinformatics analysis combined with machine learning approaches can be precious tools to automate and standardize such approaches.

PD-L1 on large extracellular vesicles is a predictive biomarker for therapy response in tissue PD-L1-low and -negative patients with non-small cell lung cancer

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Immunotherapy has revolutionized the treatment of patients with non-small cell lung cancer (NSCLC). The level of tissue PD-L1 expression (tPD-L1) positively correlates with the probability of treatment response. However, even tPD-L1 low (1-49%) and absent (<1%) patients might benefit from immunotherapy but, to date, there is no reliable biomarker, that can predict response in this particular patient subgroup. This study tested whether tumor-associated extracellular vesicles (EVs) could fill this gap. We isolated small EVs (sEVs) and large EVs (lEVs) from cell culture supernatants as well as peripheral blood of NSCLC patients (n=108), non-cancer (n=23) and healthy (n=54) controls by differential ultracentrifugation and thoroughly characterized them by electron microscopy, nanoparticle tracking analysis, and immunoblot for common EV and tumor markers. Using NSCLC cell lines, we identified a striking enrichment of the immune checkpoint protein PD-L1 on lEVs compared to sEVs. Measuring the levels of PD-L1+ lEVs in plasma using standard flow cytometry, we detected a significant increase in NSCLC patients which discriminated them from controls. PD-L1 was mainly found on a population of CD45-/CD62P- lEVs and thus did not seem to originate from platelets or leukocytes. Patients with high levels of PD-L1+ lEVs in blood showed a significantly better response to immunotherapy and prolonged survival. The predictive value of lEV-associated PD-L1 was based on a single measurement at baseline and outperformed tPD-L1, the current standard of care. This was particularly true in the subgroup of NSCLC patients with low or absent tPD-L1 expression. Our data have therefore identified PD-L1+ lEVs as a novel predictive and prognostic marker for immunotherapy in NSCLC.

Saliva-derived small extracellular vesicles as biomarker for head and neck cancer – does saliva provide better diagnostic potential than plasma?

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Background: Head and neck squamous cell carcinoma (HNSCC) is a highly aggressive disease, and early non-invasive detection is crucial due to the high prevalence of advanced disease and low survival rates. Plasma-derived small extracellular vesicles (sEV) from HNSCC patients correlate with clinical parameters. Here, we investigated the cargo of saliva-derived sEVs. By comparing sEV miRNA profiles from saliva and plasma, we identified liquid biomarker candidates for HNSCC.

Methods: sEVs were isolated from saliva and plasma by differential ultracentrifugation. sEV surface levels of immune checkpoints and tumor antigens were measured by on-bead flow cytometry. The ability to produce adenosine was examined by mass spectrometry. sEV miRNA profiles, determined by nCounter technology from paired saliva/plasma samples, were analyzed regarding their diagnostic and prognostic potential and integrated into network analysis.

Results: Saliva-derived sEVs from HNSCC patients carried high levels of CD44v3, PDL1 and CD39, and strongly produced immunosuppressive adenosine. Compared to plasma, saliva was rich in tumor-derived CD44v3+ and poor in hematopoietic CD45+ sEVs. 29 tumor-exclusive miRNAs were identified within sEVs from both biofluids and associated with tumorigenic pathways (TP53, TGFB1, CDH1). The top 10 candidates with the strongest co-expression within each biofluid emerged as diagnostic panels for HNSCC detection and potentially prognostic panels for disease-free survival. sEV miRNAs exhibited differential representation in HPV positive and negative, as well as low and high stage disease.

Conclusion: Saliva from HNSCC patients contains tumor-derived sEVs and thus resembles a liquid biopsy source. However, we propose separate sEV-based, tumor-exclusive miRNA panels from saliva and plasma, which are valuable for future biomarker studies and, upon validation in a larger cohort, could be implemented in clinical practice for diagnosis of HNSCC.

Plasma Exosomal Non-Coding RNA Profile Associated with Renal Damage Reveals Potential Therapeutic Targets in Lupus Nephritis

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Background: Despite considerable progress in our understanding of systemic lupus erythematosus (SLE) pathophysiology, patient diagnosis is often deficient and late. The aim of this study was to analyze exosomal non-coding RNA (ncRNA) to assess the molecular profile associated with renal damage to identify new potential targets.
Methods: NcRNA profile was identified by RNA-sequencing from 96 samples of SLE patients, 23 of which with LN, and 25 healthy volunteers. Biological pathways were analyzed using GO terms and KEGG analysis, and ncRNA levels were validated in podocytes under TFG-β1 stimulation.
Results: The three exosomal plasma ncRNA types with the highest number of differentially expressed transcripts in lupus nephritis (LN) were microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and piwi-interacting RNAs (piRNAs). We identified an exosomal 15-ncRNA molecular signature associated only with LN presence. The transcriptional regulatory network showed a significant role for four lncRNAs (LINC01015, LINC01986, AC087257.1 and AC022596.1) and two miRNAs (miR-16-5p and miR-101-3p), targeting critical pathways implicated in inflammation, fibrosis, epithelial–mesenchymal transition and actin cytoskeleton. Finally, podocytes stressed with TFG-β1, showed a significant increase in miR-16-5p and miR-101-3p exosome levels (p

A Selected signature of miRNAs in Urinary Extracellular Vesicles Allows Noninvasive Detection of Graft Fibrosis in Renal Transplant Patients

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Introduction: Diagnosis of interstitial fibrosis and tubular atrophy (IFTA) is closely related to the prognosis of the renal grafts. IFTA diagnosis relies on renal biopsy (RB) which is costly, invasive, and biased by sampling limitations. Thus, the implementation of non-invasive biomarkers of fibrosis is of interest.
Previously, with the use of RNAseq sequencing of urinary extracellular vesicles (uEVs) we found a differentiated miRNA signature in Kidney Transplant recipients (KTx) who showed IFTA in their grafts. In this study, we validated this signature using "digital droplet PCR" (ddPCR), a very sensitive technique with high detection potential and clinical applicability. Additionally, we analysed the urine expression of Vitronectin (VTN), a protein already described as a potential IFTA biomarker.
Methods: miRNA signature was analysed by ddPCR from uEVs in a new cohort of 20 KTx (≥3 years post-transplantation, GFR≥30 mL/min/1.73m2, proteinuria ≤1.5 g/d). The cohort was divided in two groups according to the Banff score: NO IFTA (Banff score<1, 8 patients) and IFTA (Banff score ≥1, 12 patients) as per protocol biopsy. All the urine samples were obtained immediately before RB. VTN was assessed by ELISA.
Results: ddPCR rendered absolute quantification of miRNA (copies of target miRNA / uL urine) in all samples. The previously defined miRNA signature was partially confirmed in this new KTx cohort and VTN levels pointed to a direct correlation with IFTA score (Spearman r=0.37/p=0.06). Combined analysis of all the selected miRNAs (n=6) resulted in an area under the ROC curve (AUC) of 0.877 (p= 0.001) for the detection of biopsy-proven

IFTA. The addition of VTN increased the AUC value to 0.948, (p=0.0004).
Conclusions: Detection of a specific uEV miRNA signature by ddPCR combined with urinary VTN levels is a non-invasive method that permits to diagnose the presence of IFTA in KTx recipients.

Identification of a plasma extracellular vesicle surface signature associated with nephritis in systemic lupus erythematosus

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Background: Extracellular vesicles (EVs) represent an attractive candidate for renal damage in systemic lupus erythematosus (SLE), as their cargo reflects the originating cell and its pathophysiological condition. The aim of the study was to characterize EV surface markers from plasma as biomarkers of kidney disease in SLE patients.
Methods: We characterized 37 surface antigens in plasma EVs from 76 SLE patients (of which 22 have nephritis) and 20 healthy subjects. EV surface antigenic profile was evaluated by a multiplex flow cytometric assay (MACSPlex human Exosome Kit; Miltenyi Biotec). The mean fluorescence intensity (MFI) for each sample was employed as internal normalizer to allow comparison among samples.
Results: Platelets and endothelial cells markers (CD41b, CD40 and CD31), several T and B cells markers (CD19, CD14 and CD4) and CD69, a phenotypic marker associated with tissue residence program, were higher in plasma EVs from SLE patients with nephritis compared to control group. In addition, a decreased CD49e or alpha 5 integrin, that mediates podocyte adhesion/anchoring to glomerular base membrane, was the most significance marker to distinguish patients with nephritis of those without. The CD49e marker correlated inversely with urinary albumin excretion ratio (r=-0.42, p

Altered small RNA-secretome in Huntington´s Disease neurons

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Huntington's disease (HD) is a neurodegenerative disorder caused by a CAG repeat expansion in the Huntingtin gene (HTT). Despite that most studies have been historically concentrated on the pathogenic role of the resultant mutant HTT protein, growing insights on HD indicate that altered small non-coding RNA (sRNA) are involved in the pathophysiology. Data from our lab showed that sRNAs from HD patients´ brain are sufficient to induce neuronal toxicity and mediate neuroinflammation in vivo. In the paradigm of HD, these harmful sRNAs may be released in the extracellular space as freely circulating, associated to protein/lipid complexes, and/or encapsulated in extracellular vesicles (EVs), and could mediate paracrine toxicity. Herein, we used HD human embryonic stem cells (hESCs)-derived neuron cultures (neuron-hESCs) to elucidate the possible altered release of extracellular sRNAs (exRNAs) in HD. Using an optimized method for EVs purification from cell supernatants by Size-exclusion chromatography (SEC) and Ultrafiltration (UF), we explored the exRNA composition of the vesicular (EVs) and extravesicular (NonEVs) neuron-hESCs secretome through an exhaustive analysis pipeline of sRNA sequencing data in HD and Control neuron-hESCs lines. Characterization of hESCs-EVs revealed no differences in size and morphology of EVs between HD and Control. We showed heterogeneous proportions of exRNAs biotypes between intravesicular and extravesicular compartments and we highlighted that, inside EVs, different sRNA biotypes, such as miRNAs and tRNA fragments (tRFs), are significantly differentially expressed in

HD compared to Control neuron-hESCs cell lines. Interestingly, specific miRNAs and tRFs species already described as deregulated in HD plasma-EVs, CSF and/or putamen samples, appeared similarly altered in this paradigm. These findings suggest that exRNAs release is altered in human HD neurons, and that these exRNAs may contribute to the paracrine toxicity observed in previous studies.

Synovial fluid-derived EVs in Equine Osteoarthritis – Correlation between the proteome and phospholipidome for biomarker identification

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Both humans and horses are affected by osteoarthritis (OA), which results in chronic joint deterioration, substantial morbidity, and reduced mobility. Cell-derived vesicles released in the extracellular milieu (EVs) and used by cells for intercellular communication are a new source of potential biomarkers. Here, we used a multi-omics strategy to find possible EV-based biomarkers of horse OA in synovial fluid-derived EVs.

We utilised 14 pooled synovial fluid (SF) samples (Healthy n=7, OA n=4, Advanced OA n=3), where each pooled sample consisted of three biological replicates. EVs were extracted from SF by differential ultracentrifugation followed by sucrose density gradient and characterised by quantitative single-EV analysis with high-resolution FCM. The Fusion Orbitrap was used to investigate the phospholipidome and a Triple TOF 6600 for the proteome. The proteomics and lipidomic datasets were processed separately using R or Metaboanalyst, and proteomic data was subjected to ANOVA. Multi-omics integration was performed using unsupervised sparse Partial Least Squares, thus, enabling the determination of the degree of correlation of proteomic and lipidomic data.

We observed that OA did not significantly impact the number of SF-EVs. From the phospholipidome, sphingomyelins comprised 19.9%, 35.5% and 37.5% of the lipid profile in the healthy, OA, and advanced OA groups, respectively. From the proteome, integrins (p=2.72x10^-24),), the actin cytoskeleton (p=3.88x10-32), Rho family GTPases (p=1.24x10^-19), and the clathrin-mediated endocytosis pathway (p=1.30x10^-24) were shown to be essential signalling pathways in chondrocyte dysregulation and EV biology. Moreover, the advanced OA group specifically showed an upregulation of pathways related to macrophage activity. Interestingly, integrated multi-omics and functional enrichment analyses unveiled a predominantly positive correlation between the proteome and phospholipidome connected to changes in signalling pathways involved in chondrocyte dysregulation. The findings need to be confirmed in a larger cohort, but these data suggest that SF-EVs may provide combined protein-phospholipid biomarkers for OA.

Single vesicle imaging flow cytometry approach to unravel the molecular profile of endometriosis-related extracellular vesicles as a source of potential biomarkers

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BACKGROUND
Endometriosis is a chronic gynecological disorder and despite its high prevalence, at present there are no effective treatments or biomarkers for early diagnosis. Since EVs are present in almost all body fluids, they have been recently investigated as easy accessible source of biomarkers in liquid biopsy. Given the complexity and heterogeneity of this disease, it is likely that not a single but a panel of biomarkers is necessary to diagnose endometriosis.
METHODS
EVs were isolated from plasma and peritoneal fluid (PF) of endometriosis and control patients using SEC and were verified by WB, fluorescent mode NTA, imaging flow cytometry (IFCM) and TMT-based quantitative proteomics analysis. The molecular phenotype of EVs was determined directly in plasma sample by high-throughput, multiparametric IFCM using a specially designed panel of antibodies, which included detection of antigens that are typically elevated during chronic inflammatory states (CD152), or are associated with the development of early endometriotic lesions (CD82; CD44), immune suppression (CD16; CD206), and endometrial receptivity (infertility) (CD227).
RESULTS
We detected a heterogenous collection of EVs in plasma and PF samples from endometriosis patients and controls. These vesicles exhibit general features associated with small EVs, and contained bona fide EV markers. Upon performing single EV analyses on the IFCM platform, we learned that endometriosis-derived EV populations contain a wide range of molecules and at least some of them are associated to endometriosis pathogenesis.
CONCLUSIONS
Our results, imply endometriosis-specific small EV signatures in the patients' plasma. After evaluation, these EV signatures may serve as potential non-invasive diagnostic/prognostic biomarkers or therapeutic targets in endometriosis in the future.

Physical association of low density lipoprotein particles and extracellular vesicles unveiled by complementary single particle analysis techniques

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Introduction: Extracellular vesicles (EVs) present in biofluids have potential to be used as disease biomarkers. However, plasma is a complex biofluid that contains lipoprotein particles (LPPs). In order to exploit the potential of EV biomarkers in plasma we investigated how the presence of abundant LPPs impacts the detection of single EVs.

Methods: EVs were isolated from 4T1 mammary carcinoma and breast cancer MCF7 conditioned media by density gradient ultracentrifugation and size-exclusion chromatography. CM, VLDL and LDL were purchased. Samples were characterized by immunoblot, nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), high-sensitivity flow cytometry (FC), cryo-EM, atomic force microscopy (AFM) and single EV optical trapping - synchronized Rayleigh and Raman scattering (OT-RS). For fluorescence-based FC, samples

were stained with PKH67 and/or CD9-PE and succumbed to density gradient floatation prior analysis on a BD Influx.
Results: FC-analysis revealed different profiles and peak enrichment density fractions of PKH67 stained EVs and LPPs. Spiking of LPPs into EV preparations had an impact on quantitative and qualitative FC-analysis, by shifting the CD9 antibody signal into lower densities. The profiles from spiked-in samples suggested EV-LPP interactions, which seemed most prominent with LDL particles. AFM analysis of the commercial LDL sample unveiled single LDL particles, LDL aggregates and EVs. In addition, we visualized by cryo-EM EVs decorated with LDL particles in this sample. Importantly, we confirmed by label-free OT-RS that the presence of LDL can affect the chemical signature of optically trapped EVs.
Summary/Conclusion: Using complementary single particle techniques we gained insight into the association between EVs and LPPs. Our results indicate that LPPs can interact with EVs to a different extent as we observed that LDL-EV interactions were more prominent compared to CM or VLDL interactions. Furthermore, these interactions can have direct implications for blood-derived EV biomarker profiling.

Serum Extracellular Vesicle miRNA Profiling to determine Extracorporeal Photopheresis Response in Graft versus Host Disease

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Graft versus Host disease (GvHD) is a life-threatening complication of allogeneic haematopoietic stem cell transplantation (HSCT), affecting, 40-70% of patients, leading to post-treatment mortality in 40-60%.
Steroids are frequently used as first line treatment, however they have significant adverse effects. Extracorporeal photopheresis (ECP) therapy is a safe alternative, though its precise mechanism of action is unknown, and there are no biomarkers to determine ECP response. Our aim is to investigate the role of serum extracellular vesicles (EVs) during ECP therapy for GvHD.
Serum samples were acquired from n=12 paediatric patients undergoing ECP therapy at the Great North Children's Hospital before every treatment cycle. EVs were isolated from 4 sequential timepoints (TP) by precipitation for n=6 complete (CR) and n=6 partial/non-responders (PR). Particle size and concentration were measured using Nanoparticle Tracking Analysis (NanoSight). MicroRNA expression profiling was performed using NanoString technology (n=800 microRNA).
Overall EV modal size was significantly smaller for patients who reached PR compared to CR, and this was most pronounced between TP1 and TP2. Analysing the EV microRNA composition using DESeq2 showed that in principle component analysis (PCA), three groups formed clusters; PR and two separate clusters of mostly CR with some PR. This analysis also showed that treatment cycle was not a driving factor, as there was no treatment cycle-based clustering. There were 8 microRNAs that were significantly differentially expressed in CR compared to PR patients following FDR adjustment; hsa-miR-23a-3p was downregulated and hsa-miR-144-3p, hsa-miR-548ar-3p, hsa-miR-378e, hsa-miR-21-5p, hsa-let-7i-5p, hsa-miR-579-3p, and hsa-miR-1283 were upregulated ($p<0.05$ and Log2 Fold Change range -1.04 – 24.21). Further assessment of miRNA-target interactions, EV surface markers, serum cytokines, and TCR diversity is ongoing.
Preliminary analysis shows the potential for miRNA biomarkers as an indicator of ECP response, as well as shedding light on the mechanisms of ECP action.

Uptake of extracellular vesicles by the liver and the effect of ApoB-rich protein corona on their biodistribution

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The effectiveness of therapeutic extracellular vesicles (EVs) is limited by their rapid elimination from circulation by the liver. The involvement of different liver cells in this process is not fully known. In addition, the protein corona surrounding EVs may also influence their sequestering to the liver.
HEK293T-palmGFP-derived medium EVs (mEVs) and small EVs (sEVs) were labelled with 99mTc-HYNIC-duramycin. The biodistribution of EVs was analysed by SPECT/CT one hour after intravenous injection in mice. The role of hepatocytes, Kupffer cells and liver sinusoidal endothelial cells (LSECs) in the uptake of EVs was investigated in vitro. To examine the effect of protein corona on the biodistribution, sEVs were incubated with human serum albumin (HSA) or a mixture of HSA-ApoB. We determined the effect of proteins on the size, particle number, TritonX-100 and phospholipaseA2 sensitivity of sEVs.
EVs accumulated mainly in the liver. All tested cell types were able to take up EVs. Kupffer cells were the most effective ones in the uptake of mEVs, while LSECs have taken up sEVs most efficiently. Although no difference was observed in the liver, the ApoB corona significantly reduced EV accumulation in the spleen. The lysis of sEVs by TritonX-100 or phospholipase A2 was reduced by the artificially built HSA-ApoB EV corona.
In conclusion, we provide evidence that different cell types in the liver differentially take up EVs in a size-dependent manner. According to our data, although the ApoB-rich protein corona alters the physicochemical properties of EVs, it does not affect hepatic EV uptake.

The Potential Role for Extracellular Vesicles in Primary Open-angle Glaucoma, Oxidative Stress Aspects.

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Purpose: To explore the role of exosomes derived by oxidative stress (OS) non-pigmented ciliary epithelium to modulate key signaling pathways in trabecular meshwork (TM) cells

Methods: Human NPCE cells were incubated with 10mm AAPH for 90min causing nonlethal cell damage. Exosomes were collected from the media for 24h and characterized. The OS-NPCE-derived exosomes were incubated with human primary TM cells, and changes in gene and protein expression were analyzed using PCR and Western blot analysis. In addition, miRNA microarray chip analyses of the OS-NPCE-derived exosomes were performed, and TM mRNA experimental target validation expressions were performed.

Results: The effects of OS-NPCE-derived exosomes on the activation of the Nrf2-Keap1, a major OS pathway, and of the Wnt pathway, known for its role in primary open-angle glaucoma, were evaluated. Exosomes derived from OS-NPCE cells significantly protected TM cells from direct OS. The TM cells' uptake of EVs from OS-NPCE and their cytosolic Nrf2 levels were significantly higher. Exosomes derived from OS-NPCE cells significantly attenuated Wnt protein expression in TM cells and activated major antioxidant genes as measured by qRT-PCR. TM cells exposed to exosomes derived from OS-NPCE cells exhibited significantly lower OS and higher superoxide dismutase and catalase activity.
We found that various miRNA families, including miR27, miR199, miR23, miR130b, and miR200, changed significantly. Upon pathway prediction analysis, we found that these miRNAs can regulate the genes including Nrf2, Keap1, GSK3B, and serine/threonine-protein phosphatase2A (PP2A).

Conclusions: NPCE-derived exosomes play a critical role in signaling communication contributing to the modulation of TM. These exosome-mediated effects significantly change when the exosome-producing cells are under OS. These results add another layer of understanding of exosomes' role in ocular signaling.

Immunomodulatory effects of placenta MSC-derived EVs in a novel 3D, immune-competent in vitro human small airway model of cystic fibrosis

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Recently, evidence has accumulated that the therapeutic effect of mesenchymal stem cells (MSCs) mainly depends on their paracrine action, like the secretion of extracellular vesicles (EVs). There is an increasing trend to use such MSC-derived EVs as replacement for cells in future clinical treatments to avoid the safety concerns associated with live cell therapy. Specific differences in the anatomy, physiology and pathology of the respiratory systems in laboratory animals commonly used in pulmonary research in comparison to humans and the strong support for the 3R principle in animal testing have driven the development of alternative in vitro cell culture models. Here, we are investigating for the first time the immunomodulatory properties of placenta MSC-derived EVs, in comparison to parental cells, in a human in vitro cystic fibrosis (CF) 3D-model. Human primary pseudostratified epithelium cells and fibroblasts of small airways from a homozygous (mutation ΔF508) CF donor (Epithelix SmallAir™) were co-cultured at an air-liquid interphase with alveolar macrophages. The impact of EVs alone, EVs + parental MSCs and MSCs alone, applied at the apical side, on the secretion of inflammatory cytokines/chemokines on the basal and apical side (Legendplex bead assay), epithelial cell cytotoxicity (LDH assay) and macrophage polarization and function were studied. From the data presented here, it is evident that the presented organo-typical co-culture model, so far used mostly in the field of inhalation toxicology, provides also an excellent tool to study under in vivo-like, standardized conditions the therapeutical potential of MSC-derived EVs. They can be administered by aerosol-based delivery systems with exact control dosing in contrast to in vivo administration, resulting in a better optimization of parameters for follow-up in vivo experiments. The as an example presented CF model could be a basis for the development of other physiological human tissue models to study EV-cell interactions.

Vectorised radiotherapy in oncology using milk small extracellular vesicles

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Background: Targeted radiotherapy allows the selective accumulation of high doses of radiation in tumour tissue through the use of tumour microenvironment-specific biomolecules radiolabelled with therapeutic radioisotopes, such as lutetium-177 (Lu-177). This vectorised radiotherapy enhances the destruction of malignant cells and reduces the exposure of surrounding tissue. Based on previous results of the group, in which we demonstrated the ability of sEVs from goat's milk (MisEVs) to accumulate in tumour tissue and internalise into tumour cells, we propose for first time the development of a vectorised radiotherapy based on 177lutetium-labelled sEVs (177LutsEVs).Thus, these MisEVs will act as a Trojan horse, delivering radiation precisely to tumour and preventing it from accumulating in healthy tissue. Methods: sEVs were isolated from goat milk and radiolabeled with Lu-177, testing two strategies: active Lu-177 labeling by chelator-functionalized MisEVs or Lu-177 passive incorporation into the MisEVs structure. Chelator conjugation (DOTA-NHS ester) to MisEVs was performed at 4ºC/pH 8.5/overnight, purified by size exclusion chromatography (SEC) and characterized by TEM, DLS, NTA and HPLC. Both functionalized and non-functionalized samples were radiolabeled at 95ºC/30min/pH 5. Purity after SEC and tracer stability (37ºC/PBS) were determined by radio-TLC. Biodistribution of 177LutsEVs was evaluated by in vivo SPECT/CT imaging (n=3 healthy mice, i.v., 3MBq) at 4h, 24h and 48h post-administration, and by ex vivo biodistribution (24h and 72h); Results: DOTA-sEVs were functionalized preserving original morphology and size of isolated nanovesicles (121.60±3.30nm). Both non-functionalized and functionalized MisEVs were successfully radiolabeled with Lu-177, achieving high purity (>95%). However, only DOTA-sEVs tracer remains stable over time (>95% after 24h). Its vivo evaluation showed hepatobiliary metabolism (71.47±1.94 %AI/g in liver and 45.89±9.31 %AI/g in spleen, 72h post-administration) Conclusions: We have successfully performed 177-Lu labelling of MisEVs to develop a novel vectorised nanoradiotherapy. In future experiments, we will evaluate the biological effect of this nanotracer in tumour models.

Extracellular vesicles from human iPS cells as new tool enhancing biological functions of cord blood-derived hematopoietic stem and progenitor cells

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Human iPS cells (hiPSCs) are a promising source of cells for the purposes of regenerative medicine. Apart from their utilization for cell-based therapy, there has been a growing interest in using the paracrine activity of those cells, that comprises secretion of extracellular vesicles (hiPSC-EVs). Growing evidence indicates that the bioactive cargo transferred by hiPSC-EVs may serve as potential new-generation therapeutic strategy. However, since a detailed mechanism of hiPSC-EVs activity is still not fully understood, in the current study we aimed at investigating molecular composition of hiPSC-EVs. Additionally, we evaluated functional impact of hiPSC-EVs on cord blood-derived hematopoietic stem and progenitor cells (CB-HSPCs).

Our results revealed that hiPSC-EVs contain bioactive content in the form of miRNA, that may differ from their parental cells. Additionally, hiPSC-EVs might modulate signalling pathways in CB-HSPCs on protein and gene expression level. Moreover, we demonstrated that hiPSC-EVs may improve functional properties of HSPCs in vitro, including metabolic activity, hematopoietic and clonogenic potential, as well as survival, chemotactic response and adhesion to the model components of hematopoietic niche. Importantly, hiPSC-EVs enhanced homing and engraftment of CB-HSPCs in vivo.
In conclusion, our data support the concept envisioning hiPSC-EVs as paracrine modulators of target cells, containing bioactive cargo in the form of miRNA. Particularly, we demonstrated that hiPSC-EVs may improve several functions of CB-derived HSPCs important for their hematopoietic potential following the transplantation, enhancing future applications of CB in the field of hematology.

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Clonal Immortalized Mesenchymal Stromal Cells: an ideal cell source for a reproducible and standardized production of therapeutic extracellular vesicles.

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The therapeutic potential of extracellular vesicles derived from primary mesenchymal stromal cells (MSC-EVs) has been effectively demonstrated in various animal models and pre-clinical trials. In our research group, we have successfully demonstrated the therapeutic benefits of EVs obtained from the conditioned media of cultured primary MSCs derived from bone marrow. These EVs have shown improvement in treating a patient with treatment-resistant graft-versus-host disease (GvHD), as well as in animal models of ischemic stroke and hypoxia-induced neonatal encephalopathy (HIE).
However, the significant heterogeneity among donors and the limited lifespan of primary MSCs in vitro pose significant challenges in translating primary MSC-EV products into therapies. To address this issue, we have developed strategies to enable the standardization, reproducibility, and scalable production of EVs. To achieve this, we have designed methods to immortalize primary MSCs. Through this approach, we have successfully immortalized primary MSCs, generating clonal immortalized MSC lines (ciMSCs), while ensuring that these ciMSCs retain their healthy bona fide characteristics.
Furthermore, we have demonstrated that EVs derived from ciMSCs (ciMSC-EVs) are capable of suppressing T cell activation in a multi-donor mixed lymphocyte reaction assay. Additionally, they have shown the ability to reduce the symptoms in ischemic stroke and HIE models, similar to primary MSC-EVs. Based on these findings, we conclude that ciMSC-EVs possess therapeutic potential, and ciMSCs represent an ideal cell source for the standardized, reproducible, and scalable manufacturing of EV-based therapeutics.

Manufacturing of Wharton's Jelly Mesenchymal Stromal Cells-derived Extracellular Vesicles for Clinical Application

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Extracellular vesicles derived from mesenchymal stromal cells (MSC-EVs) are gaining interest due to their regenerative and immunomodulatory properties. However, large-scale GMP-compliant manufacturing of

MSC-EVs remains challenging. Here, we aimed to comparatively test EVs derived from clinical-grade Wharton's Jelly (WJ)-MSCs grown in 2D cell culture flasks or 3D microcarrier-based bioreactors using human serum-supplemented (SER) or chemically-defined medium (CHEM). EVs were manufactured by a cleanroom-adapted semiautomated procedure combining tangential flow filtration and size exclusion chromatography. In vitro, identity and purity of produced EV batches were characterized by bead-based flow cytometry, cryo-TEM, NTA, TRPS and protein-to-lipid ratio. Immunomodulatory potency was evaluated in both inhibition of T cell proliferation and CD73 enzymatic activity assays. Safety and biodistribution of EVs once administered in vivo were analysed in healthy mice (n=8/group), while their functional activity was assessed in mice following myocardial infarction (MI) (n=10-12/group). EV batches expressed EV- and MSC-specific markers as well as low MHC-I levels. 3D-CHEM culture conditions yielded smaller EVs (median 100nm in 3D-CHEM vs. 117nm in 2D-SER; $p<0.0001$). In terms of immunomodulatory potency, EVs exclusively derived from 3D-CHEM demonstrated robust inter-batch and inter-assay reproducibility. No adverse effects, reactivity or teratogenicity were found after intravenous infusion of increasing or repeated EV doses. Infused EVs were initially detected in lungs and then rapidly accumulated in the liver and spleen, with no detection in brain or gonads. Infarcted animals with intravenously administered 3D-CHEM-derived EVs exhibited a slightly less reduction of the left ventricle ejection fraction at 21 days post-MI versus baseline compared to non-EV-treated animals, but differences did not reach statistical significance probably due to limited sample size. Taken together, in advance of more reliable efficacy studies using more direct local delivery approaches, our preliminary results suggest that 3D-CHEM-derived WJMSC-EVs show more consistent immunomodulatory potency in vitro and could be of assistance post-MI.

Incorporating Extracellular Vesicles from dendritic cells in fibrinogen and magnesium scaffolds to promote bone regeneration

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Bone fractures are common injuries that require hospital treatment, and in 5-10% of cases result in non-union or delayed union. A better understanding of cell-cell communication during the bone regeneration process is crucial for the development of new therapeutic approaches to overcome this problem. Our previous work shows that Dendritic Cells (DC) secreted Extracellular Vesicles (EV) are the main secretome component mediating MSC recruitment (1). In parallel, we demonstrated the regenerative potential of Fibrinogen (Fg) scaffolds (2), and that their modification with 10mM of Magnesium (Mg) modulates macrophage response in vitro and in vivo (3). Herein, we aim to boost the features of FgMg scaffolds by incorporating DC-EV, to promote MSC recruitment to the injury site.

Scaffolds were produced by freeze-drying and characterized (3). Primary human peripheral blood monocyte-derived DC were cultured, and their secreted EV were isolated by differential (ultra)-centrifugation and characterized (1). EV and scaffolds were combined and EV release was investigated. The scaffolds functional impact on MSC recruitment and osteogenic differentiation was assessed. Ethical permission was sought for all studies.

Results showed that DC-EV enabled scaffolds were more efficient at recruiting MSC than scaffolds alone. Moreover, MSC cultured on FgMg scaffolds showed significantly increased osteogenic differentiation, in comparison with those cultured on Fg scaffolds.

Therefore, combining Fg scaffold with DC-secreted EV acts to promote MSC recruitment, while their modification by Mg promotes MSC osteogenic differentiation. Overall, EV-enabled FgMg scaffolds are promising candidates to promote bone formation.

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Standardized cell factories for production of biologically active, clinical-grade MSC-EVs

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Human mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) are considered to have broad therapeutic applicability in age-associated diseases and tissue regeneration due to their anti-inflammatory and pro-reparative properties. However, the clinical application of MSC-EVs is hampered by reproducible and standardized EV production protocols. In particular, the use of primary MSCs as a source for EV production is limited due to their restrictive replicative life span and loss of tri-lineage differentiation capacity resulting in the inability to produce sufficient EVs of reproducible functional characteristics. Overexpression of the catalytic subunit of human telomerase (hTERT) has been shown to extend the cellular life span of various cell types ('telomerized cells') while maintaining their primary cell-like characteristics. We extended the replicative life span and differentiation capacity of human MSCs from different tissue origins, including Wharton's Jelly, bone marrow, adipose tissue, placenta, amniotic membrane and dental pulp, by ectopic expression of hTERT at early population doublings and investigated cell-type specific characteristics as well as their suitability as EV production hosts. EVs were produced using different xeno-free culture conditions and scalable hollow fiber bioreactors, and subsequently isolated by tangential flow filtration. Enriched EVs maintained typical EV-marker expression profiles and morphological characteristics, according to MISEV guidelines. EVs isolated from telomerized and primary MSCs showed similar miRNA expression patterns, and absence of full-length hTERT mRNA, demonstrating limited effects of immortalization on EV characteristics. Moreover, EVs isolated from telomerized MSCs maintained anti-inflammatory, anti-fibrotic, wound healing, and pro-angiogenic properties. This demonstrates the safe and promising use of telomerized MSC lines as hosts for standardized, reproducible, and scalable EV production.

Optimization of EV-protein tagging strategies

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Introduction. Extracellular vesicles (EVs) are increasingly being studied as drug delivery systems due to their high blood stability and low immunogenicity. However, currently there are still no standardized procedures for effective loading of therapeutic agents into vesicles. In this project we explored different methodologies for loading therapeutic affimers, small proteins mimicking-antibody activity, into EVs, both endogenous and exogenously.

Methodology. For endogenous EV membrane loading, the affimer was fused to CD63 tetraspanin. While C-terminus fusion allowed location of the affimer on the lumen of the vesicle, fusion to 2nd loop of CD63 granted external location. Endogenous luminal, no membrane-bound, loading was reached by fusing the affimer to a targeting peptide derived from EV soluble protein N-Syntenin. To test exogenous loading, the affimer was modified with a short peptide from GAPDH protein named G58 with reported affinity for the EV membrane. Endogenous loading constructs were chemically transfected into EV producing HEK293T cells and consequently recombinant proteins were expressed on vesicles from the outset. Conversely, exogenous loading was carried out by incubation of EVs with soluble G58-affimer protein for surface binding. All constructs were generated by Gibson Assembly technique including mClover3 or nanoLuc as reporters. EVs were concentrated by ultrafiltration and isolated by Size Exclusion Chromatography. Cargo loading was assessed by western blot and flow cytometry.

Results & Discussion. Affimer and reporter protein were found into EV cargo with all tested strategies, being CD63 C-terminus the construct with the best loading capacity. However, other moieties are also effective and should be considered depending on the particulars of the therapeutic protein of interest e.g. subcellular location of the target. Therapeutic efficacy assays will determine the real potential for the distinct affimer-EV conformations in a case per case approach.

Enhancing Immunomodulatory Potential of Immortalized Mesenchymal Stromal Cell-Derived Extracellular Vesicles through Overexpression CD39, key regulator of the purinergic adenosine signaling pathway

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Mesenchymal stromal cells (MSCs) possess therapeutic properties and have been extensively studied in clinical trials. Their therapeutic effects are primarily mediated by paracrine factors, with Extracellular Vesicles (EVs) playing a significant role. MSC-EVs have been shown to convert pro-inflammatory extracellular adenosine phosphates into anti-inflammatory adenosine, contributing to their immunomodulatory effects. While primary MSC-EVs contain CD73, an enzyme that converts AMP into adenosine, CD39, the enzyme responsible for converting ATP and ADP into AMP, is typically absent in MSC-EVs. However, other cell types in contact with MSCs, such as regulatory T cells, can provide CD39. In this study, we engineered clonally expanded immortalized MSCs (ciMSCs) to express CD39 by introducing the coding region of CD39 into lentiviral vectors. The immunomodulatory effects of the engineered EVs, with or without ATP, were assessed using a modified multi-donor Mixed Lymphocyte Reaction assay (mdMLR). Our findings demonstrate the successful expression of CD39 in the engineered immortalized MSC cell line following transduction with CD39-eGFP lentiviral particles. The MSC-EVs maintained their characteristic morphology, molecular cell surface phenotype, and multilineage differentiation capacity.

Furthermore, we confirmed the enzymatic activity of EVs co-expressing CD39 and CD73, as they effectively hydrolyzed ATP and generated adenosine. Moreover, the engineered ciMSC-EVs exhibited a more pronounced reduction in activated CD4 and CD8 T cells compared to unmanipulated EVs, an effect attenuated by an A2A adenosine receptor inhibitor. Utilizing High Dimensional Analysis with Uniform Manifold Approximation and Projection (UMAP), we identified a positive correlation between the immunomodulatory activity of engineered ciMSC-EVs and the promotion of an anti-inflammatory monocyte phenotype (CD14++ HLA-DR dim) as well as the induction of regulatory T cells expressing (CD4+ CD25++ CD127 dim).

Our findings demonstrate that CD39 overexpression amplifies the immunomodulatory potential of ciMSC-EVs in vitro, highlighting its pivotal role in regulating T cells within inflammatory microenvironments triggered by extracellular ATP.

Mesenchymal stroma cell-derived extracellular vesicles show altered functionality after knock out of genes related to immunomodulatory capacity using CRISPR/Cas9

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Mesenchymal stroma cells (MSCs) are largely believed to confer therapeutic benefits via their secretome, specifically through extracellular vesicles (EVs). Even though the therapeutic potential of MSC-EV preparations is increasingly supported by preclinical studies and some clinical applications, a comprehensive understanding of the mechanisms of action is still lacking. To address this, we developed a CRISPR/Cas9 based strategy to investigate the roles of selected antigens, specifically ecto-5'-nucleotidase (CD73) and the tetraspanin CD81, in the therapeutic function of EVs. Bone-marrow derived MSCs were immortalized and genetically modified using a lentiviral based CRISPR-Cas9 methodology to delete the coding region of CD73 and CD81, respectively. The successful knock out of respective genes was confirmed by flow cytometric analyses and genomic PCR. The immunomodulatory effect of resulting MSC-EV preparations was investigated in a multi-donor mixed-lymphocyte reaction (mdMLR) assay. The enzymatic activity of CD73 was determined by an ELISA based assay. Impacts on the EV secretion were analyzed by imaging flow cytometry using a panel of 24 different antibodies, some considered as specific and others as nonspecific MSC markers. Our findings show that MSCs with either CD73 or CD81 knockouts didn't exhibit changes in their morphology or proliferation behavior. Furthermore, the absence of CD73 or CD81 in MSC-EV preparations didn't cause phenotypic changes in the MSC-EV populations. Interestingly, despite lacking CD73, EVs from CD73 knockout MSCs retained the same immunomodulatory capability in the mdMLR. However, EVs from CD81 knockout MSCs showed no activity in the mdMLR. Our results support that CRISPR-Cas9 gene knockout in MSCs is an effective technique to study the role of EV associated molecules. Despite CD73 knockout not affecting the immunomodulatory potential of MSC-EVs in the mdMLR, it may be vital for therapeutic functions in preclinical settings. We intend to further explore this hypothesis using appropriate in vivo models in future studies.

Therapeutic Extracellular Vesicles (TEVs) to deliver Cas9-sgRNA ribonucleoproteins for the treatment of recessive dystrophic epidermolysis bullosa.

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Recessive dystrophic epidermolysis bullosa (EB) is characterized by mutations in the COL7A1 gene encoding type VII collagen (C7). The most frequent mutation in the Spanish patient cohort is c.6527insC1. This mutation results in a premature stop codon that synthetises a truncated, non-functional C7 and consequently produces poor adhesion between the dermis and epidermis. We have described that C7 functionality can be restored by deleting the exon 80 affected by the mutation using CRISPR/Cas9-based approaches. However, the delivery of Cas9 machinery to target cells in vitro and in vivo is a challenge and new approaches are necessary. We have generated therapeutic extracellular vesicles (TEVs) from HEK-293 cells transfected with the appropriate plasmids, which contain Cas9 nuclease and two specific sgRNAs designed to remove the exon 80 of COL7A1. In a standard production experiment >1010 TEVs per mL of cell culture are produced which contain approximately 4x10-7 ng Cas9 per vesicle, as calculated by western-blot. These TEVs are internalized by different target cells and can be used to efficiently remove COL7A1 exon 80 reframing the gene and restoring the C7 functionality. Exon deletion is dose-dependent, reaching levels of 50% when a dose of 25.000 TEVs per target cell is used. In

conclusion, therapeutic extracellular vesicles for the treatment of EB can be efficiently produced from HEK-293 cells and used as a vehicle to transport Cas9 ribonucleoproteins to target cells to restore the function of C7 protein. The system is versatile and can be adaptable to other pathologies in which gene therapy using Cas9 ribonucleopoteins to delete or inactivate a determined gene is a therapeutic option.

Improving Exosome Production and Immunogenicity for More Effective CAR T Cell Therapy

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Immunotherapy using CAR T cells has shown remarkable results in treating hematological malignancies. However, their efficacy in treating solid tumors is challenging. Exosomes have emerged as a promising complementary therapy to overcome this limitation. One such approach is the use of exosomes derived from CAR T cells (EXO-CAR-T). However, the production of homogeneous exosomes with reduced immunogenicity remains a significant challenge. A better understanding of the cellular processes controlling exosome biogenesis and their modulation can aid in producing non-immunogenic exosomes from CAR T cells, thereby enhancing their clinical applicability.

Our assessment involved genetically modifying a T cell line by overexpressing CD63 and HGS, two proteins that play a critical role in exosome formation. Furthermore, we also investigated the potential impact of HLA-I elimination on the allo-reactivity of the T cell-derived exosomes.

Our preliminary data showed that overexpression of HGS and CD63 genes in Jurkat cell lines enhances the EXOs production. On the other hand, our results showed that anti-CD19 CAR-T cells produce functional anti-CD19 CAR T exosomes that could kill tumor cells. Finally, the use of B2MKO T cells in a transwell assay result in a reduction in the proliferation of third-party PBMCs.

Based on the observed increase in exosome production resulting from genetic manipulation of Jurkat cells and the ability of anti-CD19 CAR T exosomes to kill tumor cells, along with the absence of alloreactivity estimated with the transwell assay, it is suggested that exosomes have the potential to be utilized as an off-the-shelf, cell-free approach for cancer therapeutics.

Efficient non-enzymatic isolation of brain-derived extracellular vesicles

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Extracellular vesicles (EVs) are membranous vesicles released by most if not all, cells. EVs carry proteins, lipids, and nucleic acids and participate in cell-to-cell communication. EVs have been related to crucial brain functions, such as myelin maintenance and neurotransmission, but their complete role is unknown. EVs emerged relevant in Neurodegenerative diseases pathophysiology (such as Alzheimer's (AD)) and have been described as potential biomarkers in patients' biofluids. However, the isolation of EVs from brain tissue remains challenging, often requiring enzymatic digestion steps that may introduce unwanted biases and affect EV integrity. In this study, we present an efficient non-enzymatic approach for isolating brain-derived extracellular vesicles (BDEVs) to approach the role of these vesicles in AD.

Our method employs sequential ultracentrifugation and a density-based gradient to achieve a high yield and purity of BDEVs from brain tissue without any enzymatic digestion. Characterization of the isolated EVs revealed their typical morphology and size distribution, as confirmed by transmission electron microscopy and nanoparticle tracking analysis. Furthermore, we validated the presence of known EV markers, such as Flotlin-1,

CD81, Alix, and the prion protein (PrP) through Western blot without any artificial proteolytic pattern. Finally, we find no differences in mRNA content of the non-enzymatically obtained BDEVs compared with BDEV isolated with collagenase, meaning that the intraluminal part of the BDEVs is equally intact. Similarly, our proteomic analysis shows the same BDEVs-related protein expression. However, other proteins, potentially in the BDEVs corona and membrane, are differentially expressed, highlighting the consequences of the enzymatically-based isolation protocol.

Our non-enzymatic isolation approach provides a reliable and efficient method for obtaining BDEVs without any substantial subpopulation depletion/enrichment but without artificial protein pruning. This technique holds promise for a better understanding the BDEVs' role in brain disorders with a more natural model for developing functional and descriptive analysis.

Membrane Sensing Peptides: reversible affinity isolation of Extracellular Vesicles from minimally pre-treated biological fluids

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Extracellular vesicles (EVs) isolation from complex bio-samples using affinity-based methods are typically hampered by poor recovery and frequently requires a pre-concentration step. Here we present a new, rapid, effective, and reliable procedure based on affinity isolation with Membrane Sensing Peptides (MSP). A traceless release strategy was developed providing intact EVs after isolation via magnetic beads from cell culture supernatant, urine, plasma and serum allowing downstream characterization according to MISEV guidelines and including western blotting for EV-associated markers, transmission electron microscopy (TEM) and super resolution microscopy (SRM) imaging. EV isolation is demonstrated with minimal carry over of common contaminants such as lipoproteins for the blood-based workflow and uromodulin for the urinary EVs. A comparison of yield and purity is reported with standard procedures using antibody modified beads, ultracentrifugation (UC), ultrafiltration (UF), size-exclusion chromatography (SEC), showing increased recovery yield for the MSP based method. Integrity of released EVs is demonstrated by TEM and SRM and functionality by cellular assays.
In synthesis, using minimally pre-treated biofluids (serum, plasma, and urine), we offer a new isolation methodology based on affinity to general membrane characteristics of EVs, unbiased by relative abundance of surface markers. The protocol can be applied to EV samples from any species, including animal, plant, and bacterial vesicles for which there are no currently available antibodies.

Affinity capture nanodiagnostic devices for EV biomarker detection

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Affinity capture allows the isolation of biochemical markers from complex biological samples for further analysis. Affinity nanodiagnostic devices use natural or synthetic ligands bound to nanospheres to specifically engage the target molecular entity, and thus isolate it from the rest of the sample. This group specializes in the

design and development of micro- and nanospheres multifunctionalized with all kinds of affinity ligands and detection labels to offer customized solutions for extracellular vesicles (EV) asociated biomarker detection and quantification, thus providing valuable tools for preclinical and clinical applications. The capture and isolation of a specific subset of EVs given by the specificity of the affinity ligand allows a detailed evaluation of further biomarkers in the specific EV subset. This communication summarizes the chemical procedures to synthesize these devices via multifunctionalization with a specific affinity ligand and a reporter tag, and the development of assays for the detection of EVs through colorimetry, amperometry, flow cytometry, image cytometry and mass cytometry. A special focus will be given to the generation of multiplexing analytical methods to evaluate multiple parameters and samples in a single well. In particular, mass cytometry (CyToF) is a new analytical tool now available in a few biomedical labs that allows the simultaneous detection and quantification up to 35 markers within a single event. It uses rare earth elemental tags, which provide unique fingerprint signals that are not naturally found within cells, thus avoiding background noise or spectral overlapping. Mass cytometry allows the simultaneous determination of dozens of extracellular vesicle markers and barcoding of samples from different patients for detection in a single well, thus reducing inter-experiment variability and allowing an efficient comparison of inter-individual samples and controls. Our recent advances in barcoding and multiplexing characterization of affinity isolated EVs will be presented.

Utilizing Lectins for Glycan Analysis on Extracellular Vesicles (EVs)

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The glycan structure on EVs plays a vital role in cellular processes such as immune evasion and modulation, forming a component of the currently elusive EV 'address code'. Commonly employed techniques to scrutinize glycans on heterogeneous EV populations include mass spectrometry, lectin arrays, and bead-based lectin flow cytometry. In this study, we describe an innovative approach that employs soluble human lectins with known tetraspanin antibodies to evaluate EVs in conditioned medium (CM) and enriched EV fractions from mesenchymal stromal/stem cells (MSCs) of various origins.

In our approach, we employed fluorescently labeled lectins, such as galectins (Gal-1, -2, -3, -4, -7, -8, -9), and tetraspanin antibodies (CD63, CD81, CD9), to examine the binding properties of extracellular vesicles (EVs) through flow cytometry analysis. Using flow cytometry and solid-phase assays, EVs derived from diverse cell sources (Wharton's jelly and bone marrow) were assessed for galectin and antibody binding. Galectin binding was explored independently and in combination with tetraspanin antibodies.
Our findings on MSC-derived EVs suggest that galectins bind to a significant EV population, while CD63 does not mark all galectin-positive EVs. In conclusion, galectins may be an additional flow cytometry tool for comprehensive EV analysis. This approach enhances the resolution of the EV population under study and incorporates glycostructure characteristics into the EV analysis.

Selective detection of biological nanoparticles labeled with gold nanoparticles in a heterogeneous sample

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Multiple challenges remain in characterizing optically faint colloidal particles in their native liquid environment. One of these is to distinguish between different types of submicron biological nanoparticles in a heterogeneous sample. This is especially difficult when trying to detect few specific particles against a background of many nanoparticles and large molecules, a challenge that must be overcome to successfully detect viruses or extracellular vesicles (EV) in biological samples.

We present here a novel method to detect small biological nanoparticles such as viruses, EV mimics and EVs by labeling them with gold nanoparticles and selectively detect labeled particles by using digital holographic microscopy combined with particle tracking. One advantage is that unlabeled biological particles as well as individual gold nanoparticles below the detection limit will not interfere much with the detection of complexes formed between gold nanoparticles and biological nanoparticles, enabling the use of an excess of labeling particles and also specific detection in minimally diluted biological samples. Compared with molecular based methods, this approach therefore has the potential advantage of better avoiding false positive results by directly detecting and counting intact virus and EVs. Further, compared to using fluorescent molecules, comparably few binding sites are needed for detection.

The use of this approach for detection of viruses, EV mimics and native EVs will be presented and discussed.

Cell surface area as normalization factor to study prostate cancer EV targeting to specific cell types

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Cancer cell-derived EVs are known to have organotropism. Prostate cancer can metastasize towards bone and their EVs can affect osteoblasts and osteoclasts. However, data on how efficient these EVs target different bone cells versus other potential target cells are limited. Comparative analysis of EV interaction with cell types, such as multinuclear osteoclasts, with substantial difference in cell size and morphology is challenging and refutes strategies where equal amount of EVs are added to equal cell numbers. We therefore designed an experimental strategy using cell coverage area in culture systems as normalization factor for comparing EV-targeting to bone cells and monocytes.

Osteoblasts (SAOS2), osteoclasts (differentiated from THP1), monocytes (THP1), and prostate cancer cells (PC3) were seeded in various amounts per well. Light microscopic imaging was used to calculate cell coverage area. EVs from PC3 cells cultured in EV-depleted medium were enriched using differential centrifugation, labeled with PKH26, and purified by iodixanol density gradient centrifugation. Wells with similar coverage area were used for EV incubation. Fluorescently labeled dextran was simultaneously added to explore differences in receptor-mediated binding/uptake or fluid-phase pinocytosis. Binding/uptake of EVs and dextran by cells was measured by flow cytometry.

Different numbers of the target cell types needed to be seeded to obtain equal cell coverage per well. Osteoblasts were most efficient in EV binding/uptake, followed by osteoclasts and PC3 cells. Monocytes were least efficient to interact with PC3 EVs. Whereas some of these cell types readily interacted with EVs, others more efficiently internalized fluid phase dextran.

Optimized strategies to compare EV binding/uptake to different target cell types can help to identify EV-subsets targeting specific tissues and molecules involved in this process.

The role of extracellular vesicles in COVID-19 infection disease progression

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Background and aim: The hypercoagulable state is commonly considered a major component of the pathophysiology of the infectious disease COVID-19. It is characterised by a dysregulation of multiple biological pathways, spearheaded by an abnormal immune response and a persistent pro-inflammatory state, which ultimately converge to trigger the development of a serious hemostasis disturbance in the form of localised and systemic coagulopathies and thrombotic events. The surface antigen makeup of each microvesicle subtype depends on its cellular origin and the pathophysiological processes responsible for its release. COVID-19 patients show many of the pathophysiological processes that are associated with the cellular release of EVs, including endothelial injury, platelet activation, TF-mediated procoagulant activity, and increased thrombin generation.
The aim is to isolate, by CytoFLEX-SRT cytometry, circulating plasma EVs expressing Sars-CoV-2-Nucleoprotein (NP) from COVID-19 patients and to evaluate their in vitro capability to activate human endothelial cells.
Methods: Plasma from COVID-19 patients were stained with calcein-AM and Sars-CoV-2-NP. The positive EVs (70-200 nm), were isolated by CytoFLEX-SRT cytometry. Human coronary aortic endothelial cells (HCAEC) were treated with the isolated EVs and analysed for the ability to endocytose EVs by using confocal microscopy. To evaluate endothelial cell activation by EVs, the expression of tissue factor was investigated.
Results: Our study confirms the presence of increased levels of several EV subtypes derived from cells involved in COVID-19 coagulopathy. HCAEC are able to internalise Sars-CoV-2-NP+ and calcein-AM+ EVs isolated from COVID-19 patient plasma. Endothelial activation is confirmed by the increased expression of tissue factor.
Conclusion: These results suggest that EVs are involved in the cell-cell communication and play an important role as virus protein vehicle in the progression of the disease. Further studies are needed to define the exact mechanism by which the different EVs subtypes play a role in the pathogenesis of COVID-19 coagulopathy.

Evaluation of the role of CD133-Extracellular vesicles secreted by TNBC cells in promoting cancer aggressiveness

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Breast cancer is the most frequently diagnosed malignancy and represents the leading cause of cancer mortality in women worldwide. Triple-negative breast cancer (TNBC), in particular, is the most aggressive subtype of breast cancer with a poor prognosis due to the absence of targetable receptors, such as estrogen receptor progesterone receptor, and epidermal growth factor receptor-2, accounting for approximately 15%–20% of breast cancer. The metastatic spread of cells from the primary tumor to distant organs is the primary cause of morbidity and mortality in breast cancer. In these tumors, high levels of CD133 have been correlated with adverse outcomes and chemotherapy resistance. We reported that CD133 promotes the secretion of Extracellular Vesicles (EVs) and hypothesized that CD133-EVs released by breast cancer cells may contribute to cancer aggressiveness. EVs from two triple-negative breast cancer cell lines and one hormone-dependent cell line were isolated and characterized The three cell lines secrete a heterogeneous population of EVs, that carry EV-specific markers while also bearing CD133. Moreover, our data indicate that TNBC-derived EVs, bearing CD133, promote branching and sprouting in endothelial cells, a key feature in cancer invasion. We are currently evaluating in vitro the function of EVs on migration and epithelial-mesenchymal transition potentially related to the metastatic capacity of cancer cells and in vivo the ability of EVs derived from TNBC cells to disseminate throughout an organism and promote metastasis at specific distant organs. Furthermore, we want to correlate the possible association of CD133 overexpression in primary tumors and metastases with clinical and biological parameters relevant to patient care. For this purpose, we have focused on the study of the content of EVs from healthy and TNBC patients at different stages of chemotherapy treatment.

Protein corona of EVs is essential for skin cell self organization and aids wound healing

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Introduction:
Transport of functional protein cargo via extracellular vesicles (EVs) is an important mechanism in cell communication. We investigated the distribution of active cargo proteins between EV's inside and outside to evaluate their implications for theraputic applications.

Material & Methods:
EVs from placenta-derived stromal (PLX) cells conditioned medium or from human platelet lysates were enriched by tangential flow filtration (TFF) optionally followed by ultracentrifugation or size exclusion chromatography (SEC). Following MISEV2018 guidelines EV preparations were characterized using western blot,

tunable resistive puls sensing (TRPS) , cryo electron microscopy and super-resolution microscopy. Self organization capacity in presence or absence of different EV preparations was analyzed in a highthroghput organoid assay. Contribution of EVs to woundhealing was assessed in an in vivo model transplanting human skin cells on NSG mice.

Results:
EV identity could be confirmed for both sources by enriched expression of tetraspanins in EV preparations compared to originating cells. Integrity for platelet and PLX cell derived EVs was verified by cryo electron microscopy and super resolution microscopy. Spheroid formation of human skin derived fibroblasts alone or as whole skin organoids together with endothelial cells and keratinocytes was only succesful in the presence of TFF purified EVs bearing a functional protein corona while soluble factors alone or corona depleted EVs after SEC could not initiate this process. Also in our in vivo mouse model TFF purified EVs were essential for proper organization of the human skin cells in the wound area and providing sufficient vascular suport.

Discussion:
This findings allowed us to develop a self organizing human skin model in mice and shows the important role of corona proteins for EV function. Recent calculations indicating a ‘surface-to-bulk partition of EV cargo’, for EVs < 180nm, in favor of surface cargo loading support the new concept of a functional EV corona.

Proteomic comparison between non-purified CSF and CSF-derived EVs from patients with Alzheimer's, Parkinson's and Lewy body dementia

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Extracellular vesicles (EVs) have recently been considered as a potential biomarker source for a variety of diseases, including neurodegenerative disorders. EVs are important mediators of intercellular communication due to their capacity to transfer genetic material, lipids and proteins. By means of their communication role, interesting biomarkers are often enriched in EVs compared to total biofluid. In our study, this hypothesis is tested as untargeted proteomics is performed on both CSF samples and CSF-derived EV samples from the same cohort with different dementia diseases.

CSF samples were collected from 297 patients (with Alzheimer's disease, Parkinson's disease, PD with mild cognitive impairment, PD with dementia, Lewy Body dementia and healthy controls. Those samples were, on the one hand, kept as non-purified CSF, and on the other hand, purified with the SmartSEC HT EV isolation kit (System Biosciences). On a selection of samples of both non-purified CSF and CSF-derived EVs, targeted proteomics (SIMOA) was performed within a MOVE collaboration. Both types of samples, of all 297 patients, were analysed with label-free LC-MS/MS as the discovery phase of a biomarker study.

We observed a greater number of differentially expressed proteins in CSF-derived EV samples (N = 276) compared to non-purified CSF (N = 169), with minimal overlap between both datasets. This finding suggests that CSF-derived EV samples may be more suitable for the discovery phase of a biomarker study, due to the removal of more abundant proteins, resulting in a narrower dynamic range. From the combined lists of differentially expressed proteins, we identified the most promising potential biomarkers to differentiate between the different included dementia diseases, which will be validated in a further study.

Red Blood Cells-derived Extracellular Vesicles as targetable drug delivery vehicles

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Thanks to their ability to transport functional cargoes and the possibility of modifying their surface to incorporate ligands, EVs have been recently considered valuable delivery vehicles, especially for targeted therapy. Particularly, red blood cell-derived nanoparticles, which have been found to be beneficial in terms of yield, bioavailability, and drug loading, have shown promising potential as efficient delivery systems for the targeted delivery of drugs. In this study, we propose an exogenous method for the functionalization of nanoerythrosome surface (NanoEs, artificial erythrocyte-derived vesicles) with a fluorescent peptide able to target the tumor extracellular matrix. Our strategy is based on the “classic” click chemistry reaction of an azide with an alkyne (Huisgen cycloaddition), without copper. In this method, a DBCO-NHS ester tied to the amino group of the NanoE-membrane proteins, links to an azo-fluorescent peptide which can recognize an oncofetal variant of the fibronectin (Extra Domain B(EDB)-fibronectin), absent or only minimally expressed in healthy adult tissues but upregulated in various disease states, including cancer. Flow cytometry was selected to (i) detect the fluorescent signal associated with an accomplished NanoE functionalization and (ii) evaluate whether NanoE staining with CFSE can affect the efficiency of click chemistry. Moreover, in vitro experiments, analyzed by Ligand Tracer technology and Imaging flow cytometry, were conducted on a cancer cell line to examine the interaction and subsequent internalization of anti-EDB-NanoEs. Click chemistry turned out to be a successful targeting system (about 50% of engineered-NanoEs) and functionalized NanoEs were efficiently internalized by responder cells. Our EV membrane functionalizing system represents a promising tool that can be potentially applied for both molecular imaging and targeted therapy not only in cancer, but also in other pathological conditions.

I am a MOVE fellow and thanks to the fellowship I could perform this work in Saara Laitinen’s Lab in Finland.

Development of a protocol for the isolation and characterization of extracellular vesicles from Manila clam hemolymph

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Besides their importance as food source, marine bivalves (e.g. claims) with their filter-feeding behavior are considered possible sentinels for environmental monitoring. Since extracellular vesicles (EVs) are regarded innovative biomarkers and EVs in bivalves are poorly studied, we aimed to isolate EVs from bivalve hemolymph to use them for health and environmental monitoring. Here, we present a protocol for EV-isolation from Manila clam hemolymph, describing the adaptations to standard EV-isolation protocols needed to deal with hemolymph complexity and high osmolarity.
Hemolymph was collected from living clams and stored at -80°C until use. After 10,000xg differential ultracentrifugation, hemolymph supernatant was diluted 1:2 in PBS or in ultrapure water with 3.2% of NaCl. EVs were isolated with top-down sucrose density gradient ultracentrifugation and 12 fractions collected and pooled in groups of three from both PBS and NaCl-diluted samples (PBS-fractions and NaCl-fractions). Each pool was analyzed by protein quantification (BCA assay), gel electrophoresis (Sypro-ruby staining), Nanoparticle Tracking Analysis (NTA) and transmission electron microscopy (TEM).
TEM revealed the similarity of pooled PBS and NaCl-fractions 7-9 (mean density 1.2 g/mL), where most of EVs (100-200 nm) with least contaminants were present. EVs were also present in other fractions, especially in 4-6, but with more co-isolated contaminants. Importantly, dilution of hemolymph in PBS caused the formation of precipitates. Interestingly, while protein concentration in PBS-fractions 7-9 was lower (10 µg/ml) than in NaCl-fractions 7-9 (150 µg/ml), particle concentration measured by NTA were comparable (1*1010 particles/ml).
To conclude, we report a tailored protocol for EV-isolation from Manila clam hemolymph. Importantly, diluting hemolymph in a hypertonic solution rather than in PBS avoids precipitates that hamper further EV isolation and processing. Considering the commercial and scientific importance of clams, this protocol provides a base for further characterization of clam-derived EVs in relation to health and environmental monitoring.

Characterization of mRNA loading onto mammal extracellular vesicles by STORM Imaging

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The potential future use of Extracellular Vesicles (EVs) as therapeutical delivery systems has been gaining interest in the last decade. Among the current challenges of such technology is the need of an efficient loading method to encapsulates the active principle of interest into the EVs. Most researchers have worked on the loading of drugs and proteins; however, fewer have studied the genetic material loading, which could enable the use of EVs as vaccines or as gene therapy bullets. For this reason, our aim was to set up an efficient methodology for the efficient encapsulation of big macromolecules such as mRNA.

The study has been carried out with mRNA codifying for EGFP as the reporter cargo. As the encapsulation efficiency can be low, we need a sensitive technique to analyse our mRNA loaded EVs. Thus, we are developing imaging methods based on Stochastic Optical Resolution Microscopy (STORM), a technique that allows the study of single molecules with a resolution of 20nm.

For samples imaging, EVs have been stained with the lectin WGA488, and mRNA with Cy5 fluorophore, both optimal for STORM imaging. The idea is to match the EVs signal from the 488 channel with the mRNA signal from the Cy5 channel. Images are based on different localizations of the fluorophores, captured by its photoswitchable property, which results in a higher resolution image, going beyond the resolution limits of traditional fluorescence microscopy. After solving some troubleshooting, as buffer composition and sample processing, the optimal sample preparation conditions were set up. Images were then corrected and filtered by ImageJ and analysed by MATLAB. Preliminary results showed successful mRNA encapsulation into EVs, using sonication for the encapsulation. Consequently, STORM can be an optimal technic to study the EVs loading, thanks to its resolution and sensitivity.

LC-MS/MS-based proteome profiling of CSF-derived extracellular vesicles in Alzheimer's disease

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Background. Extracellular vesicles (EVs) participate in the spread of pathological proteins between cells, contributing to the progression of neurodegenerative disorders, including Alzheimer's disease (AD). EVs from CSF constitutes a promising source to investigate novel biomarkers and therapeutic targets. We aimed to explore the unbiased proteome profile of CSF-derived EVs from individuals with AD by label-free quantitative liquid chromatography mass spectrometry (LC-MS/MS).

Methods. EVs were obtained from 500µL of CSF from cognitively healthy donors (HC,n=10,mean age=73±1.4) and patients with AD dementia (AD,n=10,mean age=76.2±0.9) by a size-exclusion chromatography (SmartSEC). EVs were characterized by nanoparticle tracking analysis (NTA). EVs were lysed and proteins were trypsinized overnight via S-Trap filters (Protifi). The peptides were separated on an Evosep nano LC system and analyzed by the TimsTOF Pro mass spectrometer. Peptides were identified using PEAKS software (1%FDR). DAVID Bioinformatics 6.8 was used to determine the gene ontology (GO) enrichment. We normalized log2 peptide intensities (quantiles) and determined the differential protein expression across groups by a linear mixed effects (fixed effects=diagnosis, random effects=peptide sequence) model using the MsqRob package in R. P-values were adjusted using Benjamini-Hochberg.

Results. NTA showed homogeneous EV size profiles (mean size=113.4±1.41nm). We identified 2096 proteins with at least 1 proteotypic peptide. GO enrichment analyses showed "extracellular exosome" as the most statistically significant enriched term (p=1.8×10-212). Statistical inference analysis revealed 18 differentially

expressed proteins between both diagnostic groups (adj.p<0.05), all of them increased in the AD group. Some of the greatest changes include CLUS (log2FC=0.61, adj.p=8.38×10-4), SPB1 (log2FC=1.15,adj.p=1.23×10-2), LRP1 (log2FC= 3.43, adj.p=1.23×10-2), KLK7 (log2FC=2.59,adj.p=1.23×10-2), SAP3 (log2FC=2.09,adj.p=3.1×10-2) and SHPS1 (log2FC=1.03, adj.p =4.25×10-3), which are proteins that have been previously associated with inflammation.

Conclusions: Our results reveal novel protein changes in CSF-derived EVs from individuals with AD, which could be explored further to their applicability in distinguishing the different clinical stages across the AD continuum.

Functional and Potency Assays for Profiling Bioreactor Derived Mesenchymal Stromal Cell Extracellular Vesicles in Chronic Kidney Disease

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Introduction: Chronic kidney disease (CKD) presents a global health challenge with limited treatment options. One potential therapeutic approach for CKD involves the use of mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) for their ability to modulate renal injury and inflammation. To support the translation of EV-based therapies into the clinical field, bioreactors have emerged as promising technology for large-scale EV production.

Bioreactors offer advantages such as increased yield, cost reduction, and minimized manual handling, while ensuring consistent quality and reproducibility. However, it's crucial to thoroughly characterize MSC-EVs from bioreactors to facilitate successful clinical translation. This characterization includes evaluating batch-to-batch reproducibility and assessing the functional parameters of EVs for therapeutic purposes. In this study, we focused on employing functional and potency in vitro assays to profile MSC-EVs derived from a hollow-fiber bioreactor within the context of CKD.

Methods: MSC-EVs obtained from bioreactor cell supernatant using size exclusion chromatography (SEC) were subjected to assays relevant to CKD, particularly to focal and segmental glomerulosclerosis (FSGS). First, we examined their immunomodulatory function by conducting T cell proliferation assays. Second, we tested their angiogenic potential in HUVEC tube formation assays. Lastly, we evaluated the ability of MSC-EVs to reduce

podocyte permeability after doxorubicin treatment, utilizing human podocyte cultures, highly differentiated cells that make the last component of the kidney filtration barrier.
Results: MSC-EVs isolated from both standard culture flasks and bioreactor cultures exhibited comparable results in the three assays performed. Specifically, MSC-EVs were effective in suppressing T cell proliferation, stimulating HUVEC angiogenesis, and abrogating the impact of doxorubicin on podocytes.
Conclusions: This study shows the potential of targeted potency assays for the functional characterization of MSC-EVs derived from bioreactor systems for downstream application and highlights therapeutic properties relevant to CKD, including immunomodulation, angiogenesis promotion, and protection against doxorubicin-induced podocyte damage.

Determining the Function of Matrix Bound and Secreted Vesicles in Mineralisation

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Introduction: Matrix-bound vesicles (MBVs) function as sites of early mineral formation yet remain insufficiently characterised. Specifically, their relationship with secreted EVs (sEVs) such as exosomes remains debated. We compared the biogenesis and pro-mineralisation capacity of MBVs and sEVs to define their composition and contribution in early osteogenesis.

Methods: sEVs and MBVs isolated from conditioned medium and ECM of mineralising MC3T3 pre-osteoblast and human MSC cultures at days 7, 9 and 14 were characterised by nanoparticle tracking analysis, western blotting, alkaline phosphatase (ALP) assays, nano-flow cytometry, super resolution microscopy (ONI) and TEM. Immunoprecipitated populations positive for ALP, a putative pro-mineralisation marker, were characterised. Binding efficiency to collagen type 1 was evaluated using fluorescence microscopy.

Results: Results were comparative across cell types. Western blots indicated expression of endosomal biogenesis markers (CD9, CD81, ALIX, TSG101) and pro-mineralising proteins (ALP, Pit1, Annexin II, Annexin V) in MBVs, with Annexin V and CD9 present in immunoprecipitated ALP-positive fractions. An ALP assay revealed higher concentrations in MBVs than sEVs, increasing from day 7 to day 14 ($p<0.05$), confirmed as EV-associated ALP by super resolution single vesicle analysis. This mirrored the pattern of electron-dense vesicles observed via TEM. Co-expression of ALP with CD9, CD63, CD81, ALIX and Annexin II was significantly upregulated in MBVs compared to sEVs ($p<0.05$). Annexin II and ALP co-expression in MBVs significantly increased from day 7 (31.5%±4.4) to 14 (44.9%±0.3) ($p<0.05$). At day 7 (88.3%±4.6), 9 (83.1%±5.9) and 14 (82.3%±4.6) a considerable percentage of ALP-positive MBVs co-expressed CD63, with more expressing at least one of CD9, CD63 or CD81 (93.3%±3.5; 97.8%±0.7; 83.4%±10.7). MBVs exhibited preferential collagen binding with minimal sEV binding.

Conclusion: Advanced imaging methods demonstrated that contrary to prevalent opinions in the field, pro-mineralising MBVs appear to possess exosomal markers and may arise via endosomal biogenesis.

Poster Presentations

Surface functionalizations for the preferential capture of extracellular vesicle

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Extracellular vesicles (EVs) are increasingly considered both as innovative biomarkers and as biomarkers carriers, gaining huge potential in terms of diagnosis, progression and therapy of diseases like cancer, cardiovascular and neuropathologies. Among EVs, exosomes are possibly the most studied as cell signaling mediators and as source of various biomarkers. Exosomes contain indeed proteins, lipids, metabolites and nucleic acids, in particular microRNAs, which are well-studied biomarkers. Methods for an efficient and reliable isolation of the different classes of EVs are therefore crucial. Standard techniques for EVs isolation exploit their physical properties such as size and density, but are in general scarcely efficient and give poorly pure vesicles. Microfluidic technologies combined with suitable biosurfaces integration could overcome these disadvantages. Here, starting from silicon oxide surfaces, different functionalizations aimed at exosome capture are developed. Positively and negatively charged surfaces, neutral and immunoaffinity surfaces are set up, characterized and tested in functional assays with exosome mimicking vesicles, pre-purified exosomes (via serial ultracentrifugations of cell supernatants) or EVs from raw samples (cell supernatants). Performances related to the capture of exosomes by surfaces modified with divalent ions (e.g. Ni2+, Mg2+, Cu2+) are also compared. The different functional surfaces showed different promising properties both as EVs capture and exosomes enrichment. The captured vesicles could be recovered and their cargo in biomarkers analyzed. Exosomal microRNAs were analyzed with RT-PCR and compared with total microRNAs of same cell supernatants. The best-performing functionalizations can be easily transferred to microdevice surfaces or microbeads surfaces for developing modular microfluidic systems for on-chip isolation of EVs. Then, these systems could be integrated in simple and fast biosensors capable of characterize the biomarkers present in EVs, helping the biomarker analysis both in clinical settings and in research.

Isolation of astrocytes-derived extracellular vesicles from brain, to decipher their influence on recovery after stroke

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Ischemic stroke is among the main reasons for long term disability. Stroke is caused by an acute occlusion of brain supplying arteries resulting in a lack in oxygen and glucose, which leads ultimately to neuronal loss in the infarct area.

Astrocytes are among the most abundant glial cells in the central nervous system and are involved in stroke pathophysiology at all stages. In the healthy brain, astrocytes communicate with their neighboring cell and are responsible for multiple functions such as regulating ion homoeostasis, maintenance of synapses, formation of the blood-brain barrier, among others. Immediately after stroke injury, astrocytes are activated. Similar to microglia, astrocytes acquire various activation states, with protective effects on the one side and deleterious functions on the other. Accordingly, astrocytes are involved in the acute damage but also the long term recovery following stroke.

Extracellular vesicles (EVs) play an essential role in cell communication in the healthy brain but also under pathophysiological conditions. EVs can deliver a complex set of molecules (lipids, metabolites, nucleic acids, and proteins) from donor to recipient cells.

In a previous study, we demonstrated in a murine stroke model that astrocytic brain-derived EVs (ABDEVs) increase in the acute phase (24h hours) after stroke-reperfusion injury when compared to sham. To study the content of ABDEVs in stroke, we recently developed a protocol that allows us to enrich large and small ABDEVs from ischemic brain tissue by magnetic sorting and immunoprecipitation. Preliminary data from mass-spectrometry showing the presence of characteristic astrocytic proteins indicates that our protocol is suitable to study ABDEVs. Our next goal is to study how the protein content in ABDEVs is changing at the different phases after ischemic damage (from acute to recovery) shedding light on the role of EVs as new players in the pathophysiology of stroke.

Genetic and metabolic factors affecting EV-secretion in Bacillus cereus

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The food-pathogen Bacillus cereus is a Gram-positive endospore-forming rod causing emesis and diarrhea. Although there has been an increase in studies on extracellular vesicles (EVs) derived from Gram-positive bacteria, the exact mechanisms of EV secretion remain unknown. By using deletion mutants of the emetic B. cereus F4810/72, the influence of seven well-known transcription factors and virulence regulators on EV secretion was investigated. Moreover, two different growth media were used to examine the role of external factors influencing EV secretion.

EVs were isolated from bacterial cultures grown in different media using differential centrifugation. To study the influence of the genetic deletion and the growth media, EV numbers and sizes were measured by NTA. In addition, bacterial growth, protein concentration and lipid composition were determined, and changes were analyzed by transmission electron microscopy (TEM), Fourier-Transform-Infrared spectroscopy (FTIR) and untargeted lipidomics.

Through these approaches, our study revealed a substantial influence of global transcriptional regulators and virulence-associated transcription factors on the secretion and vesiculogenesis of EVs. Furthermore, we show that the choice of media exerts a substantial effect on both the secretion dynamics and the compositional profile of EVs.

Consequently, we highlight the importance of establishing and employing appropriate culturing conditions to study EV secretion dynamics. Additionally, we show that knock-out mutants are suitable to unravel the mechanisms behind EV secretion, ultimately leading to a better understanding of EV vesiculogenesis in Gram-positive bacteria.

Role of Extracellular Vesicles in Müller glia-neuron crosstalk during neuroinflammation

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The retina is a complex tissue where neurons and glia orchestrate retinal homeostasis through multiple mechanisms of transcellular communication. Extracellular vesicles (EVs) are considered as mediators of this cell-to-cell communication and, by delivering their cargo, can modify the recipient cell phenotype. In the diseased retina, Müller glia –the most abundant type of retinal macroglia– are known to undergo reactive gliosis and play a dual role in the homeostasis of retinal neurons. Concomitantly, EVs can act as vehicles for the delivery of inflammatory mediators, leading to the modulation of reactive gliosis/neuroinflammatory processes and potentially influencing the phenotype of the surrounding neurons. As such, it is plausible that EVs constitute one of the main mediators of the crosstalk between Müller glia and retinal ganglion cells (RGCs) and thereby co-determine pathogenesis of retinal diseases. I hypothesize that the Müller-EVs cargo has a distinct signature depending on the presence or absence of reactive gliosis and, in turn, influences homeostasis of the recipient RGC. Towards this aim, we isolated EVs via size exclusion chromatography (SEC) from primary Müller glia stimulated with lipopolysaccharide (LPS) to induce reactive gliosis, or with PBS as control. Nanoparticle tracking analysis revealed that LPS stimulation of Müller glia results in an increased secretion of EVs into the supernatant. Subsequently, we evaluated the effects of these two EV populations on ex vivo organotypic mouse retinal explants and we show that EVs isolated from PBS-treated Müller glia promote neurite outgrowth, yet this effect is abolished when EVs derived from LPS-treated Müller glia were added. This phenotype was also accompanied by an increase in reactive gliosis in the receiving retinal explants. Finally, addition of the EV-poor, protein-rich SEC fraction to the explants does not affect neurite outgrowth or reactive gliosis, in contrast to the addition of EV-rich SEC fraction, thus confirming an EV-mediated effect.

Investigating the behavior of extracellular vesicles after a neuroinflammatory stimulus

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Over the past decade, the extracellular vesicle (EV) community has rapidly grown, investigating their role in intercellular communication during health and disease. It is becoming clear that despite their nanoscale, EVs can have a mega impact on recipient cells. EVs have emerged as important mediators of disease progression in the central nervous system by acting as vehicles for toxic protein aggregates such as alpha-synuclein, tau and amyloid-β. These diseases share a neuroinflammatory response as a common trait which drives the spreading of toxic proteins. Concomitantly, EVs can also act as carriers of inflammatory stimuli, although their exact proteomic profile and to which extent EVs contribute to the neuroinflammatory response has not fully been determined. Moreover, due to technical limitations, most studies up till now have been performed with EVs secreted from in vitro set-ups. Although these experiments have led to pioneering insights into EV biology, the cargo and behavior of EVs differs from in vivo approaches. Therefore, we evaluated how the EV dynamics and proteome changes upon neuroinflammatory stimulation in different retinal models. In this study, an inflammatory response was induced in primary retinal glia or eyes by administering lipopolysaccharide. The neuroinflammatory response in each model was profiled using longitudinal cytokine measurements. In parallel, EVs were isolated from cell medium or vitreous humor, and characterized using nanoparticle tracking analysis, transmission electron microscopy and Western blot. Finally, in an ongoing study we are performing a proteomic analysis in each set-up. In conclusion, this comparative work will provide the EV community with novel insights into the behavior of EVs in in vitro vs in vivo settings, and thereby help decide which set-up is most adequate to address specific research questions. Moreover, this study will aid in a better understanding of how cells respond to neuroinflammatory stimuli and which role EVs play herein.

Adipocyte-derived extracellular vesicles: the quest for a proper isolation protocol

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Background: In obesity, excessive adipose tissue (AT) accumulation leads to ectopic lipid deposition, insulin resistance (IR) and dysfunctional interorgan crosstalk, in which extracellular vesicles (EVs) became known as important mediators. In the obese state, adipocyte-derived EVs (adEVs) might play a distinct role in the development of obesity-related IR. However, a broad research interest yet large methodological heterogeneity and lack of prior optimization studies emphasizes the need for uniform, source-specific adEV isolation protocols. Our study aims to standardize the approach for adEV isolation in future studies.
Methods: Human mature adipocytes were isolated from paired visceral and abdominal subcutaneous AT biopsies. Adipocytes were incubated using different modalities (falcon vs membrane cultures), media compositions and timings. EV isolations were performed using either ultracentrifugation (UC) or 10kD ultrafiltration combined with size exclusion chromatography (UF-SEC). Obtained adEVs were characterized following MISEV18 guidelines, including TEM, NTA and western blot.
Results: After 3h incubation of visceral mature adipocytes, UC-based isolation of adEVs resulted in a notable lower yield as opposed to UF-SEC-based isolation (mean yield: 2,0E8 ± 3,8E6 vs 1,1E10 ± 2,8E7; mean size: 166nm vs 152nm, respectively), most likely due to the inability of UC to pellet high-lipid containing floating EVs. Isolation of adEVs from conditioned media of mature adipocytes, incubated without EV-depleted FBS supplementation, resulted in concentrations below the accuracy limit of the Zetaview. Subcutaneous mature adipocytes produced less adEVs as compared to visceral mature adipocytes (n=1; yield: 2,6E9; mean size: 162nm). However, in contrast to mature adipocytes, similar whole tissue EV concentrations were observed for visceral and subcutaneous AT (mean yield: 1E10 vs 1,4E10; mean size: 218nm vs 216nm, respectively).
Conclusion: Even though adEVs are advocated for their role in regulating metabolism, further optimization with regard to incubation modalities for EV release from mature adipocytes is required to improve quality of obtained adEV samples.

Transparent reporting and centralizing knowledge in bacterial extracellular vesicle research

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Introduction: In recent years, the role of the microbiome has been demonstrated in a plethora of disease, leading to an exponential increase of research into the topic, including the study of bacterial extracellular vesicles (BEV). These nanometre-sized membrane vesicles are released into the extracellular environment by both Gram-negative and Gram-positive bacteria. The biogenesis, components, and function of these bacterial extracellular vesicles (BEV) are increasingly studied and explored as a promising diagnostic and therapeutic platforms. With this study we conduct a systematic review to map current BEV research practices and identify knowledge gaps and possible improvements in the field.

Methods: Studies reporting BEV experiments and published between 2015 and 2021 were searched on PubMed and EMBASE. Data extraction and quality assessment of included studies was performed using the EV-TRACK knowledgebase (Van Deun et al., Nat. Methods, 2017).

Results: Evaluation of all included studies (n=843) shows that none report all necessary information according to the MISEV guidelines. The highest obtained EV-METRIC is 87%, with 38% receiving an EV-METRIC score of zero percent. Less than a third of inclusions apply the term “bacterial extracellular vesicle” (BEV) to denote EV from bacterial origin. The most implemented methods are differential (ultra-)centrifugation (DUC, 95.5%) and filtration (87%). Qualitative characterization is often omitted, with 25.6% of experiments reporting particle nor protein characterization. When protein analysis is performed, 64.5% of experiments report a protein that is

indicated as BEV-associated and only 4.6% report a contaminant.

Conclusion: Current research highlights the need for transparent reporting and development of international guidelines regarding nomenclature, separation, and characterization of BEV. This will be indispensable to stimulate BEV research and develop the necessary tools to advance the field.

Fecal extracellular vesicles: potential applications in disease diagnosing, prognosis, and monitoring

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Extracellular vesicles (EVs) are heterogeneous, membrane-enclosed, nano-vesicles released from cells that can mediate various pathological functions. Faeces are considered an easy-access, non-invasive type of sample, and EVs derived from them have a high potential to be used in disease diagnosis and prognosis. However, there is no report on enriching EVs from ruminant feces and their application in disease diagnosis and monitoring to date. Thus, this study was conducted to devise a protocol for enriching EVs from cattle faeces and characterize them. First, faecal EV isolation method was developed with cow faecal material using differential centrifugation and size exclusion chromatography and then improvised for calf samples. Afterward, EVs were isolated from feces of healthy (n = 6) and Cryptosporidium infected (n = 6) 10 days old dairy calves. Nanoparticle tracking analysis (NTA), Bradford assay, transmission electron microscopy (TEM), and western blotting were performed according to the guidelines of the International Society for Extracellular Vesicles. NTA analysis showed enrichment of particles (average of 3.45 x 1011 particles/ml) and TEM analysis revealed the presence of typical cup-shaped EVs and EV-specific protein biomarkers CD 63 and TSG 101 were identified in the western blots. Furthermore, the presence of proteins, DNA and RNA cargos in the EVs were also identified. The total nanoparticle concentration of isolated fEV samples of healthy vs. Cryptosporidium positive was 4.6 x 1011 and 3.95 x 1011 particles/ml respectively. Moreover, there was a significant difference ($p < 0.05$) in the average nanoparticle size (diameter) between the healthy and infected animals, (173 nm vs 150 nm). In conclusion, EVs can be enriched from bovine feces and there is a potential to use them as a non-invasive tool for diagnosis and monitoring gut-related pathogens and early host-pathogen interactions.

Studies of extracellular vesicle heterogeneity at the single vesicle level

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Cells release different extracellular vesicle (EV) populations with distinct biophysical properties and biological functions. Extensive proteomic analysis has shown that EVs produced by different cell types and even from the same cells may greatly differ qualitatively and quantitatively with regard to their specific tetraspanin content. In the current study, we have expressed EGFP and/or Cherry-fused CD9, CD63, CD81 as well as melanoma associated antigen MAGEA4 protein in the human osteosarcoma cells using MultiBacMam expression system, and isolated EVs from the cell media by differential ultracentrifugation. Characterization of EVs at the single vesicle level using TIRF microscopy revealed sub-populations enriched with different CD markers. Most of CD63-positive EVs contained CD9 or CD81 while only 20% of CD9-positive EVs were enriched with CD63, and only one-third of MAGEA4-positive EVs contained one of CD markers analyzed. This study showed that secreted EVs are heterogenous in nature having probably diverse functions in both physiological and pathophysiological processes.

Quantitative proteomic analysis of serum-purified exosomes identifies putative antimonial failure-associated biomarkers in immunosupressed mice with visceral leishmaniasis

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Leishmaniasis is a neglected tropical disease caused by Leishmania parasites. Visceral leishmaniasis (VL) is the most severe form of this parasitic disease. VL is more frequent among immunosuppressed patients and it has markedly increased among those patients receiving immunosuppressive therapy for autoimmune diseases. These patients do not respond adequately to conventional antimony treatment and have high risk of relapses, requiring special clinicians' attention. There is a real need to find biomarkers that facilitate their follow-up and prevent relapses from happening. Extracellular vesicles (EVs) are a vehicle for biological particles capable of modulating the functioning of host cells, and they play a key role in parasite-host communication, enriching the environment in virulence factors or molecules related to drug resistance. EVs have emerged as a promising target to identify biomarkers of failure, allowing early identification of these episodes and reducing the risk to suffer them. In order to determine some of these biomarkers, we have analysed and compared the EVs protein content from serum of BALB/c mice infected with Leishmania infantum and immunosuppressed at clinical doses with TNF antagonist or methotrexate, followed by pentavalent antimonial treatment for VL.
EVs were isolated using a commercial qEV size exclusion chromatography followed by a concentration process with ultracentrifugation. Size, concentration and morphology of EVs were determined by nanoparticles tracking analysis and transmission electron microscopy. Finally, we analysed the protein content of EVs by liquid

chromatography-tandem mass spectrometry.

When compared to the non-immunosuppressed infected group, the immunosuppressants altered the serum EVs content and we could identify proteins linked to lower treatment efficacy. These analyses have allowed us to better understand the high susceptibility to antimonial treatment failure occurring under pharmacological immunosuppressive conditions. In addition, the study of EVs from clinical strains of patients with leishmaniasis can shed light about new factors of resistance and virulence.

Identification and characterization of plasma-derived extracellular vesicles in burn-septic shock patients

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Introduction. Septic shock is the main cause of mortality in patients with severe burns. Extracellular vesicles (EVs) have emerged as novel cell-to-cell mediators and promising non-invasive biomarkers that may aid in the diagnosis of septic shock. However, the pathophysiological mechanisms of EVs as pro-inflammatory mediators remain unclear in burn-septic shock patients.

Methods. We enrolled twenty-nine burn patients, including burn-septic shock patients (BSP, n=23) and burn non-septic patients (BnSP, n=6). Ten healthy subjects (HS) were used as controls. Plasma-derived EVs were isolated by ultracentrifugation and characterized following the MISEV 2018 guidelines by nanoparticles tracking analysis, transmission electron microscopy (TEM) and flow cytometry. EV surface antigens were investigated by bead-based multiplex flow cytometry. In vitro, we reproduced the effects of EVs on platelet function by adding EVs from BSP or HS to platelet-rich plasma by healthy donors.

Results. Plasma-derived EVs were successfully isolated and their presence was confirmed by TEM and flow cytometry analyses of small EVs-tetraspanin markers that showed enrichment in CD63 and CD9. When compared to controls, BSP exhibited increased plasma-derived EVs concentration. BSP EVs phenotyping revealed a pattern of cell surface markers associated with septic shock and identified platelets, leucocytes, lymphocytes, and endothelial cells as EVs-parental cells. We found a significant increase of CD42 that discriminated BSP from HS and BnSP. Moreover, EVs isolated from septic shock patients primed platelet aggregation.

Conclusions. Plasma-derived EVs are potentially related to septic shock in burn patients. In particular, septic shock in burn patients might be reflected in a pool of heterogeneous circulating EVs that contribute to inflammatory-related signaling mechanisms.

Evaluation of different fluorescent labelling strategies to track exosome biogenesis, loading, and delivery

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Extracellular vesicles (EVs) are cell-derived nanoparticles essential in cell-to-cell communication. Among EVs, exosomes represent membrane vesicles formed by inward budding of endosomal membrane and secreted by fusion with the cell membrane, thus containing thousands of different biomolecules originated from the parent cell and delivered to host cell.

Although the exosomes' biological characteristics have been extensively defined, the investigation of the biogenesis and delivery of exosomes is a still unclear crucial point. In particular, exosomes bioengineering represents an attractive approach to producing vesicles with specific targeting and/or loading features.

In this study, exosomes were produced by HEK293T cells, isolated by size exclusion chromatography and characterized by western blot and dynamic light scattering. Their loading during biogenesis and delivery were monitored exploiting different methods to simultaneously label the exosome surface and their cargo. In particular, transient transfections with a DNA plasmid encoding for green fluorescent protein (GFP) or for farnesyl GFP (F-GFP), a membrane-targeted GFP protein, were performed in HEK293T cells by lipofectamine protocol. Also, exosomes were labelled by the addition of a lipophilic membrane dye (VybrantTM DiD). Then, HEK293T-derived exosomes were delivered to recipient cells in different amounts and for diverse incubation times. The obtained preliminary results highlighted that both GFP and F-GFP allow to track exosome delivery, although at a different extent. We are currently performing a comparison of the performance of the GFP and F-GFP constructs with DiD probe by confocal microscopy. Furthermore, we are planning to label the nucleic acids which are embedded into the exosomes, in order to simultaneously track the shell and the cargoes therein.

Overall, the current work aims to propose a simple and systematic workflow to optimize a method to track the exosome route from biogenesis to delivery, especially in the presence of exogenous nucleic acids as cargoes.

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A different perspective on EV surface engineering: the protein corona “variable”

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In the last years, advancements in EV surface engineering methods have contributed to the development of EVs with augmented targeting performances towards specific tissues. EV surface engineering by covalently bonding tissue-specific protein ligands has been widely exploited among others. However, in these works, the protein corona (PC) “variable,” namely, the spontaneous adsorption of extrinsic proteins on the EV surface, has never been considered, even if its role in determining the biological and functional role of EVs is becoming widely accepted.

In this contribution, we show for the first time the formation of a surface engineering-derived PC and its relative molecular and biological implications. We compared two edge cases of EVs engineered with the antibody Cetuximab (CTX): (i) by chemisorption (covalent binding of CTX via biorthogonal click-chemistry) and (ii) by physisorption (formation of a CTX corona). The two cases were mainly compared regarding surface composition, morphology, mechanical properties, stability, on-chip affinity, and in vitro uptake. Both cases showed a PC of comparable CTX with a similar binding affinity towards the Epidermal Growth Factor Receptor (EGFR), the main CTX target ligand. However, only the EV set engineered by chemisorption showed improved cellular uptake compared to the pristine EVs. Thus, even though EVs could be engineered by physisorption, covalent bonding is required to improve EV cellular uptake.

Multi-platform ligands for Extracellular Vesicles integrated isolation and analysis

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Extracellular Vesicles (EV) play significant roles in intercellular communication and are gaining increasing prominence as potential biomarkers and therapeutic agents. Due to their nanometer size, low refractive index, intrinsic heterogeneity and the high sensitivity required in detecting low abundant disease-specific subpopulations, EV pose significant challenges to the analytical sciences. High performance EV characterization methods, possibly integrating isolation from complex samples are urgently needed to meet clinical requirements in diagnostics.

Innovative affinity probes and ultra-high sensitivity detection, capable of reaching single-molecule sensitivity, can meet this demand. Our recent research has identified a unique class of membrane-sensing peptides (MSP) as novel molecular ligands for integrated small EV separation and analysis. The mechanism of membrane recognition and binding rely on complementary electrostatic interactions between the peptide and the phospholipids on the outer membrane leaflet, which might result in the insertion of hydrophobic residues into membrane defects. Notably, small EVs (< 180 nm) have quite unique membrane characteristics in the extracellular environment that may be regarded a 'universal' identifier, either in place of, or in addition to, typical surface-associated proteins. As a result, our MSP are pan-specific, interspecies, interkingdom and minimize co-isolation of contaminants, representing a diverse class of ligands with additional benefits in terms of stability and synthetic versatility. Here we present the implementation of MSP into several platforms for integrated EV separation and analysis, including microchips for SP-IRIS (Single Particle Interferometric Imaging Sensor) and microbeads for Single Molecule Immunoassays (SiMoA) and multiparametric flow cytometry. We will show their application into different workflows for liquid biopsy in urine and blood by measuring the differential expression of putative surface antigen biomarkers opening avenues to concrete and plausible clinical applicability in diverse contexts.

B cell receptor engagement boosts the secretion of extracellular vesicles that modulate activation capacity in human B lymphocytes

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B lymphocyte functions are determined by their activation status where the most important activation trigger is specific antigen binding to the B cell antigen receptor (BCR). Critical for B cell activation is the spatiotemporal regulation of various vesicle populations; however, the detailed functions of these is only superficially understood and in hyperactivated pathologies e.g. B cell lymphomas, totally unknown. In this study, we aim to characterize the activation mechanisms of B cells, focusing on extracellular vesicle (EV) functions, in health and disease. EVs carry various biological cargo like bioactive enzymes, regulatory RNAs and lipids with the help of which they modulate the environment and mediate intercellular communication. In B cells, EV release mediates antigen extraction at immunological synapses and they may shield lymphoma cells from rituximab (anti-CD20) treatment with their robust CD20 surface expression. However, basic research data on the characteristics and function of B cell EVs is largely unknown. Our emerging unpublished data reveals that upon malignant human B cell activation (minutes) i) EV secretion is boosted and the EV sizes remodelled; also, preliminary lipid and protein mass spectrometry (MS) data suggest ii) altered cargo and lipid species in the activated cell EVs and ii) functional modulation, by the activated cells' EVs in the recipient B cells. To sum up, we will characterize B cell EV functions in detail, by state-of-the-art techniques such as lipid and protein MS and RNA-Seq and analyse their functional impact on the target cells. We have also developed a fluorescence method to visualize EV markers by high resolution using standard confocal microscopy.

Production and characterization of Saccharomyces cerevisiae extracellular vesicles containing recombinant PQLC2, a lysosomal amino acid membrane transporter.

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Extracellular vesicles (EVs) are membrane-enveloped vesicles that are released by all types of cells. Due to natural EV biogenesis, membrane proteins are embedded in a natural microenvironment, well folded, fully mature, and with their original membrane topology. Here, we want to explore the use of EVs released from S. cerevisiae as a platform to characterize the functional activity of PQLC2, a lysosomal membrane transport protein produced recombinantly in yeast. GFP-tagged PQLC2 is localized in the vacuolar membrane of S. cerevisiae. Notably, EVs isolated from the yeast culture media also contains PQLC2-GFP as judged by nanoparticle flow cytometry and western blot. In order to enrich the isolated EVs with PQLC2-GFP several approaches were tested, including the use of different culture media composition and temperature of PQLC2 expression. In addition, the effect of two drugs in EV release were also tested. While treatment with Bafilomycin1, a vacuolar V-type H+-ATPases reduces the release of PQLC2-containing EVs, the cell wall synthesis inhibitor caspofungin enhances the release of EVs by 10-fold. Finally, we demonstrated that the C terminal end of PQLC2 is located in the EV lumen, an essential information to study the transport activity of PQLC2 in EVs.

Detection of tumor-derived extracellular vesicles interactions with immune cells is dependent on EV-labelling methods

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Cell-cell communication within the complex tumor microenvironment is critical to cancer progression. Tumor-derived extracellular vesicles (TD-EVs) are key players in this process. They can interact with immune cells and modulate their activity, either suppressing or activating the immune system. Deciphering the interactions between TD-EVs and immune cells is essential to understand immune modulation by cancer cells. Fluorescent labeling of TD-EVs is a method of choice to study such interaction. This work aims to determine the impact of EV labeling methods on the detection by imaging flow cytometry and multicolor spectral flow cytometry of EV interaction and capture by the different immune cell types within human Peripheral Blood Mononuclear Cells (PBMCs). EVs released by the triple-negative breast carcinoma cell line MDA-MB-231 were labeled either with the lipophilic dye MemGlow-488 (MG-488), Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE), or through ectopic expression of a MyrPalm-superFolderGFP reporter (mp-sfGFP), which incorporates into EVs during their biogenesis. Our results show that these labeling strategies, although analyzed with the same techniques, led to diverging results. While MG-488-labeled EVs incorporate in all cell types, CFSE-labeled EVs are restricted to a minor subset of cells and mp-sfGFP-labeled EVs are mainly detected in CD14+ monocytes which are the main uptakers of EVs and other particles, regardless of the labeling method. Furthermore, our results show that the method used for EV labeling influences the detection of the different types of EV interactions with the recipient cells. Specifically, MG-488, CFSE and mp-sfGFP result in major observation of, respectively, transient EV-PM interaction that results in dye transfer, EV content delivery, and uptake of intact EVs. Consequently, the type of EV labeling method has to be considered as they can provide complementary information on various types of EV-cell interaction and EV fate.

Development of EV tools labelled with fluorescent or luminescent cargos

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Several assays have been done to standardize the study of extracellular vesicles (EVs) using, for instance, MISEV guidelines. However, there is still a need for standard EVs, labelled or not, as tools to track and quantify EVs in vitro and in vivo.

Currently, labelling EVs by adding fluorescent/luminescent cargos is done by either i) merging these cargos to regular EV protein, like CD63, that will be overexpressed; or ii) chemically grafting a fluorophore through covalent bond randomly affecting any EV surface proteins; or iii) anchoring a fluorophore in EV membrane via a hydrophobic domain. All these methods present issues and bias that may hamper experiment results and analyses, i.e. overexpression and modification of a regular EV protein may affect EV fate (case i), uncontrolled modifications of EV surface proteins (case ii) and leakage of the fluorophore during investigating experiments (case iii).

To address these issues, we used our technology of EV bio-engineering to sort any protein cargos, of which labelled proteins, inside EVs. Two families of labelled cargos were developed, either bound under the EV membrane, or free in the EV lumen. Thanks to this technology, there is no modification of EV surface proteins, like tetraspanins, and therefore, there are no or minimal modifications of the natural EV properties (interactions, targeting, …).

A wide range of cargos, eGFP, mCerulean, CyoFP1 and NanoLuc proteins, were successfully targeted in the EV lumen. According to MISEV guidelines and our internal quality controls, EVs were characterized for their size, number, EV markers and their cargo contents using Nanoflow cytometry, Western blot, ELISA and fluorimetry. Cargo protein functionality was also assessed.

In conclusion, we produce large batches of highly purified and characterized EVs, either regular EVs or labelled EVs, that are promising reference tools that can be used by the scientific community for EV research.

Spatio-temporal analysis of the intercellular transfer of extracellular vesicles between mammary cells

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Extracellular vesicles (EVs) are small lipid vesicles secreted by cells that originate from the cell surface (typically enriched in CD9) or from multivesicular bodies (typically enriched in CD63). While current methods for studying EVs involve concentrating and purifying EVs, they do not provide information about the distance or amount of EVs that may transfer from one cell to another. Here, we adapted a coculture assay previously developed (Burtey et al. FASEB 2015) in which MCF-7 cells - a model for human mammary epithelial cells – were color-coded by expressing EV-markers hCD63 and/or hCD9 or hCD81 fused with fluorescent mCherry or GFP with untransfected "acceptor" cells labelled with Cell-Tracker-Blue, a non-transferrable dye. By 3D quantitative high-resolution confocal fluorescence microscopy, we observed the direct transfer EV markers between cells, quantified the distance and the rate of transfer for the different EV markers. Results showed that CD81 did not transfer far away from the donor cells while CD9 reached more distant acceptor cells. Similar results were obtained for endogenous CD81, CD9 and CD63 whose transfer were analyzed by immunofluorescence on cocultures of wt MCF-7 cells to CRISPR KO CD63, CD81 or CD9 MCF-7 cells. Simultaneously monitoring of endogenous CD9 and CD81 transfer was done by coculturing wt cells with double KO CD9/CD81 cells and revealed that EV markers colocalized in acceptor cells suggestive of their cotransfer. Interestingly, 3D analysis indicated the abundance of punctate structure containing CD81 and CD9, or CD63 at the basal level, below acceptor cells or on the substrate. Altogether, these results suggest that this coculture approach is highly suitable for direct qualitative and quantitative imaging of EV-transfer, in living or fixed cells, and is also suited to screen for EVs cargoes or regulators and further decipher the mechanisms underlying EV transfer.

Multiparametric characterization of the secretome produced by mesenchymal stromal cells: towards regulatory compliance

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Extracellular vesicles (EVs) are nanometer-sized subcellular particles naturally produced by cells. As EVs can replicate the properties of their parent cells, mesenchymal stromal cell (MSC) derived EVs represent a promising alternative to regenerative cell therapy. However, the ability to manufacture EVs meeting regulatory requirements is a major bottleneck hampering the progress to the clinic. According to the guidelines on investigational biologicals, active substance and drug product, specifications are mandatory for the following quality attributes (QA): quantity, identity, purity, microbiological quality and biological activity. The aim of this investigation is to perform a multiparametric study on MSC EVs to create a standardised characterization toolbox complying with regulatory requirements.

Several characterization methods assessing quantity, identity and therapeutic efficiency were investigated and, based on industrial needs, three of them were selected to be then validated analytically according to ICH Q2. Standard reference material and EV samples were tested for size and concentration measurement to evaluate the linearity, precision, accuracy, and limits of the device.

The ZetaView was selected for the characterization of EV quantity QA. The ZetaView demonstrated its superiority for quantity analysis due to its simplicity and working range (4.6E7-1.5E8 part/mL). It also showed a high linearity (R-squared ≥ 0.98 for silica and ≥ 0.96 for EVs), great accuracy (100 ± 10%) and precision (intermediate precision ≤ 5%, interlaboratory reproducibility ≤ 15%) but also its capacity to detect change of nanoparticles subpopulation size. At a research grade level, the Exoview was selected as it can distinguish different identity profiles of EVs in correlation with their production process. Finally, scratch tests were combined to confirm EVs in vitro biological activity.

This work is of outmost importance for the release of EV batches intended for clinical use to make the EV field's promises come closer to patients while maintaining quality, safety, and efficacy.

Workflow optimization for the isolation and characterization of human milk extracellular vesicles

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Human milk extracellular vesicles (HMEVs) are a bioactive component of HM, encasing specific compounds for delivery from mother to infant. Due to the complex composition of HM, the isolation of pure HMEVs remains a challenge. We present a workflow for HM collection, storage, HMEVs isolation and characterization. A HM sample was collected and aliquoted recreating three scenarios: (i) directly subjected to defatting by centrifugation (3000xg, 22°C, 10') and EV isolation; (ii) defatted and stored until further processing (i.e., EV isolation); and (iii) stored until further processing (i.e., fat removal and EV isolation). From 5 mL of the defatted HM, casein was removed with acidic precipitation prior to EV isolation by size exclusion chromatography (SEC) with a qEV10/35nm column (IZON®). The first 20 mL eluted were discarded and fractions of 5 mL (i.e., fractions 5-11) were collected and preconcentrated using Amicon 30 KDa filter or ultracentrifugation (175000xg, 4°C, 2h). Fractions 5th-11th were characterized by: total protein content (BCA), nanoparticle tracking analysis (NTA), Tunable Resistive Pulse Sensing (TRPS), Attenuated Total Fourier Transform Infrared (ATR-FTIR) spectroscopy, Western Blot (WB), and immunofluorescence assay (ExoView®).

Isolation of HMEVs was confirmed by tetraspanins detection (CD9, CD81, CD63) by WB and ExoView® and of cytosolic proteins (Alix, HSP70) by WB, and also complemented with ATR-FTIR (peptide bands at 1651 and 1540 cm-1). The total protein concentration across SEC fractions varied between the 5th-11th fraction with a maximum of 12.4 mg/mL detected in fraction 11. The TRPS and NTA assay indicated highest particle concentration in 6th and 7th fraction ranging between 1.6·1011 and 1.9·1011, respectively. A loss of particles of 69% was detected when HM was not defatted immediately. The developed workflow will be employed in future studies focusing on the characterization of the HMEV lipidome and its role in the prevention of necrotizing enterocolitis in preterm infants.

Analysis of protein and miRNA cargo of human conjunctival EVs

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Purpose: The purpose of this study is to characterize the protein and miRNA cargo of EVs isolated from human conjunctival epithelial and stromal cells.
Methods: This study followed all ethical requirements. Three different human cell types were used: the immortalized human conjunctival epithelial cell line IM-HConEpiC, conjunctival mesenchymal stromal cells (Conj-MSCs) isolated from cadaveric donor tissue, and adipose tissue mesenchymal stromal cells (AT-MSCs) obtained from lipoaspirates, that were used as control. Cells were cultured under standard conditions and EVs were isolated from 48h secretomes by ultracentrifugation. EV size was characterized by dynamic light scattering and morphology was evaluated by atomic force microscopy. Protein cargo of EVs was analyzed by mass spectrometry. Micro RNAs (miRNA) were purified from EV samples and analyzed by Next Generation Sequencing (NGS). Three different samples from each cell type were studied.
Results: EVs isolated from the three human cell types were round with sizes of 330.1 ± 63.1 nm for IM-ConEpiC-EVs, 230.0 ± 37.0 nm for Conj-MSC-EVs, and 196.4 ± 28.6 nm for AT-MSC-EVs. Mass spectrometry revealed the presence of 1,892 proteins in the analyzed EVs. From them, 918 were found in the 3 cell types, whereas 300 were exclusively found in IM-HConEpiC-EVs, 19 in Conj-MSC-EVs, and 40 in AT-MSC-EVs. 1,111 mature miRNAs were detected in the EVs by NGS analysis. 312 miRNAS were present in the three cell type EVs, whereas 365 miRNAs were exclusively detected in IM-HConEpiC-EVs, 89 in Conj-MSC-EVs, and 87 in AT-MSC-EVs. Detected proteins and miRNAs are mainly involved in signal transduction, cell communication, metabolism, and cell growth.
Conclusion: We carried out the first exhaustive analysis of conjunctival EVs cargo. Conjunctival epithelial cell derived EVs contain more proteins and miRNAs than stromal ones. Conj-MSC-EVs and AT-MSC-EVs share many characteristics that could imply that Conj-MSC-EVs might also have important therapeutic properties.

Study of host-symbiont communication mediated by extracellular vesicles in Blattella germanica

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Organisms do not live in isolation but rather interact with individuals from other species. Yet, mutualistic stable relationships require an “entente cordiale” among the partners leading to a better fitted life form in which all of them benefit. Symbioses are widespread in eukaryotes, especially in insects. Many of them live in obligate relationship with different ecto- and endosymbiotic bacteria needed to maintain host fitness (1). It is the case of the cockroach Blattella germanica, with two symbiotic systems in separated compartments: the endosymbiont Blattabacterium in specialized bacteriocytes located in the fat body, and a complex microbiota in the gut lumen. The presence of small RNA molecules (sRNA) has been systematically reported in extracellular environments, including extracellular vesicles (EVs), and has been associated to cell-to-cell communication, as an additional layer of regulatory complexity (2,3). Our goal is to investigate whether sRNAs produced by the insect and/or by the endosymbiont exert a regulatory role related to endosymbiosis, either locally (in the bacteriocyte) or in distant target tissues, reached through the hemolymph inside EVs. In this sense, we have isolated EVs from insect’s hemolymph and characterized them by Nanoparticle Tracking Analysis (NTA) and Transmission Electron Microscopy (TEM). Current studies of their cargo by -omics technology will be also presented and their possible role in bacteria-insect communication will be discussed.

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"Effects of Growth Medium and Incubation Time on Fungal Extracellular Vesicles: Insights into Differential Composition and Characteristics"

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EVs in fungi have gained recognition as significant carriers for transferring bioactive substances across cells, facilitating interactions that have been associated with various physiological functions. It is widely established that the growth conditions have a strong impact on the composition of the fungal EVs. We conducted a comparative analysis of the vesicles secreted by the fungus C. albicans in different growth media and at different time points. As expected, differences in the size of secreted EVs and more importantly variations in their protein composition, were detected by DLS and through a preliminary proteomic study using mass spectrometry, respectively. Overall, a higher number of proteins was observed in EVs secreted over longer incubation periods of 72h compared to 16h periods across all tested media. Furthermore, the proteomic data suggests that growth in a specific culture medium promotes the enrichment of EVs secreted with proteins from a specific cellular compartment or related to a specific biological function. In this way, EVs from SC5314 strain grown in yeast-restricted medium (YNBS+ Tartaric acid) showed an increased presence of cell wall proteins, while those grown in a hypha-restricted medium (YNBS+ MOPS+N-acetyl glucosamine) exhibited an enrichment of cytoplasmic proteins. Interestingly, the addition of fetal bovine serum to YPD, which also induces the morphological transition from yeast to hyphae, resulted in a distinct protein composition of the EVs vesicles compared to that obtained with the hypha-restrictive YNB medium. Studying all these differences in the protein patterns of the vesicles according to their environment constitutes the first step in elucidating the truly important roles of the fungus EVs in each case.

Protection Against Invasive Candidiasis with Vesicle Immunization: Promising Advances towards an Effective Vaccine

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EVs possess the unique ability to elicit immune responses while safeguarding the cargo from degradation. We previously described how prior infection of BALB/c mice with the C. albicans ecm33 cell wall mutant, completely avirulent, achieved over 80% protection against a subsequent intravenous infection in a model of systemic candidiasis. Given the tendency to substitute cellular vaccines with acellular ones, we performed a characterization of the C. albicans ecm33 EVs and an immunization experiment using them. Furthermore, considering the significant role of the dimorphic transition in the pathogenesis of this fungus, as well as our previous findings demonstrating substantial differences in protein composition between EVs derived from yeast and hyphal forms of the SC5314 parental strain, we also included these vesicles in the immunization assay. We observed that mice immunized with EVs secreted by SC5314 yeasts exhibited a remarkable protection rate of over 80% compared to non-vaccinated. Mice immunized with EVs secreted by ecm33 mutant showed a lower but also remarkable level of protection of approximately 60%. Interestingly, 35% of mice immunized with EVs secreted by SC5314 hyphal forms died even at shorter time intervals than those in the control group, while approximately 35% survived until the end of the experiment. The cytokine profiles, total IgG antibody titers, and IgG2a subtype in the serum of mice immunized with the different vesicles are nicely aligned with the level of protection attained in each respective case. The remarkable protection reached by SC5314 yeast-derived EVs can be attributed to their higher abundance of cell wall proteins, as revealed by the proteomic analysis.

Optimization of EV isolation and their separation from lipoproteins by size exclusion chromatography

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Interest in the use of extracellular vesicles (EVs) as biomarkers of disease is rapidly growing. However, one main unsolved issue in the EV field is finding a technique able to eliminate non-EV contaminants present in biofluid samples in a one-step isolation protocol. Due to the expansion and value of size exclusion chromatography (SEC) as one of the best EV isolation methods, we have tested several agarose resins with different agarose percentages, bead sizes and crosslinking features to optimize EV isolation. For this optimization of SEC, we first employed conditioned media from a melanoma cell culture, a simpler sample in comparison to biological fluids, but which also contains abundant contaminants such as soluble protein and lipoproteins (LPPs). The distinct agaroses, and the combinations of resins with different agarose percentage in the same column were tested. Soluble protein, EVs and LPPs levels from the different eluted fractions were quantitated by immunodetection or absorbance measurements. Samples were also analysed by NTA and TEM to verify the yield and the LPP contamination. Different percentages of agarose resins (2%, 4% and 6%) yielded samples with increasing LPP contamination respectively, which was not improved in the columns that combined them. Crosslinking of the agarose did not affect EV isolation yield nor the LPP contamination. In contrast, reducing the bead size greatly improved EV purity. We thus selected 4% Rapid Run Fine agarose beads from ABT as the resin that more efficiently isolated EVs with almost no contamination of other particles. Using blood plasma samples, this resin also demonstrated an improved capacity in the isolation of EVs from LPPs in comparison to the agaroses most commonly used in the field (2BCL and 4BCL).

ELECTROCHEMICAL SENSOR FOR POINT-OF-CARE QUANTIFICATION OF EXTRACELLULAR VESICLES

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Extracellular vesicles (EV) are vesicles membranous structures naturally released by cells. Their complex cargo (proteins, lipids, or nucleic acids) and their role in intercellular communication are make them an interesting source of suitable biomarkers in the diagnosis of diseases, and quick and easy to use quantification methods are urgently needed. [1]. Tunable resistive pulse sensing or nanoparticle tracking analysis (NTA) are some popular options.

In this work, we have developed an electrochemical sensor for extracellular vesicles quantification based on the use of nanochannel membranes. Nanochannels are pores whose depth is much larger than the diameter where samples can diffuse. Different research was published based on the modification of nanochannels with several types of biomolecules (proteins, DNA), [2]. An electrochemical probe was used as indirect readout. The sensor was optimized by using Hansa BioMed EV standards (HBM-PEP-100/5). It allows the quantification of nanoparticles and nanovesicles using small volumes of sample (1-4 µL). The concentration can be calculated by comparison of the signal obtained with a previous calibration. The total time for the measurement is less than 15 minutes, with a portable and user-friendly device. This work has been submitted for a patent [3].

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Comparative analysis of extracellular vesicle-derived protein extraction methodologies for mass spectrometry analysis

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In recent years, extracellular vesicles (EVs) have emerged as a promising tool with potential applications in the clinical field. These nanometer-sized membranous particles are secreted by cells and their molecular cargo comprises a wide variety of bioactive molecules, including lipids, proteins and nucleic acids. As a result, EVs constitute key elements of cellular communication and an important source of biomarkers that correlate with physiological and pathological processes in human health. Mass spectrometry (MS) technology allows the investigation and identification of protein biomarkers associated with EVs, although the selection of an optimal protein extraction method for its subsequent analysis is essential. To this end, we present a comparative study involving three different methodologies for the purification and digestion of EV-derived proteins prior to MS analysis. EVs were isolated by size exclusion chromatography (SEC) from human platelet lysates (hPL) and

characterized through the determination of specific markers by western blotting, size distribution and concentration via nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) imaging. Protein isolation was performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), organic solvent precipitation (OSP) or magnetic beads (MB), followed by trypsin digestion and LC-MS/MS analysis. Principal component analysis (PCA) reveals a clustering of the samples according to the performed technique. Altogether, a total of 275 proteins were identified - 258 using MB, 269 using SDS-PAGE and 268 using OSP - of which 91.3% are common to all three methodologies. Furthermore, the minimal number of differentially detected proteins and their distribution pattern indicate that all three techniques are optimally functional for the isolation of EV-derived proteins and could be considered in accordance with the research requirements. Remarkably, OSP method allows to perform an additional metabolomic analysis of the same samples without compromising the proteomic results, since the generated supernatant could be used for this alternative omic approach.

COMPARISON OF METHODS FOR ISOLATING EXTRACELLULAR VESICLES FROM HUMAN PLASMA

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Extracellular vesicles (EVs) are small lipid membrane vesicles secreted by almost all types of cells, playing an important role in intercellular communication and participating in physiological, pathological processes, and homeostasis maintenance. For this reason, EVs have great potential in the clinical setting, serving as a source of biomarkers for the detection, diagnosis, and monitoring of various diseases, including colorectal cancer (CRC). In recent years, there has been a growing interest in EV research, however, there is no consensus on the most suitable protocol for their isolation and characterization. In this work, a comparative analysis of four methods for isolating EVs from plasma samples was carried out: size-exclusion chromatography (SEC), ultracentrifugation (UC), precipitation using two available commercial kits, as well as a combination of several of them: SEC + precipitation and UC + precipitation. Our results showed that size-exclusion chromatography is the most efficient and appropriate method for further analysis, achieving the recovery of a greater number of extracellular vesicles without detectable presence of lipid contamination. In addition, the combination of methods has not improved performance or purity, and it increased the EV enrichment process times. Therefore, its application in a clinical setting is not considered a suitable approach for subsequent EV biomarker analysis.
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In vitro binding of seminal extracellular vesicles (sEVs) to porcine sperm increases with co-incubation time.

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Seminal EVs interact with spermatozoa and modulate their function, raising the possibility of using them to modify sperm functionality in vitro. To define suitable in vitro conditions for co-incubation of spermatozoa and sEVs, this study evaluated the efficacy of two subsets of sEVs (large [L-sEVs] and small [S-sEVs]) and three pHs of the co-incubation medium (6.5, 7 and 7.5). The sEV subsets were isolated from two seminal plasma pools (five ejaculate/pool) by sequential centrifugation and size exclusion chromatography. Seminal EVs were characterized by concentration (total protein), size distribution (DLS), morphology (TEM) and flow cytometry analysis of EV marker proteins (CD63 and HSP90B) and purity (albumin). Seminal EV subsets (5 mg/mL) were labelled with DiD and DiI (Vybrant™ Cell-Labelling Solutions) and co-incubated separately with 5x10^6 spermatozoa/mL in Tyrode medium at the different pHs. Co-incubation was for 24 h at 37ºC and 5% CO2 in a 24-well plate. Binding of sEVs to spermatozoa was analyzed by imaging flow cytometry (ImageStream®X Mk II Amnis®) at four co-incubation times (30 min, 3 h, 6 h and 24 h), with 10,000 spermatozoa analyzed for each experimental condition. Neither sEV subset nor medium pH affected sEV-sperm binding. Co-incubation time influenced (p

Proof of concept of using a membrane-sensing peptide for sEVs affinity-based isolation

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One main limitation in biomarker studies using EVs is the lack of a suitable isolation method rendering high yield and purity samples in a quick and easily standardized procedure. Here we report an affinity isolation method with a membrane-sensing peptide (MSP) derived from bradykinin.

We designed a protocol based on agarose beads carrying cation chelates to specifically bind to the 6His-tagged membrane-sensing peptide. This approach presents several advantages: i) cation-carrying agaroses are widely used and standardized for His-tagged protein isolation, ii) the affinity protocol can be performed in small volumes, feasible and manageable for clinical routine and iii) elution with imidazole or EDTA allows a gentle and easy recovery without EV damage, facilitating subsequent characterization and functional analyses.

The optimized final procedure incubates 0.5mg of peptide for 10 minutes with 10µl of Long-arm Cobalt agarose before an overnight incubation with concentrated cell conditioned medium. EV downstream analyses can be directly performed on the agarose beads adding lysis or nucleic-acid extraction buffers, or gently eluted with imidazole or EDTA, rendering a fully competent EV preparation.

This new isolation methodology is based on the recognition of general membrane characteristics independent of surface markers. It is thus unbiased and can be used in any species EV sample, even in samples from animal or plant species against which no suitable antibodies exist. Being an affinity method, the sample handling protocol is very simple, less time-consuming, does not require specialized equipment and can be easily introduced in a clinical automated routine. We demonstrated the high purity and yield of the method in comparison with other commercially available kits. This method can also be scale up or down, with the possibility of analyzing very low amounts of sample, and it is compatible with any downstream analyses thanks to the gentle elution procedure.

Characterisation of circulating microvesicle content in horse plasma in the search of biomarkers for the diagnosis of equine metabolic syndrome.

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Metabolic syndrome is an endocrine multifactorial disorder affecting both humans and animals and for which pathogenesis is still not well understood. Its diagnosis presents a challenge due to the lack of specific symptoms. In horses, affected animals often exhibit obesity, insulin resistance, regional adipose infiltrate and predisposition to laminitis, a severe disease of the horse's foot. The increased risk of laminitis in equine metabolic syndrome (EMS) makes this condition a major welfare problem. Laminitis is a severely debilitating condition, extremely painful, and potentially life-threatening in severe cases. It often causes such extreme suffering to the animal that euthanasia is recommended for animal welfare considerations.

Metabolic syndrome has been associated with increased generation of EVs secreted from endothelial cells, adipocytes, leukocytes and platelets. Therefore, the study of these microvesicles has great potential as a source for the discovery of diagnostic and prognostic biomarkers and as a vehicle for the transport of active molecules for the treatment of metabolic syndrome. Our working hypothesis is that EVs protein and small RNA cargo in plasma show detectable changes correlating with the evolution of equine metabolic syndrome and that these changes inform about the pathophysiology of this disorder.

In this communication we will show our latest results on the optimization of two isolation methods to study the cargo of EVs in the search for biomarkers of EMS.

Study of the in vivo biodistribution of hepatocyte-released extracellular vesicles in mice models for metabolic syndrome progression

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Introduction

Metabolic syndrome (MetS) groups different diseases such as obesity, hypertension, insulin resistance and dyslipidemia. It is known that liver has an important role in the establishment and progression of obesity in MetS. However, the mechanisms that disseminate the disease throughout the body are not completely understood. Hepatic EVs (HepEVs) are active metabolic vesicles that can extend hepatocyte activities outside the liver to other target organs as a response to different stimuli. In MetS, HepEVs could be part of the vectors that propagate the disease by having a different biodistribution, pattern of secretion and cargo.

Methods

To follow and study HepEVs in MetS, we have generated in house two genetically modified mice that express a reporter protein only in the hepatocytes due to liver specific-driven recombination. One expresses a membrane-bound GFP (TMCRE strain) and the other one expresses luciferase (LSCRE strain).
We induce obesity and liver damage by feeding these mice during four months a high fat diet (45% kcal or 60% kcal). Liver damage is controlled by following the levels of transaminases in serum over time.
For TMCRE mice, we used CytoFLEX to measure the levels of HepEVs in serum at different time points. Biodistribution and cargo of EVs is studied through histology and immunoblotting. For LSCRE mice, we used IVIS to measure luciferase signal in vivo and ex vivo.

Results

We were able to induce obesity to mice by feeding them with high fat diet. In serum samples from TMCRE mice, we are able to detect an average of 2.3E6 HepEVs/mL from a total of 2.5E9 particles/mL. For LSCRE mice, liver luciferase signal masked any other possible signal coming or induced by HepEVs.

Conclusions

Our in vivo models are a proper system to study the secretion of HepEVs in the progression of metabolic syndrome.

RNA-seq in blood circulating EVs shows the presence of bacterial RNAs in multiple sclerosis patients.

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The presence of bacterial extracellular EVs (outer membrane vesicles(OMV)) has been reported in several papers, but their presence in human fluids is not well studied. The human gut microbiome is becoming increasingly important in the context of disease, as has been demonstrated in multiple sclerosis.

In this project we want to study the presence of this OMV in circulating blood of patients with multiple sclerosis (pwMS) and healthy control(HC) by studying the presence of bacterial RNA in their cargo.

Plasma-derived EVs were isolated by differential centrifugation in 20 pwMS and 8 HC and after characterization of the EVs, RNA was extracted. 2 ugr of RNA was sequenced from 12 pools (4 recurrent-remitting pools, 4 secondary progressive pools and 4 healthy control pools) using Illumina Hiseq X Ten.

An average of 40-50 million reads per pool was obtained. FastQC was used on the Fastq files obtained to check the quality, after STAR and SALMON was used to align and quantify the reads against the human genome 38. The unmapped reads were used to perform the rest of the analysis to check for the presence of bacterial RNAs.

To ensure the results, 4 different algorithms (kraken2, krakenuniq, centrifuge and kaiju) were used. Taxpasta was used for data aggregation. We want to be restrictive in the analysis and therefore 4 different filters have been used to filter out low quality data.

We have been able to obtain RNA corresponding to bacteria (124-571 species) and fungi (3-15) finding differences between the different types of MS in the number of different species of bacteria and fungi.

Our results suggest that bacterial EVs (OMVs) are present in circulating blood and may be involved in the mechanism of MS.

CD44 may be a universal protein marker for the identification of porcine seminal extracellular vesicles

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Porcine seminal plasma (SP) contains a heterogeneous population of seminal extracellular vesicles (sEVs) that differ in origin source and function. Currently, there are no universal markers for the identification all sEV subtypes. This study evaluated the suitability of CD44 for the identification of six sEV subpopulations isolated according to their size (small [S-sEVs] and large [L-sEVs]) from three different porcine ejaculate fractions (the first 10 mL of the sperm rich fraction [SRF-P1], the remaining SRF [SRF-P2] and the post-SRF [PSRF]) of different origin sources. Seminal EVs were isolated from six SP pools of each ejaculate fraction (five ejaculates/pool) using a base SEC protocol. sEVs were characterized by total protein (BCA™assay), morphology (transmission electron microscopy), size distribution (dynamic light scattering, DLS) and purity (albumin by Western blot). The CD44 expression (anti-CD44-FITC, MCA4703F, Bio-Rad) was analyzed by flow cytometry (CytoFLEX S). The optical setup of the flow cytometer was modified using the side scatter information from the 405 nm laser and calibrated using polystyrene beads of known size (80-300 nm). The sEVs were further discriminated by CFSE labeling and 10,000 events/sample were acquired at a low flow rate (10µL/min). Controls included 0.1-µm filtered PBS (fPBS), fPBS with CFSE or CD44 and unstained sEVs. Results showed that sEV subpopulations were similar in total protein concentration, morphology, and purity (albumin-free). DLS revealed differences in sEV-size ($P < 0.0001$), with L-sEVs being larger than S-sEVs, and those from PSRF being larger than those from SRF-P1 and SRF-P2 fractions. The proportion of sEVs expressing CD44 was higher in all sEV subpopulations (ranging from 90.87% to 98.50%), with no differences between them. These results suggest that CD44 may be a universal protein marker for the identification of porcine sEVs, independent of their origin source and size. Fundings: MCIN/AEI/10.13039/501100011033 (PID2020-113493RB-I00, RYC2021-034546-I), NextGenerationEU/PRTR, Seneca Foundation (21935/PI/22).

Small extracellular vesicles but not microvesicles from the parasitic liver-fluke Opisthorchis viverrini promote cell proliferation in human cholangiocytes

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Infection of humans with the liver fluke Opisthorchis viverrini is a major risk factor for the development of bile duct cancer, or cholangiocarcinoma (CCA) in the Lower Mekong River Basin countries, including Thailand, Lao PDR, Vietnam and Cambodia. However, the precise mechanisms by which O. viverrini contributes to CCA development remain largely unknown. In this study, we characterized different extracellular vesicle populations released by O. viverrini (OvEVs) using proteomic and transcriptomic analyses and investigated their potential role in host-parasite interactions. While 120k OvEVs promoted cell proliferation in H69 cholangiocyte cells at different concentrations, 15k OvEVs did not produce any effect compared to controls. The proteomic analysis of both populations revealed compositional differences that might account for the differential effects observed. Additionally, we analyzed the miRNAs present in the 120k EVs and used computational target prediction to explore their potential interactions with human host genes. Several pathways implicated in inflammation, immune response, and apoptosis were identified as potential targets of the miRNAs present in this particular population of EVs. This study represents the first evidence of distinct roles played by different EV populations in the pathogenesis of a parasitic helminth, providing valuable insights into the mechanisms involved in opisthorchiasis and liver fluke infection-related malignancy.

Attenuation of SASP through sEV biogenesis interruption

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Cells have the capacity to modulate the microenvironment through secreted molecules and factors like cytokines, chemokines, small extracellular vesicles (sEV). Senescent cells are characterized by a specific secretome phenotype known as SASP, an inhibition on the cell cycle, and an increased β-galactosidase activity. SASP leads the microenvironment to a more pro-inflammatory state and induces senescence in the neighbouring cells, triggering with time age-related diseases. To study this paracrine senescence transmission, we focused on the role of sEV. In this work, we pretend to find a proteomic target for the SASP mediated by sEV to reveal pathways associated with the senescence transmission.
We knocked-down the expression of RAB27A and RELA, proteins implicated in sEV biogenesis and in paracrine senescence respectively, in mesenchymal stem cells. It was accomplished to reduce the paracrine senescence

transmission in the knock-down cells after the addition of sEV from senescent cells, validated by -galactosidase and proliferation assays. We also performed a shotgun proteomic study of the recipient cells, identifying several proteins statistically significant dysregulated involved in the Golgi traffic and network. In conclusion, the silencing of RAB27A prevents the paracrine senescence transmission and Golgi apparatus-associated proteins could be future targets for the development of senomorphic drugs.

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CHARACTERIZATION OF EVs FROM APOPLASTIC FLUID OF CAROB PULP: ANTI-INFLAMMATORY AND ANTIBACTERIAL ACTIVITY

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In recent years, extracellular vesicles (EVs) have gained the interest of many experts since different functions have been discovered that make them very attractive for their applications, both in pathological states and for health maintenance and promotion. Moreover, the valuation of the waste from the food industry could satisfy the need for scalability in obtaining EVs, thus contributing to the current model of circular economy. With this sustainable production approach, we have identified and characterized EVs from the Carob (Ceratonia siliqua L) pulp, a by-product of the extraction of locust bean gum with beneficial properties such as antioxidant, anti-inflammatory or anti-aging. Its use would have an economic impact, since it is a widely spread crop in the Mediterranean area.

The apoplastic fluid (AF) was first extracted using a vacuum infiltration method, optimizing the pH buffer and time of infiltration. Then, AF was subjected to differential centrifugation to remove cellular debris and EVs were isolated by size exclusion chromatography (SEC). Nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) showed the presence of EVs in the apoplastic washing fluid of carob pulp (CbEVs) with a mode size of 216.3 nm. Proteomic analysis identified 782 proteins. In in-vitro studies, CbEVs showed no cytotoxicity in THP-1-XBlue™-CD14 human macrophages by the MTT assay, and suppressed Nf-Kb expression in LPS-stimulated macrophages.

Moreover, CbEVs showed to have an impact on the growth of microorganisms such as Salmonella spp, E. coli, Staphylococcus spp. and Enterococcus spp.

In conclusion, the first description of isolation of CbEVs opens up a large field of study that can explain the properties of this by-product.

INVOLVEMENT OF EXTRACELLULAR VESICLES SECRETED BY INFECTED CELLS ON THE PERSISTENCE OF THE DISEASE. LONG COVID, DIAGNOSIS AND PROGNOSIS.

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INTRODUCTION: Long-COVID is a recently identified condition affecting some individuals who have been infected by the SARS-CoV-2 virus. Despite the progress made, there are currently no reliable diagnostic methods or specific treatments available for this condition.

Our hypothesis is that during infection, cells release abundant extracellular vesicles (EVs) containing viral genetic material, possibly higher amounts than virus produced. Concentration of those EVs could be useful to diagnose difficult COVID patients, such as long-COVID and their prognosis. As we hypothesized that EVs secreted by viral infected cells might be implicated on the development of long COVID, we will also study that involvement

RESULTS: In a previous work, we successfully diagnosed COVID patients who had received a negative result from the conventional PCR test (over 30% of the tested patients, n=600). Furthermore, we found that the percentage of the genetic material present in the sample could be correlated with the infection stage and the prognostic of the patient. This gave us enough evidence that diagnosis of the infection through EVs isolation and DNA amplification is feasible and it could be used also for other viruses.

Moreover, we demonstrated that isolated EVs with the viral genetic material inside were capable of forming viral particles and become infective under appropriate culture conditions.

FUTURE RESEARCH: We aim to extend the numbers of our new diagnostic test, for patients who are difficult to detect (long-COVID patients) and other viral infections. This will allow us to diagnose on a more reliably way and on earlier times for those who cannot be accurately detected solely based on symptoms. Additionally, we will study the implication of the transfer of viral genetic material carried in the EVs during viral infection and in the application of possible therapies aimed at reducing the production of these EVs, in vitro and in vivo.

Characterization of Mesenchymal Stromal Cells derived Extracellular Vesicles from 3D Hollow-fiber Bioreactor culture

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Introduction: Mesenchymal stromal cells (MSC) present high potential as cell therapy, exhibiting anti-fibrotic, regenerative, and immunomodulatory functions. Remarkably, their secretome, and specifically their extracellular vesicles (EVs), have been shown to present advantages above cells. Therefore, large-scale production of MSC-derived EVs (MSC-EVs) in controlled and reproducible conditions is considered for their clinical application. The use of bioreactors is a promising strategy for large-scale production of functional and high-quality EVs. They offer trustworthy three-dimensional environmental conditions and increased surface area for cell growth, reducing volume output. In this work we evaluated and characterized the culture of a MSC line in a hollow-fiber bioreactor to produce and isolate EVs using a clinically applicable approach.
Methods: Conditioned medium was collected daily during the four weeks of MSC culture in chemically defined medium in the bioreactor (3D) or from 48h MSC cultured in cell culture flasks (2D). EVs were isolated by size-exclusion chromatography (SEC) and EV fractions were pooled according to the presence of EV (CD63) and mesenchymal cells (CD90) markers. Subsequently, EVs were characterized based on their protein and lipid content and analyzed for the presence of possible contaminants. Finally, phenotyping and cryo-TEM studies were performed to better delineate EV characteristics.
Results: Our findings indicate that the features of bioreactor-derived EVs (3D) are comparable to flask culture-derived EVs (2D). The protein/lipid ratio of the EVs and the presence of surface markers did not reveal any differences between both culture methods. Finally, cryo-TEM studies showed clean EVs, sized between 80-500 nm in diameter. Of note, EV-yield increased in 3D-EVs compared to 2D-EVs, isolating EVs from the same cells for a longer period of time with similar features.
Conclusion: These results show that the use of a hollow-fiber bioreactor allows the large-scale production of iMSC-EVs, maintaining EV characteristics and providing purified and well-defined EV pools.

Proteomics profile of peritoneal EVs from CD38-deficient lupus mice reflects a defective expression of the immunoproteasome and increased abundance of proteins related with inflammatory-resolving processes.

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Introduction: A chronic graft-versus-host reaction (cGVHD) induced in non-autoimmune C57BL6 mice (B6) by the adoptive transfer of Ia-incompatible bm12 spleen cells results in a syndrome that closely resembles Systemic Lupus Erythematous (SLE) in the spectrum of autoantibodies and immunopathology. This is a suitable model to study the role of CD38 in autoreactive B cells using CD38-deficient mice versus B6 WT mice as recipients (doi: 10.3389/fimmu.2021.713697). Objectives: To analyze the protein composition and function of EVs released in the peritoneal cavity of cGvHD mice, to identify predictive or diagnostic biomarkers of the disease Methods: EVs were isolated by qEV size-exclusion-chromatography (SEC) from peritoneal exudates of cGVHD lupus-mice, two and four weeks after the adoptive transfer of bm12 cells. Protein from isolated EVs were analyzed by LC-MS/MS. The protein identification was performed with ProteinScape, and MASCOT data searching using Swiss-Prot database. To quantify protein abundance, the emPAI-based method was used. We used ClueGO and CluePedia apps within the Cytoscape software for functional analysis, and Principal Component Analysis (PCA) to discriminate the different conditions analyzed. Results: At 2 weeks upon lupus-disease induction signaling pathways associated with an activated innate immune system within B cells and with increased immunoproteasome expression were predominantly associated with WT-EVs vs CD38ko-EVs, while at 4 weeks inflammatory-resolving terms associated with neutrophils, platelets and other cell types were predominantly associated with CD38ko-EVs. Moreover, PCA analyses on the abundance of common proteins to the four conditions analyzed and two different qEV fractions showed a clear clustering of the groups according with time (2-weeks vs 4-weeks) and EV source (CD38ko vs WT). In summary, peritoneal EV proteomics provide novel candidate proteins to investigate lupus pathogenesis and/or to be used as EV-based diagnostic biomarkers of the disease.

A method compatible with a clinical setting to isolate extracellular vesicles from cerebrospinal fluid

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Extracellular vesicles (EVs) found in cerebrospinal fluid (CSF) are an important source of biomarkers for neurodegenerative diseases. Since its discovery, several techniques have been used to investigate and improve EV separation in CSF, but more practical and efficient isolation techniques suitable in a clinical environment are still missing.

Here, we describe a method to isolate EVs from CSF by using ExoGAG (Nexotech), a commercially available precipitating agent based on its affinity to the glycosaminoglycans present on the EV surface. Then, we compare it side by side with the conventional ultracentrifugation technique based on successive centrifugations at different rotation speed. Our workflow included optimization of the ExoGAG protocol, isolation and characterization of the EV profile by cryogenic electron microscopy (cryo-EM), protein estimation, Western blot of specific EV markers, and nanoparticle tracking analysis (NTA).

Cryo-EM analysis revealed that ExoGAG precipitates intact vesicles with the expected size and morphology that are free of cell debris. Of note, filaments corresponding to the precipitating agent were also observed in the EV preparations. NTA showed that the majority of the particles were within a size between 50 to 165 nm in diameter. Remarkably, ExoGAG yielded a larger amount of EVs than ultracentrifugation as seen by total protein estimation and the relative amount of CD81 analyzed by Western blot.

Taking together, our data indicate that ExoGAG can be used to isolate EVs from the CSF with advantages over the ultracentrifugation method: more efficient in terms of EV yield, less time consuming and suitable for application in a clinical setting without the need for specialized equipment such as ultracentrifuges. Therefore, the implementation of ExoGAG as a method of choice has the potential to accelerate the search of CSF biomarkers needed to monitor and characterize brain pathologies in neurodegenerative diseases.

ORGAN-ON-A-CHIP TECHNOLOGY TO STUDY EXTRACELLULAR VESICLES-MEDIATED CRYPTIC INFECTIONS IN MALARIA

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Intercellular communication mediated by extracellular vesicles (EVs) plays a key role in the pathophysiology of Plasmodium falciparum and Plasmodium vivax, the two species that account for the major burden of malaria cases worldwide. Our group has recently shown that P. vivax gametocytes are always present in the bone marrow (BM) of infected patients and recent reports have shown that P. falciparum gametocytes fully develop in the BM causing defects in erythropoiesis. We hypothesize that circulating EVs from natural Plasmodium infections signal the BM to favor gametocyte differentiation, thus facilitating transmission from this cryptic niche.

To overcome the ethical and technical difficulty of working with the human bone marrow, we have designed organs-on-a-chip (OOC), mimicking functional 3D units of this human tissue. The two-channel microfluidic devices were fabricated using polydimethylsiloxane (PDMS) with a 3D printer with stereolithography

technology. The BM channel houses ex vivo generated erythroblasts and BM stromal cells embedded in a fibrin-collagen hydrogel mimicking the extracellular matrix of the organ. The vessel channel contains endothelial cells, emulating an endothelium-lined blood vessel. Medium, blood or transgenic parasites expressing fluorescent reporters will be perfused through the vessel channel and functional assays to study adhesion and migration to the BM channel will be addressed.

This bioengineered device will facilitate studies of EV roles at a space and velocity that will ensure interactions with all cells and will contribute to unveil molecular insights of parasite cryptic infections in this hemopoietic tissue. Moreover, it will reduce the use of animals in human experimentation. As the bone marrow is an immune-privileged site, these studies will discover alternative control strategies ultimately contributing to malaria elimination.

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Sepharose CL-2B is more efficient than CL-6B for isolating porcine seminal extracellular vesicles in purity by size exclusion chromatography.

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The use of size-exclusion chromatography (SEC) to isolate extracellular vesicles (EVs) has become widespread. It is based on differential elution of different sized particles through a polymeric stationary phase. The aim of this study was to compare the efficacy of Sepharose CL-2B® (CL-2B) and Sepharose CL-6B® (CL-6B) polymers in isolating two subtypes of porcine seminal EVs (sEVs) that differ in size, namely large (L) and small (S) sEVs, at higher purity. Three pools of porcine seminal plasma pools (three ejaculates/pool) were centrifuged at 20,000 xg to separate the pellet containing L-sEVs and the supernatant containing S-sEVs. The pellet and supernatant were separately subjected to SEC using CL-2B and CL-6B and twenty 0.5-mL fractions were collected. DLS and TEM determined that the fractions containing EV-compatible particles were 7 to 11 for S-sEVs and 6 to 10 for L-sEVs using CL-2B, and 7 to 10 for S-sEVs and 6 to 10 for L-sEVs using CL-6B. Fractions containing EV-compatible particles were incubated with CFSE and analyzed by high-resolution flow cytometry (FC), extending FC-analysis to EV protein markers (CD63, HSP90β). The purity of sEVs was assessed by FC by measuring albumin, a very abundant protein in porcine seminal plasma. Isolation purity (IP) of sEVs (CFSE+ events per µL/total particle count x 100) of S-sEVs was 84.41% ± 4.01% (mean ± SD) (range from 64.44% to 92.71%) using CL-2B, while it was 74.81% ± 5.66% (58.49% to 86.00%) using CL-6B (P =0.0108, paired t-test). The IP of L-sEVs was 88.74% ± 1.57% (83.22% to 93.53%) using CL-2B, while it was 70.04% ± 12.14% (51.30% to 90.51%) when using CL-6B (P =0.0926). These results showed that CL-2B was more effective than CL-6B for SEC isolation of pure porcine sEVs.
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Regulation of of cell envelope remodeling to mediate EV production in the human pathogen Mycobacterium tuberculosis

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Our studies and others have clearly established a role of mycobacterial extracellular vesicles (MEVs) in immunomodulation and shown that MEVs deliver factors that impair macrophage effector functions, inhibit T cell activation, and modify the response of host cells to infection. Although the importance of MEVs has been recognized, hardly anything is known regarding the molecular mechanisms underlying vesicle formation in mycobacteria and how they contribute to its survival strategy within the host. Understanding the molecular mechanisms of MEV formation can lead to novel therapeutic or prophylactic interventions urgently needed to strengthen TB control efforts. We have identified two distinct conditions that stimulate vesiculogenesis in Mtb: iron limitation and deletion of the gene encoding the surface protein VirR. Common transcriptional responses indicate upregulation the iniBAC operon, that have been previously involved in the response to drugs targeting the mycobacterial cell envelope. We demonstrate that two genes encode for dynamin-like proteins and function at the level of cell membrane to release membrane stress. Moreover, we uncover that alterations in the homeostasis of peptidoglycan (PG) and arabinogalactan (AG) synthesis, two key components of the mycobacterial cell envelope, lead to hypervesiculation. These studies shed light on how unconventional cell-walled bacteria of great clinical relevance produce EVs.

The profiling of extracellular vesicle subpopulations in human Huntington's disease brains identifies Alix as a novel neuropathology marker

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Huntington's disease (HD) is an inherited autosomal dominant disease characterized by progressive degeneration of the striatum and the cerebral cortex caused by a mutation in the HTT gene, which comprises an aberrant expansion of a CAG repeat. The mutant HTT protein accumulates in the endosomal compartments and alters the endosomal-lysosomal trafficking, which can affect the secretion and content of exosomes, extracellular vesicles (EVs) with an endosomal origin. Therefore, we investigated whether the levels and content of small EV subpopulations, including exosomes, are altered in postmortem brains of HTT-mutation carriers.

We isolated and characterized Annexin-2-enriched and Alix-enriched EVs from the striatum and the dorsolateral frontal cortex of HD brains, at two stages of degeneration, and control brains by differential ultracentrifugation followed by an iodixanol density gradient. Additionally, we explored the secretion of EVs by fibroblasts from HD patients compared to healthy individuals.

Our data indicate that the level of Annexin-2-enriched EVs was increased in the HD cortex, whereas the level of Alix-enriched EVs was reduced in both the HD striatum and cortex compared to controls. The Annexin-2 and Alix loading per EV were also increased and reduced in the disease, respectively. Moreover, the altered cargo loading of Alix in EVs was consistent with a progressive decrease of Alix protein levels in the cortex and striatum of HD patients as the brain pathology worsens. In our in vitro studies, we found that HD fibroblasts secreted fewer Alix-positive EVs compared to control fibroblasts, despite having similar levels of Alix protein in the cell lysates.

Taken together, our data provide new insights into the pathophysiology of brain EVs in HD and identifies Alix as a novel marker of neuropathology, opening up new venues to explore Alix in peripheral brain-derived EVs as a potential biomarker of disease progression.

Fida: A novel technique for determination of protein binding on Extracellular Vesicles

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The surface composition of extracellular vesicles EVs is important for their intercellular communication. Understanding the binding of EVs to specific proteins on cells and in body fluids is essential for unraveling their biological functions and find potential diagnostic and therapeutic applications. In this study, we introduce a novel technique, Flow Induced Dispersion Analysis (FIDA), for the precise determination of EV-protein interactions.

By utilizing fluorescently labeled proteins and target EVs, FIDA provides a highly sensitive and specific method to detect and quantify these interactions. This technique offers several advantages, including minimal sample requirements, high throughput capability, and compatibility with various sample types.

We applied Fida 1 to investigate the binding dynamics of EVs derived from MSCs from different sources (Umbilical cord WJ and Bone Marrow)with specific target proteins. Our results demonstrate the successful detection and quantification of EV-protein interactions, revealing diverse binding affinities. Moreover, Fida 1 facilitated the identification of key factors influencing EV-protein binding, such as protein concentration, EV subtype, and cellular conditions.

In summary, FIDA represents a powerful tool for studying EV-protein interactions, shedding light on the complex mechanisms underlying intercellular communication mediated by EVs. Moreover, FIDA has promising applications in identifying biomarkers and therapeutic targets for various diseases. Overall, this study demonstrates the significance of FIDA technology in advancing our knowledge of EV biology and its potential medical implications.

Small particles carrying great potential – Extracellular vesicles in Parkinson's disease research

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Parkinson's disease (PD) is an incurable neurodegenerative disorder, imposing a serious burden in today's aging population. Up to today, the basis of the diagnosis of PD is the presence of the characteristic motor symptoms, often leading to its recognition only in an advanced stage. However, gastrointestinal symptoms accompanying the motor symptoms often precede those by decades, giving hope to the identification of an early disease biomarker.

Recently extracellular vesicles (EVs) emerged as promising candidates for fulfilling the role of such a biomarker. In PD-derived EVs, pathological forms of alpha-synuclein (aSyn; a protein which under pathological conditions forms aggregates, causing the loss of neurons in PD) have been detected.

Our aim is to establish a diagnostic method for PD, based on the detection of pathological aSyn species in peripheral blood-derived EVs with the use of an in vitro polymer formation assay, termed seed amplification assay (SAA).

We utilize various approaches for the enrichment of human blood derived EVs and subtypes of the vesicles based on their cellular origin. For aSyn-SAA optimization, we tested various assay conditions and established positive and negative controls.

We have successfully extracted blood derived EVs, isolated vesicles positive for the neuronal marker NCAM-L1, and characterized the EVs following the latest guidelines of EV-research. Furthermore, we show that SAA is a sensitive method, which is strongly influenced by various assay conditions and sample quality. With the optimization of those and the establishment of a standard protocol, aSyn-SAA can be a valuable tool in PD biomarker research.

Unraveling signaling mechanisms of renal extracellular vesicles in ciliopathies

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Extracellular vesicles (EVs) play a crucial role in kidney development and disease, influencing key signaling pathways. Recent research highlights the significance of the primary cilium, a highly conserved signaling organelle, in coordinating the release and composition of renal small EVs (smEVs), which differ in terms of their content, particularly regarding signaling molecules. Changes in EV-mediated signaling due to ciliary dysfunction could thus impact tissue homeostasis leading to the formation of renal cysts and other pathological conditions. In our study, we focus on the functional significance of ciliary smEVs to unravel the mechanistic defects underlying cell communication in kidney diseases.

We were able to show a Wnt-attenuating effect of smEVs released by kidney medullar (KM) cells in recipient cells. This appears to be predominantly attributable to their protein content. In both mouse model and patient-derived samples, we observed distinct readouts between ciliary mutants compared to control groups. Looking more closely at the specific protein content of smEVs from our renal mutant models, we found an enrichment of proteins involved in multiple signaling pathways associated with renal homeostasis, such as Wnt signaling and proteasomal degradation. We further evaluated the functionality and specificity of these smEVs on downstream target cells, with particular emphasis on proliferation, migration and the induction of cellular responses related to polarization and cyst formation.

Our results provide insights into the communication pathways of renal cells via ciliary EVs, shedding light on their influence in processes that may underlie renal ciliopathies including cystic kidneys.

Pro-coagulant extracellular vesicles mediate smoking-induced pulmo-vascular inflammation

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Background: Chronic obstructive pulmonary disease (COPD) is a leading cause of death worldwide, with smoking as major risk factor. Tobacco smoke increases the risk of thrombosis, resulting in dysregulation of tissue factor (TF)-mediated extrinsic coagulation that further impacts downstream intracellular signaling via protease activated receptors (PARs). Here, we investigated the impact of bronchial epithelial cell (BEC)-derived TF+-extracellular vesicles (EVs) on human lung endothelial cell (EC) activation as mediators of pulmo-vascular inflammation.

Results: We could show that human BECs secrete pro-coagulant TF+-EVs when stimulated with tobacco smoke extract. Those TF+-EVs are secreted towards the lung lumen and basolateral side by polarized BECs to potentially target PAR1 and PAR2 expressing cells from the lung lumen and tissue side. COPD-specific regulation of TF and PAR1 was further demonstrated by re-analysis of public RNA sequencing data showing upregulation of these factors in lung tissue from COPD patients. With respect to potential TF+-EV target cells, we demonstrated that especially lung ECs express PAR1 and PAR2. Stimulation of these cells with TF+-EVs induced EC activation as monitored by proinflammatory gene expression of IL-8, ICAM 1 and VCAM-1 or intracellular calcium release. Additionally, EC activation can be blocked by PAR1 and PAR1/PAR2 siRNA knockdown as well as the clinically relevant thrombin-inhibiting anti-coagulants Dabigatran and anti-thrombin III.

Conclusion: In conclusion, tobacco smoke induces secretion of procoagulant TF+-EVs by BECs to stimulate human lung ECs via PAR1 and PAR2. Stimulated lung ECs express pro-inflammatory mediators, thereby potentially promoting COPD-associated EC dysfunction and thrombotic events.

Bacterial extracellular vesicles repress the vascular protective factor RNase1 in human lung endothelial cells

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Background: Sepsis is one of the leading causes of death worldwide and characterized by blood stream infections associated with a dysregulated host response and endothelial cell (EC) dysfunction. Ribonuclease 1 (RNase1) acts as a protective factor of vascular homeostasis and is known to be repressed by massive and persistent inflammation, associated to the development of vascular pathologies. Bacterial extracellular vesicles

(bEVs) are released upon infection and may interact with ECs to mediate EC barrier dysfunction. Here, we investigated the impact of bEVs of sepsis-related pathogens on human EC RNase1 regulation.
Results: bEVs from the sepsis-associated bacteria Escherichia coli, Klebsiella pneumoniae and Salmonella enterica serovar Typhimurium were isolated via ultrafiltration and size exclusion chromatography and used for stimulation of human lung microvascular ECs. Thereby, gram-negative bEVs from E.coli, Klebsiella and Salmonella significantly reduced RNase1 mRNA and protein expression and activated ECs, while TLR2-inducing bEVs from Streptococcus pneumoniae did not. These effects were mediated via LPS-dependent TLR4 signaling cascades as they could be blocked by Polymyxin B. Additionally, LPS-free ClearColiTM had no impact on RNase1. Further characterization of TLR4 downstream pathways involving NF-κB and p38, as well as JAK1/STAT1 signaling, revealed that RNase1 mRNA regulation is mediated via a p38-dependent mechanism.
Conclusion: Blood stream bEVs from gram-negative, sepsis-associated bacteria reduce the vascular protective factor RNase1, opening new avenues for therapeutical intervention of EC dysfunction via promotion of RNase1 integrity.

Extracellular vesicles cargo of the proteolytically active ADAM10 or ADAM17 shape the inflammatory response and drive disease severity

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Extracellular vesicles (EVs) like-exosomes play an essential role in orchestrating the immune response and influencing the performance of immune cells. The released exosomes from immune cells can load pro-inflammatory signals and molecules that stimulate immune cell activation and promote the release of cytokines (e.g., TNF-α, IL-6R) and other signaling molecules involved in the inflammatory process. Members of the 'a disintegrin and metalloproteinase' (ADAM) family, particularly ADAM10 and ADAM17, have the capability to proteolytically cleave transmembrane molecules close to the plasma membrane, a process called ectodomain shedding. Thus, we could show that infection of alveolar epithelium with Pseudomonas aeruginosa induces an exosomal release of proteolytically active ADAM10, predisposing to cleavage in trans orientation. Furthermore, our investigations showed that the catalytic activity of ADAM10 or ADAM17 on exosomes drives the disease severity in hospitalized viral and bacterial pneumonia patients. Pre-clinical pneumonia model of conditional ADAM10 or ADAM17 knockout in leukocytes and detailed in vitro mechanistic investigations of primary human neutrophils revealed that leukocytes represent a major source of the exosomes pool. This exosomal release was associated with a differential immune response such as leukocyte recruitment to the alveolar space and the lung tissue, edema formation, cytokine release, macrophage polarization, reactive oxygen generation, bacterial clearance and sepsis. Our results highlight the significance of ADAM10 and ADAM17, as exosomal cargo, for developing new prognostic and therapeutic tools that need to be addressed in further preclinical and translational studies.

Enhance small RNA NGS analyses with caRNAge - Bias detection, ncRNA isoforms and improved validation chances

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Small RNA species have become the major focus of transcriptomic EV studies in recent years due to their high potential as prognostic and diagnostic biomarkers in addition to their relevance in almost all physio- and pathophysiological pathways. At the same time, continuous progress in Next-Generation sequencing technologies has revealed a formerly unexpected complexity of small RNA transcripts in biofluids especially liquid biopsies. This heterogeneity stems not only from different RNA species like miRNA, tRNA, snRNA, snoRNA and many others but encompasses functionally active isoforms and fragments such as isomiRs and tRFs as well. Further complicating accurate analyses are batch effects commonly introduced by EV isolation methods and correct identification of stable reference genes which have severe implications for the success of downstream verification of findings by independent methods. Unfortunately, the wealth of information afforded by current sequencing technologies is not yet fully exploited most of the times due to the complexity of transcripts as well as the involved bioinformatics.

We established an easy to use computational pipeline comprised of well-established tools and custom reporting scripts for experienced as well as beginning analysts. Incorporating all major steps including quality control with batch effect identification, trimming, alignment with regard to transcriptional isoforms and functional fragments, differential gene/transcript expression, unsupervised and supervised clustering and pathway analysis of over represented targets, caRNAge is designed to deliver comprehensive outputs that cover a vast variety of scientific questions especially in regard to EVs. The pipeline is freely available at https://www.physio.wzw.tum.de/caRNAge/ and runs locally on all Linux distributions. Users can directly input raw sequencing data and only need a meta data file containing information on experimental setup to start the analysis. All results are presented as explorative html files including visualizations to allow easy sharing and browsing on any computer without the need of further programs.

Role of extracellular vesicles in the pathophysiology of Alzheimer's disease; modulation of amyloid β aggregation

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Introduction:
Extracellular vesicles (EVs) are excellent cargo vehicles for cell-to-cell communication and are involved in crucial brain functions, such as myelin maintenance and neurotransmission. EVs have a high abundance of cellular prion protein (PrPC) on their surface. PrPC is a glycosylphosphatidylinositol-anchored glycoprotein with highest expression in the nervous tissues; and in the Alzheimer's disease (AD), EV-associated PrPC along with other extracellular PrPC fragments is suggested to sequesters Aβ oligomers (Aβo) hence reducing the Aβo neurotoxicity, however formal evidence is still lacking. Here, we aim to study the role of PrPC expressing EVs in Aβ fibrillization and to highlight physiological alterations associated with EV in AD.
Materials and Methods:
PrPC-expressing (WT) and -deficient (KO) EVs were obtained from WT and PrPC-KO Neuro-2a cells and also from WT and PrPC-KO mice brain. Human brain-derived EVs were isolated from frontal cortices of AD and age-matched controls.
EVs were characterized using Nanoparticle tracking analysis (NTA), immunoblotting, and negative stain TEM.
To further the study objectives, small angle X-ray scattering (SAXS), super-resolution microscopy (SRM),

Cryo-EM, proteomic profiling, and associative biochemical and biophysical methods were employed.
Results:
SAXS, EM, SRM and aggregation assays highlighted potent Aβo-sequestering activity of the WT-EVs. Lipidomic and proteomic profiling of N2a-derived EVs pointed towards marked differences, however no variations were found for the Aβ binding proteins between WT- and KO-EVs.
EV isolated from AD brain tissues show alterations in PrPC expression compared to those of controls. Omics data from human EVs also highlight compositional differences specific to AD.
Conclusions:
Our data suggest crucial role of EVs in AD by their involvement in Aβ aggregation, a related potential rescue mechanism against Aβo toxicity, and AD-specific EV-mediated intercellular communication.

Bacterial vesicles block viral replication in macrophages via TLR4-TRIF-axis

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Gram-negative bacteria naturally secrete nanosized outer membrane vesicles (OMVs), which act as a means of communication. OMV uptake by host cells activates TLR signalling via transported PAMPs. As key resident immune cells, alveolar macrophages are located at the air-tissue interface, where they comprise the first line of defence against inhaled microorganisms and particles. To date, little is known about the interplay between alveolar macrophages and OMVs from pathogenic bacteria. Immune responses to OMVs and underlying mechanisms are still elusive.

Here, we examined the response of primary human macrophages to bacterial vesicles (Legionella pneumophila, Klebsiella pneumoniae, Escherichia coli, Salmonella enterica, Streptococcus pneumoniae) and observed comparable NF-κB activation across all tested vesicles. In contrast, we describe differential type I IFN signalling with prolonged STAT1 phosphorylation and Mx1 induction blocking influenza A virus replication only for Klebsiella, E.coli and Salmonella OMVs. OMV-induced antiviral effects were less pronounced in endotoxin-free Clear coli OMVs and Polymyxin-treated OMVs. LPS stimulation did not mimic this antiviral status, while TRIF knockout abrogated it. Importantly, supernatant from OMV-treated macrophages elicited an antiviral response in alveolar epithelial cells (AEC), suggesting OMV-induced intercellular communication. Finally, results were validated in an ex vivo lung infection model.

Collectively, Klebsiella, E.coli and Salmonella OMVs induce antiviral immunity in macrophages via TLR4-TRIF signaling, thereby limiting viral replication in macrophages, AECs and lung tissue. These gram-negative bacteria induce antiviral immunity in the lung through OMVs, potentially affecting bacterial and viral coinfection outcome.

TFF and SEC may lead to loss of a pro-angiogenic effect in Müller cell-conditioned media

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Introduction

Chronic retinal vascular diseases characterized by the break-down of the inner blood-retinal barrier and endothelial cell proliferation remain the leading cause of vision loss in the western world. As the major macroglial cell type in the retina, Müller cells (MCs) contribute to retinal vascular development and homeostasis. This project aimed to investigate the angio-modulatory effect of MCs on vascular endothelial cells and particularly MC-derived extracellular vesicles (EVs).

Methods

Primary murine MC cultures were derived from C57BL6/J mice at P10 by incubating retinal cells in EGF-supplemented growth medium. At passage 2, medium was changed to EV-depleted differentiation medium for 7 days, followed by preparation of MC-conditioned media for 72h. To analyze the angio-modulatory effect of the MC-secretome, large and small EVs as well as an EV-depleted fraction, rich in free secreted proteins were isolated by tangential flow filtration (TFF) and size exclusion chromatography (SEC). Isolated EVs were characterized by NTA, MicroBCA, TEM, ELISA and western blot. The angio-modulatory effect was analyzed in vitro with a spheroid sprouting assay using human umbilical vein endothelial cells (HUVECs).

Results

In the spheroid sprouting assay, unfractionated, MC-conditioned media revealed a significant pro-angiogenic effect compared to unconditioned controls ($p<0.05$). When separated by TFF and SEC into fractions of small EVs, large EVs or a protein rich, EV-depleted fraction, none of the samples induced a pro-angiogenic effect. Characterization of the isolated fractions revealed CD9, ALIX and AQP4-positive EVs.

Conclusions

Müller cells exert a pro-angiogenic effect on ECs in vitro. TFF and SEC are useful to isolate MC-derived EVs from MC-conditioned media. However, the proangiogenic mediators of the MC-conditioned media either seem to be lost due to this isolation procedure or require a synergistic interaction. Alternative EV-isolation methods may be less prone to the loss of angio-modulatory mediators.

ROLE OF THE PRION PROTEIN IN EXTRACELLULAR VESICLES UPTAKE

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How extracellular vesicles (EVs) are taken up by recipient cells is still unknown. Most of the studies point at endocytosis as the main uptake mechanism, which leads to the question of how EVs can escape from the endocytic compartments. We have shown that the prion protein (PrP), a GPI-anchored glycoprotein, and its C1 fragment which exposes a hydrophobic domain, are enriched on the surface of murine brain-derived EVs (BDEVs). The presence of PrP on BDEVs influences their uptake in primary neurons, as PrP KO BDEVs rapidly colocalized with lysosomes, whereas WT BDEVs were more present at the plasma membrane.
Our aim is to exactly pinpoint the role of PrP and C1 in the uptake of EVs. We have isolated WT and PrP KO BDEVs, and labelled them with R18, a fluorescent dye that is quenched while packed on EVs and that emits signal when fused with a larger membrane. We incubated labelled WT or KO BDEVs either with N2a cells (murine neuroblastoma cell line), or primary neurons, both also WT or KO for PrP, and measured the fluorescence intensity. WT BDEVs showed an increased fluorescence signal, but only when incubated with WT cells. Our preliminary experiments highlight the importance of PrP´s presence in both, EVs surface and on the cell membrane for fusion events. To define C1´s role, we will perform the same experiment, but using EVs isolated from N2a cells expressing only C1. To exactly pinpoint whether the fusion events occur directly at the plasma membrane or in endocytic compartments, we will check the colocalization of fluorescently tagged endosomal markers, such as Rab5, and PrP-EGFP expressing EVs.
We postulate that PrP and C1 regulate EV fusion with recipient cells. In the view of EVs as therapeutic carriers, the manipulation of their PrP load could influence cargo delivery efficiency.

The presence of cell-free DNA in plasma-derived small extracellular vesicles

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Cell-free DNA (cfDNA) is one of the novel and promising biomarkers in liquid biopsy. Small extracellular vesicles (sEVs) have emerged as safe carriers of cfDNA in circulation, as the lipid bilayer of sEVs can protect the cfDNA against degradation by nucleases. The DNA packaged inside sEVs can mediate intercellular communication in health and disease. Therefore, it is essential to characterize the nature of DNA fragments present in the sEVs. In the current study, we aim to investigate the origin of DNA associated with plasma sEVs and elucidate its localization. We isolated the pure sEVs population by using iodixanol-density gradient ultracentrifugation from healthy. In plasma-derived sEVs, approximately 60 – 75 % of DNA is associated with the surface, and about 25 – 40% is protected inside the sEV membrane. Comparing DNA's nature, we observed mitochondrial DNA (mtDNA) in all plasma sEVs, whereas nuclear DNA (ncDNA) was present in 9 out of 18 sEV samples. We further confirmed that, indeed, the majority of mtDNA is protected from DNases treatment in sEVs. This finding suggests that mtDNA in healthy samples Evs can potentially have normal physiological functions in maintaining homeostasis in an organism. Whether the ratio of protected mtDNA versus ncDNA changes in pathological states and how it impacts cellular processes remains to be elucidated.

Metagenomic profiling of fecal-derived bacterial membrane vesicles in non-alcoholic fatty liver disease and drug-induced liver disease

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Introduction: Extracellular bacterial vesicles (EVs) are mediators of intercellular communication. In the context of gut, EVs released by bacteria can play a role in facilitating communication between different bacterial species, as well as between bacteria and host cells. EVs may play a role in the development and progression of non-alcoholic fatty liver disease (NAFLD) and drug-induced liver injury (DILI).

Objectives: To compare the fecal microbiota composition with the composition of fecal-derived EVs in both control subjects and patients with liver disease.

Materials and Methods: This study included 27 fecal samples from 3 groups: healthy volunteers (n=9), DILI patients (n=5), and NAFLD patients (n=13). NAFLD were divided in two groups: (F≤1) with no-significant fibrosis and (F≥2) with significant fibrosis. We implemented a validated procedure to isolate and purify EVs from feces, and we investigated the microbial composition using metagenomic analysis.

Results:Significant differences in alpha-diversity (p≤0.0001) and evenness (p=0.0042) were observed between feces and EVs samples. At the genus level, EVs showed a significant decrease in the abundance of 21 bacterial groups and an increase of 8 bacterial groups compared to feces. Similarly, when we compared EVs and feces by patient type, we found significant differences in all groups (control: 4 genera; NAFLD (F≤1): 8 genera; NAFLD (F≥2): 21 genera; DILI: 42 genera). Anaerotroncus increased in EVs of both types of NAFLD, while in the EVs of DILI patients we found increased proportions of Abiotrophia, Alloprevotella, Actinomyces and Actinobacillus,among others.

Conclusion:This study demonstrates differences in the microbial composition of EVs in relation to the composition of feces, which also varies for each type of liver disease studied.

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A novel spectral flow cytometry workflow to assess the RNA topology of submicron particles

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Introduction:
Extracellular vesicles (EVs) carry different types of cargo, including RNA, providing candidate biomarkers for disease. However, there is no consensus or experimental proof on the exact topology (location) of EV-RNA. This knowledge gap leads to irreproducible results and hampers standardization in EV-RNA research. Within the RNA-top project we aim to decipher if EV-RNA is located on the surface of EVs, inside EVs, or both. Currently, there are no workflows to explore single EV-RNA topology and no model systems to validate such workflows. Here, using a liposome model system, we developed a spectral flow cytometry (sFCM)-based workflow to detect RNA on the membrane surface.

Methods:
Positively charged- and negatively charged liposomes (100 nm, DOTAP:HSPC:Chol=1:4:3 and DSPG:HSPC:Chol=1:4:3) were prepared using a hydration, freeze-thaw and extrusion method. Total RNA (negatively charged) was isolated from a BxPC3 cell line, and bound to the membrane surface of the liposomes based on electrostatic interactions. A fluorescent RNA dye (SYTO) was used to detect the RNA. The concentration of liposomes and RNA-bound liposomes was measured using calibrated sFCM (Cytek Northern Lights, diameter >145 nm).

Results:
The total concentration of positively charged- and negatively charged liposomes was 1.9E13 and 2.4E13 liposomes/mL (based on side scatter), respectively. The concentration of liposomes containing fluorescent RNA on the membrane surface was 3.4-fold higher for the positively charged liposomes, compared to the negatively charged liposomes or the control sample (RNA in water). These results indicate that unbound RNA is below the detection range of the instrument, and can only be detected by sFCM upon electrostatic binding to the liposomes.

Conclusion:
We have developed a workflow to detect fluorescent RNA on the membrane surface of a liposome model system using sFCM. Further research will focus on the development of workflows to detect intra-vesicular RNA, and extrapolation of these workflows to EV samples.

CHARACTERIZATION OF EXTRACELLULAR VESICLES FROM NEURONAL PRIMARY CULTURES

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Background: Brain-derived extracellular vesicles are involved in the neuron-glia communication in the central nervous system. EVs and their cargo have gain growing attention due to their potential involvement on the onset and progression of neurodegenerative disorders, such as Alzheimer's disease or Parkinson's disease. However, the study of the metabolic cargo of EV has not yet been fully explored. In this work, we isolated and characterized EV secreted by primary cultures of granule neurons from rat cerebellum.
Methods: EVs were isolated from primary neuronal cultures by ultracentrifugation or using a polymer-based precipitation method. Depolarization was evaluated as an activator of EV secretion. EV fractions were analyzed by nanoparticle tracking analysis and transmission electron microscopy. The metabolite content of EV was analyzed with gas chromatography-mass spectrometry (GC-MS).
Results: We found that the number of EV secreted upon depolarization decreased in comparison with those released under physiological conditions. Stimulation with KCl resulted in the secretion of a greater number of EVs with sizes ranging between 50 and 100 nm. The metabolomic analysis of EV fractions showed that amino acids and carbohydrates were more abundant in EV released upon depolarization when compared with the control conditions.
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Developing a scalable method for isolating and purifying milk-derived extracellular vesicles

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Foods are promising extracellular vesicle (EVs) sources, which might overcome the scalability and sustainability problems associated with using EVs in clinical practice. Among various EVs, bovine milk EVs have attracted considerable attention as a scalable source of EVs to replace human exosomes due to their multiple advantages for biotherapeutic applications, such as biocompatibility between cross-species, inert toxicity, good BBB permeability, high stability in digestion, and nanocarrier ability. However, a standard, reproducible, and cost-effective protocol for EV isolation from dairy-derived products is lacking. Here, a scalable method for isolating highly pure dairy industry-derived EVs was assessed.

With this purpose, EVs were first isolated from milk. Acidification was tested to modify the yield and purity. Ultracentrifugation, size exclusion chromatography (SEC), tangential flow filtration, ultrafiltration, or polymer precipitation methods were tested either alone or in combination. Dairy industry-derived EV isolation was confirmed by Transmission Electron Microscopy, EV concentration was measured by Nanoparticle Tracking Analysis, and protein markers and contaminations were measured by Western blot. Moreover, whey, a by-product of the dairy industry, was tested as a suitable source of EVs.

Results support that dairy industry-derived products and by-products are a cost-effective natural source of highly pure EVs, which can be used as potential nanocarriers as delivery vehicles for therapy.

The miRNA cargo of jejunal extracellular vesicles depends on the level of insulin resistance in patients with morbid obesity

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Introduction

Extracellular vesicles (EVs) contain bioactive molecules such as miRNAs, which can modulate the immune system cells, the gut microbiota, and the intestinal barrier. These factors could in turn influence the function of peripheral tissues.

Objetives
To analyze by Next Generation Sequencing (NGS) the content of miRNAs in EVs from jejunal samples of patients with morbid obesity (OM), with high and low insulin resistance (IR).

Methods
Jejunal biopsies were obtained from two groups of OM patients (with high and low IR) during bariatric surgery. Briefly, samples were disrupted and digested, and the lysate was subjected to serial centrifugations. The supernatant was ultracentrifuged to precipitate the EVs. The RNA was extracted from the EVs and NGS of the total miRNA content was performed.

Results
NGS data from jejunal EVs revealed nine differentially expressed miRNAs (DE-miRNAs) between the two groups of morbidly obese patients, which may be involved in the development of insulin resistance. Except for hsa-miR-433-3p, the remaining miRNAs (hsa-miR-372-3p, hsa-miR-570-3p, hsa-miR-10392-5p, hsa-miR-193a-3p, hsa-miR- 362-3p, hsa-miR-548ah-3p, hsa-miR-548p, hsa-miR-590-3p) were upregulated in the group with low IR compared to the high IR group. These miRNAs could modulate the expression of genes associated with the development of endocrine, gastrointestinal and immunological pathologies, and related to different canonical pathways such as STAT3, Aryl hydrocarbon receptor (AhR), IGF-1, epithelial adherens junction signaling, and PPARα/RXRα activation signaling.

Conclusions
These DE-miRNAs found in jejunal EV could modulate the expression of genes associated with various pathologies and signalling pathways that are involved in inflammation and in the development of IR induced by obesity.

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The effects of fecal-microbe-derived extracellular vesicles on intestinal mRNA expression depend on the type of patient

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Background: Crohn's Disease (CD) is a chronic inflammatory bowel disease whose pathogenic mechanisms are multifactorial and still unclear. Among all of them, it is known the important role of the gut microbiota. Bacteria can interact with their host by producing extracellular vesicles (EVs) that transport different types of molecules. For this reason, these EVs can regulate molecular pathways and the host cellular response. However, their impact on different cell types involved in the pathogenic mechanisms of CD need to be studied. In this study, we analyzed the effects of fecal-derived EVs of CD patients and healthy subjects on intestinal inflammation and fibrosis processes.

Methods: EVs were isolated and purified from feces samples of healthy subjects and CD patients. The effect of purified fecal EVs suspensions were tested in vitro by adding different protein concentrations (1 and 10 ug/ml) in Caco-2 and myofibroblast cell cultures for 24h. Gene expression of different genes was determined by using RT q-PCR in Caco-2 (RELA, TGFB1 and IL1B) and myofibroblast (RELA, TGFB1, IL1B, CXCL12 and COL1A1).

Results: In Caco-2 cells, 10 ug/ml of VEs from CD produced a greater increase of TGFB1 and IL1B expression compared to healthy subjects. In myofibroblast, 10 ug/ml of VEs from CD produced a greater increase of RELA, TGFB1 and COL1A1 expression and a lower CXCL12 expression that those from healthy subjects.

Conclusion: Our findings suggest that there are differences in the effects produced by fecal-derived EVs in genes related to intestinal inflammation and fibrosis according to their concentration and origin (CD or healthy subjects). This study contributes to understanding the molecular players (VEs) connecting gut microbes to health.

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The inflammatory microenvironment generated in acute pancreatitis modify the surface of circulating exosomes increasing their inflammatory activity

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Background: Exosomes are small extracellular vesicles that plays a role in the progression of systemic inflammation in acute pancreatitis. Although the pancreas can produce exosomes with inflammatory capacity, the microenvironment generated around the damaged tissue could also modify circulating exosomes, particularly through the binding of new proteins to the surface protein corona of these vesicles. In this study we evaluated the effect of the inflammatory microenvironment induced during pancreatitis on the biological activity of exosomes.
Methods: Acute pancreatitis was induced in rats by intraductal administration of 5% sodium taurocholate and, 3 h after induction, ascitic fluid was collected, diluted and ultracentrifuged for 16 h to remove endogenous exosomes. On the other hand, pancreatic cells of the BXPC3 line were cultivated to obtain the exosomes they secreted. These exosomes were incubated with the ascitic fluid or with PBS as a control group. We evaluated the effect of treatment with ascitic fluid on the uptake of exosomes by different cell types as well as on the induction of the expression of inflammatory cytokines by macrophages.
Results: Exposure of exosomes to an inflammatory microenvironment, such as ascitic fluid associated with pancreatitis, increased the level of uptake by macrophages and endothelial cells but did not modify this in the case of pancreatic epithelial cells or in keratinocytes. It also increased the effect of exosomes promoting an M1 inflammatory phenotype in macrophages.
Conclusions: The inflammatory microenvironment that surrounds the pancreas during acute pancreatitis modifies the characteristics of exosomes that circulate through it. Changes in the surface corona increase the uptake of exosomes by some cell types and also increase their ability to induce an inflammatory phenotype in macrophages.
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Proteomics of Circulating Extracellular Vesicles in Patients with Bacterial sepsis

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Background: Sepsis is a result of the body´s dysregulated reaction to an infection which may lead to multiple organ failure. High incidence and mortality rate makes sepsis a global health threat, and access to more specific biomarkers is desirable to achieve early and accurate diagnosis. Extracellular vesicles (EVs) play key roles in intercellular communication, transporting proteins, nucleic acids and lipids between cells, and are suggested to be involved in the pathogenesis of sepsis. The general aim of the project was to characterize the EV proteom in plasma from patients with bacterial E.coli-sepsis, gain new knowledge on their role in host pathogen interactions, and search for potential biomarker candidates.

Methods: EDTA-plasma EVs, from two patient groups with positive E. coli blood culture 1) combined with sepsis (n=4), and 2) without sepsis (n=2), and a group 3) with negative blood culture but organ dysfunction (n=3) collected from a Sepsis Biobank (Oslo University Hospital), were SEC-isolated. EV proteomics was performed with high resolution LC-MS/MS analysis and data was processed by MaxQuant, Perseus and IPA to identify, quantify and compare protein patterns during sepsis. EVs were characterized using NTA, Flow cytometry, Western blotting and TEM.

Results: Proteomic analysis of plasma EVs identified 1200 different proteins, 70 of these being statistically different ($p<0.05$) in patients with E.coli-sepsis compared to patients with organ dysfunction. In E. coli-sepsis patients, IPA predicted that a number of differential distributed EV proteins (Z-score $\geq|\pm 2$) may modulate several canonical pathways and biofunctions with changed activity. The complement system showed the most enriched pathways (C3, C4, C5) and several upstream regulators (NFE2L2, RELA, LEP) were predicted activated.

Conclusion: A significant quantitative difference in the EV proteome was identified in plasma from patients with E. coli -sepsis compared to patients with organ dysfunction. Some theoretical evaluated biomarker candidates were suggested.

MARCO as a potential human biomarker present in plasma-derived extracellular vesicles from a patient co-infected with Leishmania and HIV

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Introduction: Leishmania-HIV co-infection has been an emergent problem in the last twenty years. HIV-infected people are especially vulnerable to visceral leishmaniasis (VL), and VL accelerates HIV replication and advancement to AIDS. VL and HIV co-infection is characterized by significantly lower cure rates, higher relapse risk and mortality when compared with HIV-negative VL patients. The current disease management options are limited and require improvement to control infection progression. Therefore, new biomarkers are required to address complex situations like asymptomatic disease, co-infection and treatment assessment. In this context, extracellular vesicles (EVs) are still an untapped resource with great potential for biomarker development. Thus, plasma-derived EVs from a patient with active Leishmania-HIV infection were recovered at 6 months intervals since May 2019 (five time points) and compared with plasma EVs from two age and sex-matched groups of individuals without evidence of Leishmania infection with and without HIV for possible biomarker identification.
Methods: Plasma-derived EVs were recovered size exclusion chromatography (SEC) and fractions of interest selected by bead-based flow cytometry with specific EVs markers, CD5L, CD71 and CD9. The characterization of plasma-derived EVs was performed by TEM, NTA and LC-MS proteins characterization. The peptides identified were compared against protein-databases for HIV, Homo sapiens sapiens and Leishmania infantum.
Results: No significant differences in overall EVs size were detected. The proteomic analysis confirmed the presence of EVs biomarkers, and also common plasma contaminants. A qualitative comparative analysis identified the macrophage receptor with collagenous structure (MARCO) as consistently detected in the patient. MARCO was also identified in plasma-derived EVs from 5 other VL patients. Single peptide protein identifications associated with Leishmania proteins were also detected in the infected patient and absent in non-infected controls.
Conclusions: Overall, plasma-derived EVs have the potential as a source of biomarkers for VL. The potential of the identified molecules is undergoing evaluation.

The liver stage of Plasmodium infection – written in extracellular vesicles?

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The liver stage of Plasmodium infection is the initial, asymptomatic phase of the malaria parasite's life cycle in the mammalian host, and obligatorily precedes the blood stage of infection, responsible for disease symptoms. Therefore, detection of Plasmodium parasites during hepatic infection is crucial to hamper the ensuing pathology. To this end, we propose to identify a Plasmodium hepatic infection-specific fingerprint by identifying parasite-specific biomarkers of liver infection associated with extracellular vesicles (EVs). Proteomic identification of EV-associated rodent P. berghei parasite proteins in the plasma of mice with an ongoing liver infection was performed using size exclusion chromatography followed by mass spectrometry. Additional methods for EV isolation are currently being explored. We will further perform small RNA sequencing to identify EV-associated plasmodial RNA. In the future, we will apply the same principles as those used in the rodent model system to investigate and characterize plasma samples from Plasmodium-infected human subjects.

Functional characterization of hiPSC-derived endothelial cells as an in vitro model for studying EV-mediated tissue repair in myocardial tissue

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Human induced pluripotent stem cells (hiPSCs) are an important source of cells for disease modeling, including cardiac diseases. Importantly, as endothelial cells (ECs) play a pivotal role in heart perfusion and repair, hiPSC-derived ECs (hiPSC-ECs) may represent a useful model for mimicking ECs present in the myocardium in in vitro conditions. However, hiPSC-EC functional properties when compared to primary cells remain poorly understood, which is essential for their future applications.

Thus, in this study, we aimed to compare the biological characteristics of hiPSC-ECs and primary cardiac ECs in vitro, in inflammatory and non-inflammatory conditions, by examining: phenotype, gene expression, endothelial-monocyte interactions in response to TNF-α stimulation, and angiogenic potential. Importantly, we aimed to distinguish differences in their response to treatment with hiPSC-derived extracellular vesicles (hiPSC-EVs), since we have previously shown therapeutic potential of the EVs in cardiac repair and protection.

Our results indicate similarities on phenotypical and functional levels between hiPSCs and primary ECs including expression of endothelial identification markers on both mRNA and protein levels. However, some differences between these cells emerged in the response to pro-inflammatory cytokine (TNF-α) stimulation depending on passage. We observed different level of endothelial activation-associated markers expression such as CD62E (E-Selectin), CD106 (V-CAM), CD54 (I-CAM) after treatment with TNF-α with various doses. We noticed that the observed phenomena occurs between passages P3 and P4 suggesting progressing maturation of hiPSC-ECs. Importantly, we found similar response of maturated hiPSC-ECs to treatment with EVs when compared to primary ECs.

We confirmed that hiPSC-ECs may represent a valid in vitro model of ECs. This inquiry may contribute to the development of an optimal tissue-specific ECs model system for studying mechanisms accompanying tissue regeneration, including myocardial repair EV-mediated treatment.

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The impact of graphene-based substrates on proangiogenic properties of human mesenchymal stem cells and their extracellular vesicles - significance for cardiovascular repair

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According to statistical data of World Health Organization, cardiovascular diseases are one of the most prevalent diseases and the leading cause of death globally in recent years. Therefore, it is justified to search for new, effective therapeutic methods. Mesenchymal stem cells (MSCs) and their extracellular vesicles (MSC-EVs) are one of the therapeutic candidates for cardiovascular repair. To modulate the regenerative properties of MSCs and MSC-EVs new biocompatible scaffolds for cell culture are still being sought for. An interesting material for biomedical applications due to their unique physicochemical properties are graphene oxide (GO) and reduced graphene oxide (rGO).

The main goal of this study was to investigate the potential of GO and rGO substrates as scaffolds that can promote angiogenic properties of MSCs and MSC-EVs in in vitro and in vivo studies.

The influence of the selected graphene-based scaffolds on the biological and functional properties of MSCs were investigated. In addition, the effect of culturing MSCs on graphene substrates on the biological properties of their MSC-EVs was investigated. The amount and distribution of secreted MSC-EVs was assessed by using NTA method. The MSC-EVs characterization was performed by flow cytometry (Apogee Flow System). Moreover, capillary tube formation assay was performed to investigated the proangiogenic capacity of MSC-EVs secreted by MSCs cultured on graphene-based scaffolds. The regeneration potential of MSCs cultured on rGO surfaces and their MSC-EVs was also was evaluated in murine model of hind limb ischemia.

The conducted research indicates that selected rGO substrates, can promote proangiogenic properties of MSCs and MSC-EVs, which could contribute to their future use in cardiovascular repair. However, further studies are required to analyze these phenomenon.

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Manganese overexposure induces the release of manganese-loaded extracellular vesicles in microglia

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Microglial cells play an essential role in the defense and maintenance of the central nervous system, being able to respond to a variety of stimuli. They can release different molecules involved in intercellular communication to coordinate or amplify their responses. Recently, it has been shown that microglia can also release extracellular vesicles (EVs), but the differential release of EVs according to initial stimuli is not fully understood. In this sense, we are interested in the effect of high doses of manganese in microglia, since occupational manganese overexposure induces its accumulation in the brain causing mangansim, a pathology that shares symptoms with Parkinson's disease. In this work, we studied the putative contribution of microglial EVs in this process by using the microglia BV2 cell line and isolating EVs after manganese overexposure. Transmission electron microscopy and dynamic light scattering analysis showed two EV populations with different sizes and stable over time in non-stimulated and stimulated cells, which amount was increased with manganese stimulus. We also analyzed the EVs by scanning electron microscopy and energy dispersive X-ray spectroscopy, revealing a high amount of manganese in the EVs that were released after manganese overexposure compared with controls, suggesting their participation in manganese detoxification in microglia. In addition, we analyzed the effect of these EVs on cultured primary microglial cells, showing a significative cytotoxic effect of manganese-loaded EVs, that was corroborated by microscopy. This points out to a participation of EVs also in the spreading of neurodegeneration found in manganism.
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Modifications in the protein corona of exosomes after exposure to the inflammatory microenvironment generated during acute pancreatitis

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Background: When exosomes move through different biological fluids, they encounter proteins that can quickly bind to their surface. This fact generates a protein corona that confers them new biological properties, possible modifying their biodistribution and target delivery and, consequently, their biological effects. This can be especially relevant in pathologies whose progression is accompanied by the generation of a particular microenvironment. Here we aimed to evaluate the changes that the protein corona of exosomes undergoes when they come into contact with the microenvironment that is generated during acute pancreatitis.
Methods: Acute pancreatitis was induced in rats by intraductal administration of 5% sodium taurocholate and, 3 h after induction, ascitic fluid (PAAF) was collected, diluted, and ultracentrifuged for 16 h to remove endogenous exosomes. On the other hand, we obtained exosomes from BxPC3 cell line cultures grown in serum-free conditions for 48 h, by filtrating and ultracentrifugating collected supernatants. Exosomes were incubated with PAAF or phosphate-buffered saline as control. Changes in the hydrodynamic size and z-potential of differentially incubated exosomes were evaluated and a proteomic analysis was carried out, by shotgun proteomics in a high resolution mass spectrometer, in order to identify the changes in the proteins attached to their surface.
Results: Exposure of exosomes to PAAF resulted in changes in their z-potential and a slight increase in their hydrodynamic size. We identified 126 proteins incorporated on the surface of PAAF-incubated exosomes but not on the control, including apolipoproteins and serine protease inhibitors.
Conclusions: The microenvironment generated around the pancreas during acute pancreatitis can substantially modify the protein corona of the exosomes that circulate through it. This could have effects on exosome properties and functions and, with it, eventually in the progression of the disease.

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Transport of RNA from mouse to bacteria via extracellular vesicles: probing specificity and function

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Extracellular vesicles (EVs) are lipid-bound vesicles that are secreted by cells into the extracellular space which can carry out important roles in intercellular communication. EVs also mediate inter-organismal communication in a range of living systems. In the mammalian gut, EVs and miRNAs have been shown to mediate diverse forms of communication: plant nanoparticles were shown to be taken up by gut bacteria, and the miRNAs present in these vesicles proposed to affect bacterial gene expression (Tang et al.,) and miRNAs released by intestinal epithelial cells were shown to enter bacteria and impact bacterial gene expression and microbiome composition (Liu et al., 2016).

However, the mechanisms by which host miRNAs are transported into gut bacteria remain largely unknown. In this work, we examine whether EVs derived from intestinal epithelial cells could serve as a transport mechanism for mammalian miRNAs into specific bacteria. We applied fluorescently labelled EVs obtained from either Mode-K cells (a mouse intestinal epithelial cell line) or Heligmosmoides bakeri (a gastrointestinal nematode parasite that naturally infects mice). Our data show that Mode-K EVs are taken up by Salmonella Typhimurium SL1344 while H. bakeri EVs are not. The uptake of Mode-K EVs was not observed with Escherichia coli W3110. Furthermore, we found Mode-K EVs had a promotion effect on the growth of SL1344 but this was dependent on nutritional composition of the media. We are currently employing RNA FISH to understand whether miRNAs can be detected inside of bacteria and using the Cre-loxP system to investigate whether there is functional transmission of RNA cargo from Mode-K EVs to Salmonella.

Our initial findings suggest that EVs may enable specific targeting of RNA cargo to some bacteria which has implications for the development of new strategies for targeted drug delivery against bacteria.

Identification and characterization of Trypanosoma cruzi proteins present in circulating EVs from patients with chronic Chagas Disease

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Chagas Disease, caused by Trypanosoma cruzi, is a prevalent parasitic infection in Latin America, causing millions of cases annually. However, in recent years, migratory movements have contributed to the emergence of this tropical disease in non-endemic regions, especially in southern Europe, where it has become a public health concern. One of the main clinical problems during chronic Chagas disease (CCD) is the lack of effective biomarkers for therapeutic response and disease progression. In that context, circulating extracellular vesicles (EVs) in CDD patients represent a promising source of new biomarkers for the disease.

In this study, plasma samples were collected from CDD patients with different clinical manifestations before and at different time points after benznidazole treatment. Circulating EVs were isolated by direct CD9/CD81/CD63-immunocapture and analyzed by mass spectrometry. Proteomic analysis identified 62 parasite proteins that represent potential biomarkers for diagnosis, therapeutic response, and prognosis of Chagas Disease. Moreover, a group-specific profile of human proteins was also detected, including proteins associated with the inflammatory response, providing insights into Chagas Disease pathology. Remarkably, six parasite proteins associated with infection, therapeutic response, or the cardiac form of the disease were successfully cloned and expressed in a wheat germ cell-free protein expression system. These proteins were included in a

Luminex kit, and their immunogenicity was tested using plasma samples from patients in the same cohort with chronic Chagas Disease before, just after treatment, and at 6 and 12 months after the end of treatment. Results demonstrated that the proteins were immunogenic, albeit variably, thus indicating their value in developing multiplex diagnostics.

Overall, the study highlights the potential of EVs as novel biomarkers for Chagas Disease and paves the way for further validation of these biomarkers and the development of a diagnostic tool to monitor disease progression and treatment efficacy in patients with chronic Chagas Disease.

Proteasome activity is increased in Extracellular Vesicles isolated from the plasma of Fasciola hepatica-infected cattle

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The trematode Fasciola hepatica is the causative agent of liver fluke disease in mammalian species, causing significant economic losses in the livestock industry. Modulation of host immunity is partly due to the release of extracellular vesicles (EVs), which can be internalized by host cells. The proteasome is a protein complex essential for the degradation and turnover of proteins, and its activity is crucial for maintaining proper cellular function. The dysregulation of proteasome activity is associated with some diseases, and interestingly, increased levels of the proteasome complex, particularly in the extracellular space, have been observed.

In the present study, we have identified proteasome proteins present in EVs isolated from the plasma of cattle infected with F. hepatica. We used a fluorometric assay to measure proteasome activity in these EVs, observing higher proteasome activity compared to EVs from uninfected cattle. This result was confirmed by measuring in-gel proteasome activity, where differential activity between 20S and 26S subunits was observed.

These findings suggest that EVs from F. hepatica may play a role in modulating the host immune response by regulating the proteasome activity. Further studies are needed to elucidate the specific mechanisms involved in this regulation and the potential implications for the development of diagnostic tools and therapeutics for F. hepatica infection.

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Comparative proteomics of canine osteoblast and osteosarcoma cell-derived extracellular vesicles enriched by different methods

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Extracellular vesicles (EVs) have emerged as promising candidates for diagnostic and therapeutic applications due to their cell-specific characteristics. Deciphering their proteome can aid in elucidating their biogenesis and functional roles, as well as in identifying potential tumor markers for diagnostic purposes. In this study, we cultivated canine osteoblasts and osteosarcoma cells (D-17) in vesicle-free medium and enriched EVs using two different methods: Differential centrifugation and ultracentrifugation and size exclusion chromatography, each performed in triplicate. Western blot analysis confirmed the presence of common EV markers in all isolates, while nanoparticle tracking analysis provided evidence about particle size and concentration. Extracellular vesicle protein isolates were prepared by S-Trap technology and subjected to mass spectrometry analysis. Our results revealed variations in the number and purity of EVs depending on the isolation method used. The proteomic analysis unveiled differences between EVs derived from osteoblasts and osteosarcoma cells and further highlighted an influence of the EV enrichment method on the sample protein composition.
The findings of this study underscore the importance of selecting an appropriate isolation method for obtaining EVs with representative proteomic profiles. The observed differences in the EV proteome between the two isolation techniques suggest that the choice of method can significantly impact the composition and functional characteristics of the isolated EVs. Further exploration of the specific protein signatures of EVs derived from canine osteoblasts and osteosarcoma cells can deepen our understanding of the pathophysiological functions of these extracellular signaling hubs and potentially contribute to the discovery of novel diagnostic and therapeutic targets for osteosarcoma and related conditions.

Engineered Extracellular Vesicles for Targeting and Activation of Lymphatic VEGFR-3

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The lymphatic vasculature has not received overwhelming scientific attention for many years, although it is fundamental for the development and homeostasis of higher organisms. To re-establish true tissue homeostasis after traumatic injuries and disease, therapeutic strategies need to consider the regeneration of our lymphatic system. Vascular endothelial growth factor C (VEGF-C) is a key mediator of lymphangiogenesis upon binding and activation with the lymph-specific vascular endothelial growth receptor 3 (VEGFR-3). It has mainly been investigated in settings such as lymphedema and cancer. Nevertheless, most tested applications of this receptor ligand did not consider its highly complex biosynthesis process. Different proteolytic processing gives rises to different variants showing varying levels of receptor specificity and affinity which influences all of its applications. To enhance the pro-lymphatic effects of VEGF-C and its systematic availability, the possibility of linking it to proteins such as tetraspanins (e.g. CD9, 63, 81), which are enriched in extracellular vesicles (EVs), has been proposed recently. In this project, we aim to establish stable adipose derived stromal/stem cell (ASC) lines which express different variants of VEGF-C linked to CD81. This fusion protein should exert a bimodal function of receptor activation and tissue-specific homing. Expression and subcellular localization of the fusion protein was confirmed via western blotting and immunofluorescence respectively. Using a BaF3 cell line expressing a VEGFR3/EpoR chimera, we tested different CD81-VEGFC variants for differences in their activation activity. In this study, we provide proof of principle that we can generate different ligand variants of fusion proteins on EV surfaces, test their bioactivity in subsequent receptor activation assay and pave the way for testing their therapeutic potential for enhancing lymphangiogenesis in various in vivo models of traumatic injuries as the next steps.

Differential effect of cytotoxic therapy on primary and metastatic clear cell sarcoma and their EVs

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Extracellular vesicles contribute to tumor growth, drug resistance, metastasis, and remodeling of the tumor immunological microenvironment. Using patient-derived autologous tumor models that mimic the tumor microenvironment, the impact of EVs on drug resistance can be investigated. In the present project, we focus on Ewing-like sarcomas (ELS) .Cytotoxic chemotherapy is usually only used for metastatic Ewing-like sarcomas, as surgery is still the golden standard for primary tumors. However, metastatic ELS often exhibit a drug-resistant phenotype that differs from the characteristics of the primary tumor. Although experimental studies have shown that chemotherapy can have metastasis-promoting effects, the role of extracellular vesicles and tetraspanins in ELS resistance remains unclear. In this study we generated a patient-derived in vitro model of the Ewing-like clear cell sarcoma (CCS) harbouring the characteristic EWSR1::ATF1 oncogenic fusion consisting of cell lines derived from the primary tumor and the metastatic lesion with an acquired resistance to crizotinib. Cell lines and respective extracellular vesicles of both entities were comprehensively characterized based on their morphology, transcriptomic signatures and tetraspanin colocalization before and after treatment with different classes of cytotoxic therapy for sarcoma including the kinase inhibitor crizotinib. Interestingly, a triple colocalization (CD63+, CD81+, CD9+) of all three tetraspanins could only significantly be detected in EVs from tumor-associated cells, whereas normal healthy fibroblasts did not exhibit this feature. Cytotoxic treatment with crizotinib altered the tetraspanin colocalization patterns of the primary tumor EVs but showed no effect on metastatic EVs, implying a potential resistance mechanism. Triple colocalization unique to tumor-associated EVs decreased in a dose-dependent matter after crizotinib treatment but also resulted in an increase in single CD9+ EV populations in all entities, cancerous and healthy, allowing potential monitoring and improving chemotherapy treatment in CCS

Surface Plasmon Resonance for Sensitive Detection of Chemokine Receptors CXCR4 and CXCR7 on Extracellular Vesicles: Implications for Cancer Diagnosis and Beyond

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Surface plasmon resonance (SPR) has emerged as a powerful technique for studying biomolecular interactions due to its label-free and real-time monitoring capabilities. Here, we highlight the application of Biacore SPR technology in detecting chemokine receptors CXCR4 and CXCR7 on extracellular vesicles (EVs). Chemokine receptors are involved in cell migration and immune responses. Therefore, the accurate and sensitive detection of chemokine receptors on EVs is of significant interest in biomedical research and clinical diagnostics. Furthermore, CXCR4 and CXCR7 are known to be overexpressed in specific cancer types, making them potential targets for diagnostic and therapeutic applications.

Using SPR, we observed distinct differences in the expression of EV biomarkers (CD9, CD63, CD81) among EVs isolated from different cell types, including HEK293T, U87, MCF7, MOLT4, MT4, and HUVEC. Notably, the overexpression of recombinant CXCR4 in HEK293T and U87 cells resulted in an increased expression of CXCR4 on the EVs. Furthermore, endogenous CXCR4 expression was detected in EVs from most cell types, with MT4 and MOLT4 showing the highest response. Surprisingly, our results revealed that CXCR7 was not endogenously expressed in MCF7 cells, contrary to our initial expectations. However, upon transfection with CXCR7, U87 cells expressed CXCR7 on their EVs. Additionally, EVs isolated from HUVEC cells exhibited endogenous expression of both CXCR4 and CXCR7.

Our developed bioassay successfully detected cancer cell-derived EVs expressing high levels of CXCR4. Moreover, the detection of endogenous CXCR4 in EVs from various cell types suggests its potential as a novel EV biomarker. As SPR detects only binding events within the evanescent wave (~150 nm from the surface), it fills the gap of the current technologies (NTA, MRPS, nanoFCM, SP-IRIS) that fails to detect EVs smaller than 50-70 nm. With its real-time, label-free analysis and very low sample consumption rate, SPR will be an added value to exploring EV markers.

Contribution of endothelial progenitor cell derived extracellular vesicles in bone regeneration

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Objectives: Bone regeneration is a complex, well-orchestrated process based on the interactions between osteogenesis and angiogenesis. Tissue engineering, through mesenchymal stromal cells (MSCs) and endothelial progenitor cells (EPCs), foster bone regeneration. This study aims to evaluate if adipose tissue mesenchymal stromal cells (AT-MSCs) treated with HUVEC-derived Extracellular Vesicles (EVs) could contribute to form more bone and faster than AT-MSCs alone.
Material and methods: After obtention of HUVEC-derived EVs by ultracentrifugation, we have treated AT-MSCs with these EVs at different concentrations: 200, 400, 10^3 and 10^4 EVs/cell. We have performed qualitative and quantitative assessments of bone formation by alizarin red staining, calcium measurement and alkaline phosphatase (ALP) activity. Moreover, we have measured several osteogenic genes: ALP, osteocalcin (OCN) and bone sialoprotein (BSP).
Results: The treatment of AT-MSCs with HUVEC-derived EVs fosters the osteogenic differentiation of MSCs after only one week. More calcium deposits were highlighted with higher concentrations of EVs after staining with alizarin red. A significant increase of calcium level was objectified in the 10^3 and 10^4 EVs/cell groups, and a significant increase of ALP was evidenced in the 104 EVs/cell group. These results were confirmed by a significant increase of ALP, OCN and BSP gene expression in the 10^4 EVs/cell group.
Conclusion: Together, MSCs and EPCs contribute to bone regeneration. Their synergy leads to a greater and faster bone formation than when each population is considered separately. Interestingly, we highlighted the communication between EPCs and AT-MSCs via EVs, resulting in the uptake of EVs in AT-MSCs and the stimulation of osteogenesis translated by an increased expression of osteogenic genes. However, the underlying mechanisms are yet undetermined but could involve extracellular vesicles and their cargo such as microRNAs.

Keywords : adipose tissue stromal vascular fraction, bone regeneration, endothelial progenitors, mesenchymal stromal cells, extracellular vesicles.

Extracellular vesicles from chronic lymphocytic leukemia cells induce the differentiation of monocytes into tumor-associated macrophages

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Bidirectional interactions exist between chronic lymphocytic leukemia (CLL) cells and the cells of their microenvironment. CLL cells indeed received several stimuli such as BCR stimulation but they can also interact with the surrounding cells by extracellular vesicle (EV) release.
The microRNA profile of CLL-EVs isolated by serial ultracentrifugation from leukemic cells cultured with/without BCR stimulation (n=4) was performed by NGS and validated by qPCR (n=25). CLL-EV integration was monitored using PKH67. Leukemic EV effects on purified monocytes were analyzed at different levels.
NGS highlighted 31 microRNAs differentially expressed between BCR stimulated/unstimulated conditions and qPCR confirmed the increase of miR-146a-5p, miR-132-3p, miR-155-5p and miR-17-3p in EVs (P

Scalable production of extracellular vesicles from dental pulp stem cells cultivated in a hollow-fiber bioreactor

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Dental pulp stem cells (DPSC) are highly proliferative stem cells extensively studied in regenerative medicine. Preclinical research shows that these cells reduce acute inflammation and enhance long-term recovery due to their potential to stimulate tissue healing and angiogenesis. Since their mode of action is greatly attributed to the paracrine factors, extracellular vesicles (DPSC-EV) are known to play an important role. Nonetheless, DPSC-EV are yet to be clinically validated since low production yields hamper their use. In this study, DPSC cultivation in a hollow fiber bioreactor (HFB) was investigated. We hypothesized that using two different pore sizes of HFB can lead to differences in DPSC-EV production and functionality. To test this hypothesis, 5 and 20 kDa bioreactors were used, and the EV production was evaluated for 26 days. DPSC cultivated in HFB produced a high amount of EV in the first two weeks (1010 particles per harvest), slowly decreasing after each harvest. EV grown in both conditions were positive for Flotilin-1, CD63, and CD81, and TEM images showed a cup-shaped form. DPSC-EV pro-angiogenic markers were evaluated using a protein array, and endothelin-1 and artemin are highly expressed regardless of the pore size. Our study provides insights into DPSC cultivation in an HFB by showing that pore size does not affect DPSC-EV production and functionality.

Towards clinical translation of exosome-based mutation analysis using next-generation sequencing

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Extracellular vesicles (EVs) are of great interest to improve the sensitivity of biomarker detection in liquid biopsies. In order to implement small EV or exosome-based assays in clinical practice an easy, quick and reliable workflow is required. Moreover, whereas highly sensitive digital PCR (dPCR) methods are well suitable to detect very low concentrations of a targeted mutation associated with EVs, new-generation sequencing (NGS) methods are often preferred in clinical settings. They can screen a very broad panel of gene mutations although it remains challenging to detect mutations present at low variant allele frequencies. Our objective was to study how enrichment of small EVs prior to DNA analysis can contribute to early mutation detection in blood samples.

Blood was collected from healthy volunteers using RNA complete blood collection tubes (Streck) and processed to prepare platelet-poor plasma samples. The plasma samples were spiked with human NCI-H1975 non-small cell lung carcinoma cell line-derived small EVs, alone or in combination with short DNA fragments containing the epidermal growth factor receptor gene mutation L858R and T790M. Cell-free DNA was extracted using the QIAamp Circulating Nucleic Acid kit, directly from the spiked plasma samples or after EVs were separated by size exclusion chromatography.

Mutation analysis was performed by digital PCR and NGS, and the mutant allele frequency was found to be significantly higher for samples obtained when EVs were separated before DNA extraction. In addition, for digital PCR, the highest mutant allele frequency was obtained when both EVs and DNA fragments were spiked, indicating that co-isolated circulating tumor DNA can also contribute to the mutation enrichment in the size exclusion chromatography-based workflow.

Colorectal cancer cells during chemotherapy treatment release EVs that induce cancer associated fibroblasts reprogramming

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Despite the advancement of cancer therapies, colorectal cancer remains one of the leading causes of death worldwide. Chemotherapy is the main treatment for these patients, even if a great effort is needed to increase efficacy of therapies, and to improve patients survival. A better understanding of crosstalk between cancer cells and tumor microenvironment during the administration of chemotherapy is crucial to develop more effective therapeutic approaches. Extracellular vesicles (EVs) play a crucial role in the intercellular communication and can induce a reprogramming that occurs in cancer and tumor-stroma cells, supporting tumor cell growth. Aim of this study was to analyze the effects of EVs isolated from HT29 cells treated with 5-fuoracil and oxaliplatin (5-Fu and Oxa) on CAFs reprogramming. Our data demonstrated an increase of number of EVs released from HT29 treated 5-Fu and Oxa (EVs-5-Fu and EVs-Oxa), when compared to EVs released by untreated cells (EVs-CTR), suggesting a putative increase of their biogenesis. Moreover, we showed an increase of proteins involved in glycolysis (as PKM2) and cancer progression (such as CD147 and β-catenin) in EVs-5-Fu or EVs-Oxa compared to EVs-CTR. Finally, CAFs treated with EVs-5-Fu or EVs-Oxa, showed an increased expression of Vimentin and α-SMA, markers of myofibroblast differentiation. In conclusion, our preliminary data showed that EVs released by CRC cell line, subjected to chemotherapeutic treatments, could induce a reprogramming of CAFs.

The Role of JAK-STAT1 in Exosome Biogenesis and miRNA Regulation in A549 Lung Cancer Cell Line

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Exosomes play a critical role in intercellular communication, including cancer-related processes. However, the involvement of the JAK-STAT1 signaling pathway in exosome biogenesis and miRNA regulation in lung cancer remains unclear.

This study investigated the role of JAK-STAT1 in exosome biogenesis and miRNA regulation using wild type A549 cells and STAT1 knockout A549 cells. Cells were seeded at 2x10^6 cells per T175 flask and incubated for 24 hours. The culture media was replaced with serum-free media, and cells were cultured in DMEM with 2% FCS for 24 and 48 hours to facilitate exosome production.

Quantitative analysis revealed that STAT1 knockout A549 cells produced significantly more exosomes than wild type cells. After 24 hours, STAT1 knockout cells showed a 1.5-fold increase in exosome production compared to wild type cells. Similarly, after 48 hours, STAT1 knockout cells exhibited a 1.5-fold increase in exosome secretion. The presence of exosomal biomarkers (CD9, CD63, CD81, TSG101 AND Flotillin) were confirmed through Western blot and flow cytometry.

Furthermore, the study explored the impact of JAK-STAT1 on miRNA regulation within exosomes. RT-PCR analysis revealed downregulation of miRNA-21-3P and Let-7-5p in both STAT1 knockout cells and exosomes.

In conclusion, this study highlights the significant role of the JAK-STAT1 pathway in exosome biogenesis and secretion in A549 lung cancer cells. Moreover, it provides evidence that JAK-STAT1 may affect the regulation of specific miRNAs within exosomes. These findings contribute to our understanding of the interplay between JAK-STAT1 signaling, exosome-mediated communication, and miRNA-based mechanisms in lung cancer. Further research is necessary to elucidate the underlying molecular mechanisms and explore the therapeutic potential of targeting the JAK-STAT1 pathway in lung cancer treatment.

Dissecting exosomal-miRNAs as key-players for the early identification of an aggressive subtype of lung adenocarcinoma

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Non-Small Cell Lung cancer is the most frequently diagnosed lung cancer type (80-90% of all cases) and lung adenocarcinoma (LUAD) is the major subtype (~40% of NSCLC). Treatment of lung cancer is difficult due to high pathological, cellular and molecular heterogeneity. Therefore, gain new knowledge about mechanisms which drives lung cancer progression is urgently needed. Recently, we discovered a tumor molecular subtype, namely C1-LUAD, using a 10-genes prognostic signature which correlates with a high-plasticity cell state, increased tumor mutational burden, immune evasion phenotype, and infaust prognosis. We also found that C1-LUAD cells released exosomes loaded by miRNAs (aka exo-miRs) which can be eventually investigated as non-invasive biomarkers for the early identification of aggressive disease both at diagnosis and during treatment. Coherently, we first investigated in plasma samples from LUAD patients a subset of exo-miRs which interact with C1-LUAD transcriptome. We demonstrated that a model of 6-exo-miRs can accurately diagnose aggressive C1-LUAD tumors with an AUC=0.73. We found also that C1-LUAD tumor cells are eager to internalize both self and exogenous exosomes compared with other tumor cells, thus suggesting a differential uptake kinetics. C1-LUAD cells showed also a peculiar exo-miRs profile, with miR-223-3p being the top significantly enriched in their exosomes but depleted in intracellular compartment. Interestingly, when we re-expressed miR-223-3p in targeted C1-LUAD cells, we observed a reduction in cell migration and invasion. Notably, C1-LUAD exosomes enriched in miR-223-3p reduced T-cells viability and proliferation as well as IFN-gamma production. This data suggests that C1-LUAD tumors secrete exosomes enriched in miR-223-3p to scavenge its anti-migration/invasion effect and favor an immunoevasion phenotype by dampening cytotoxic T-cells. Overall, this study identifies a 6-exo-miRs signature for the early identification of aggressive lung cancer and provides insights into the role of exo-miRs in disease progression.

EVs from solid and fluid tissues face each other as multiscale biomarkers for Intensive Care Unit Acquired Weakness

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Critically ill patients frequently acquire muscle weakness during Intensive Care Unit (ICU) stay, named Intensive Care Acquired Weakness (ICU-AW). This condition is present in up to 50% of critically ill patients, affects limb and respiratory muscles, and is associated with prolonged ICU stay and increased mortality. ICU-AW pathophysiology remains incompletely understood, but comprises complex functional alterations within the central nervous system, microcirculation, peripheral nerves and myofibers. At present, no specific biomarkers have been identified, thus limiting the bedside management of patients.

To fill this gap, we adopted a broad-spectrum strategy: i) to characterize extracellular vesicles (EVs) isolated from skeletal muscle tissue biopsy and from plasma obtained from healthy subjects and ICU-AW patients, ii) to compare tissue-EV with plasma-EV features, in order to find specific signatures of ICU-AW that can be detected by liquid biopsy.

In order to analyze EV of different origin, it is necessary to set methods for their comparison, minimizing bias due to different separation techniques.
To this aim, we developed a protocol to extract EVs from skeletal muscle tissue that combines mechanic and enzymatic approaches which converges with a protocol to extract EVs from plasma, based on serial (ultra)centrifugation and sucrose density gradient.
In addition, we optimized methods to grade EV preparation using multidisciplinary techniques, dissecting EV characteristics at multi-scale levels:
- from the molecular scale (using spectroscopic methods to collectively fingerprint EV, –omics approaches to investigate metabolite and lipid content, immunoassays to define their biochemical composition),
- to the meso-scale (determining EV colloidal properties in terms of size, stability, purity from exogenous proteins and other nanoparticles, nano-mechanical features).
Analyses on ICU-AW and control subjects are ongoing and preliminary results will be disclosed during the meeting.

Proof-of-concept study on the use of tangerine-derived nanovesicles as RNAi delivery vehicles toward mammalian cells

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In the last years, innovative approaches based on drug delivery are emerging for the treatment of several diseases, including cancer. Nanovesicles (NVs) have several advantages over conventional synthetic carriers, such as low toxicity, high biocompatibility, and low immunogenicity. Among NV types, those from plants (Plant-derived nanovesicles, PDNVs) have attracted significant interest due to their high production yield and ability to interact with mammalian cells. This project aimed to develop an RNA interference (RNAi) delivery system using PDNVs.

To achieve this aim, nanovesicles isolated from tangerine juice (TNVs) were isolated and characterized. Notably, TNVs exhibited the presence of the traditional EV marker HSP70, and metabolomic analysis showed the presence of flavonoids, organic acids, and limonoids within TNVs. To investigate the potential of TNVs as RNAi delivery system, we evaluated different approaches to load a small interfering RNA (siRNA) in TNVs; sonication and co-incubation of the siRNA with the vesicles was not effective, while TNVs were successfully loaded through electroporation (transfection efficiency of 13%). The electroporated TNVs effectively delivered the siRNA into mammalian cells, resulting in the reduction of the expression of the target gene.

As a result, our study provides proof of concept for the use of PDNVs as RNAi carriers in mammalian cells.

EXTRACELLULAR VESICLE RELEASE AND CARGO ARE ALTERED BY CAVEOLIN-1-OVEREXPRESSION AND MODULATE TUMOR MICROENVIRONMENT IN A MODEL OF RHABDOMYOSARCOMA

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Background. Extracellular vesicles (EVs) are lipid-bound vesicles secreted by cells into the extracellular space which have a pivotal role in cancer disease. Caveolin-1 (CAV1) is an integral membrane protein required to generate caveolae and cholesterol-enriched lipid rafts. In cancer, CAV1 has a controversial role: it is widely accepted that the loss of CAV1 correlates with early-stage tumor progression, while its over-expression and phosphorylation are associated with metastatic disease. In particular in rhabdomyosarcoma (RD), CAV1 has been shown to cooperates with tumor growth and metastatic potential.

Aim. The present work aims to investigate if the increased aggressiveness of RD cells overexpressing CAV1 (RD-CAV1) correlates with an altered extracellular vesicle release and cargo and if RD-CAV1 EVs contribute to the cancer dissemination.

Methods. EVs were isolated from RD-ctrl and RD-CAV1 conditioned media by sequential ultracentrifugation and characterized by Nanoparticle Tracking Analysis (NTA), Western Blot Analysis and Flow Cytometry Analysis.

Proteomic Analysis has been performed on EV subpopulations.

Results. The obtained data show that RD-CAV1 cells release more EVs compared to RD-Ctrl cells. Western Blot analysis highlighted that small extracellular vesicles (sEVs) exhibit the tipical exosomal markers, whereas large extracellular vesicles (lEVs) are positive for Calnexin. Interestingly, the tetraspanins CD63, CD81, and CD9 were not detectable in RD-CAV1 EVs, unlike RD-Ctrl EVs. All these data suggest that CAV1 overexpression induces an alteration of EV biogenesis and secretion. Moreover, the treatment of HUVEC with RD-CAV1 EVs shows an increase in cell proliferation and migration in a dose-dependent manner.

Conclusions. Taken together, these data demonstrate that CAV1 overexpression critically affects RD-EV release and cargo; moreover, RD-EVs can alter the behaviour of the tumor microenvironment cells. Future studies will focus on the characterization of RD-EV cargo in terms of lipid- and miRNA-loading and on the evaluation of RD-EV effects in other cells, typical of tumor niche.

Study of extracellular vesicles from accessible biological fluids to characterize individual responses to physical activity

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Background: Regularly performed exercise promotes skeletal muscle health through the modulation of muscle regeneration, repair and remodelling. Unfortunately, not all individuals respond the same to an exercise protocol; some are highly trainable while others respond poorly or only marginally. To investigate the molecular mechanisms involved in interpersonal training variability, extracellular vesicles isolated from accessible biological fluids would represent a complex and dynamic diagnostic tool.
Aim: The present project aims to develop a protocol for the isolation of EVs from athletes' plasma, serum and saliva to characterize individual responses to physical activity.
Methods: To isolate EVs, plasma and serum samples were collected from marathoners pre- and post-race and analysed by SEC, while saliva samples from football players pre- and post-training were processed by serial ultracentrifugation. NTA was utilized to measure EV size distribution and concentration, and BCA assay was performed for total protein content. Finally, Dot Blot analysis was employed for the antibody-based detection of different EV markers.
Preliminary Results: For all athletes' samples, NTA analysis showed typical EV size and distribution. Concerning marathoners' samples, Dot Blot showed that most analysed targets (CD63, CD9, HSP60, CD171, CD56) increased in post-race. Regarding football players' salivary EVs, Dot Blot revealed higher CD63 and HSP60 positivity levels in EXOs compared to MVs, suggesting that the protocol was able to properly isolate EV subpopulations. Moreover, CD63 and HSP60 increased in most post-exercise samples.
Conclusions: The obtained results highlight an increase in EVs in response to physical activity, which could reveal metabolic changes and muscle adaptation to exercise.
EVs could therefore represent an innovative source of exercise biomarkers that would allow the development of standardized exercise protocols both in a clinical and elite athlete setting.

Comparative Analysis of Cell Surface Markers CD38, CD138, and CD269 on mm plasma cells-Derived Extracellular Vesicles: Plasma cells-derived EVs identification in Liquid Biopsies?

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Multiple myeloma (MM) is a hematologic malignancy characterized by clonal proliferation of plasma cells (PC). Identification of reliable biomarkers of extracellular vesicles (EVs) derived from neoplastic PC could be useful for liquid biopsies in MM.

The study aimed to compare the expression of surface markers on PC and PC-derived EVs using CD38, CD138, and CD269 through flow cytometry (Cytek North-Lights) of three MM cell lines (RPMI8226, OPM2, and U266; provided by J. Moreaux, IGH, France). Experiments were performed in triplicate. EVs were harvested from the supernatants of cell lines cultivated in RPMI1640 supplemented with 10% FBS-depleted EVs.

High levels of triple-positive expression (CD38, CD138, and CD269) were observed across all three cell lines with the exception of CD38 expression on U266 cells, which was dim The intensities of CD269 and CD138 expression on EVs were lower than on parent cell lines. By contrast CD38 expression remained consistently high on EV-derived from RPMI8226 and OPM2 cell lines, but not on U266-derived EVs, which is consistent with the supplier data of the cell line.
CD38 is also present on red blood cells and their EVs, which may introduce potential confounding factors if used in patient blood samples. One limitation is the viability of two of the three cell lines (around 50%), which could impact the emission of EVs and marker expression and intensity.

A potential explanation for the reduced expression of CD138 on EVs. could be heparanase, an enzyme known to cleave heparan sulfate moieties on CD138 , and its potential influence on CD138 levels in the EVs supernatant. Currently, we are investigating the role of this enzyme.

γ-Secretase has been shown to cleave membrane-bound CD269 into its soluble form (shedding). Presence of this enzyme could explain minimal expression of CD269 on EV surfaces.

Upstream and downstream innovations in large-scale manufacturing of mesenchymal stem cells EVs in stirred tank bioreactor

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In collaboration with MSCmed laboratory (UMR7057), EVerZom has developed a disruptive, patented technology. This scalable process stimulates the release of extracellular vesicles (EVs) by exerting controlled mechanical stimulation, via turbulent flow, on the cells during their 3D culture in bioreactors, up to a volume of 10L and 10^13 EVs produced. However, the clinical application of EVs is coming up against several bottlenecks that are hampering the spread of this innovation. These include upstream challenges, such as growing billions of adherent cells or controlling mechanical stimulation throughout scale-up, and downstream challenges, with the concentration and purification of litres of conditioned medium.

This study presents the results of several innovations developed to overcome these challenges. For the upstream process, thawing cells directly on microcarriers (µCs) in stirred tank bioreactors; bead-to-bead transfer, consisting of adding fresh µCs to the culture using the ability of cells to migrate from a confluent µC to a new µC to increase the surface area available for cell growth without the need for enzymatic detachment; characterization of turbulent flow inside the bioreactor using the Kolmogorov equation and fluid dynamics simulation; study of cellular mechanotransduction pathways to understand the impact of shear stress on EV release from cells. And for the downstream process, the implementation of a closed system comprising double clarification and tangential flow filtration.

All these innovations, combined with in-depth characterization of the cells at each upstream stage and of the secretome during downstream processing, including in vitro tests, mean that the process is robust and can be applied to several cell sources. EVerZom's ambition is to produce EVs of clinical/GMP quality in order to develop therapies that are as effective as cell-based therapies for tissue regeneration and diseases such as stroke, heart failure and arthritis, while being safer and more convenient for supply chains.

Using Raman spectroscopy for biochemical characterization of extracellular vesicles generated by thyroid tumor cell-fibroblast interplay: preliminary reports

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Carcinomas are complex societies of mutually interacting cells, with tumor-stroma crosstalk leading to tumor progression. Extracellular vesicles (EVs) are heterogeneous nanosized lipid vesicles actively implicated in intercellular communication.

We previously demonstrated that thyroid tumor cells-fibroblasts (Fb) interaction induces the secretion of metalloproteinases (MMPs) to culture supernatants (CMs) and promotes a migratory phenotype in the tumor cells. Thyroid tumor cell-Fb crosstalk liberates EVs that are functionally involved in extracellular matrix remodelling. In the present work we utilized Raman confocal microspectroscopy (Raman-CM) to explore the chemical content of individual secreted-EVs and further elucidate the impact of tumor-stroma interplay in its phenotype and ultimate biological function.

Thyroid tumor cells (TPC-1) or thyroid non-tumor cells (NThyOri) were co-cultured with normal human Fb as a simulation of the thyroid tumor microenvironment (TM). EVs obtained by ultracentrifugation of thyroid cells, Fb and thyroid cell-Fb CMs were characterised by Raman-CM. The Raman spectra of the EVs secreted by thyroid cells either pure or co-cultured with Fb, were compared to distinguish specific biochemical patterns.

Comparison of the average spectra revealed many differences between EVs obtained from pure and co-cultured TPC-1 cells. Raman peaks in the 1000–1550 cm-1 range, congruent with carotenoids, and 2150-2700 cm-1 (C≡C, C≡N, S-H groups) range were observed in TPC-1-derived EVs while they were apparently absent in EVs secreted in TPC-1-Fb context. A set of peaks in the spectral range 2800-3000 cm-1, which is greatly influenced by lipid molecules, is only apparent in TPC-1-Fb derived EVs. There are no noticeable differences between the spectra taken from EVs from pure and co-cultured NThyOri cells.

Taken together these findings suggest an important role of Fb in EV-phenotype from thyroid tumor. They also open a new approach to study the thyroid TM and its role in thyroid tumor progression.

Finding new EVs associated biomarkers of early post-menopausal osteoporosis in a Minipig model.

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Osteoporosis, the most prevalent bone metabolic disease with more than 300 million affected patients worldwide, is called a “silent disease” since no symptoms are detected until the first fracture occurs. In order to find new early diagnostic biomarkers of the disease we have created a minipig model of early postmenopausal

osteoporosis. To induce osteoporosis, animals were fed with a low-calcium diet and underwent ovariectomy. Eight months after surgery animals were sacrificed and large bones, serum and plasma samples were collected. Osteoporosis was confirmed by microcomputed tomography (μCT) analysis. Decellularized osteoporotic and control bone scaffolds were seeded with human bone marrow mesenchymal stem cells (BM-MSCs) and their RNA was analyzed (RNAseq) to identify the genes differentially expressed in osteoporotic bone. From plasma and serum samples, extracellular vesicles (EVs) were isolated by Size Exclusion Chromatography (SEC) and characterized (Bradford, NTA, CryoEM and Western blot). Both, EVs and non-EVs fractions were analyzed by proteomics and miRNAseq. We found the expression of several proteins altered in osteoporotic condition that have already been described together with new potential biomarkers in the EVs of serum and plasma. In addition, we identified 6 miRNAs, 1 of them in serum EVs, to be upregulated in osteoporosis which are implicated in bone metabolism pathways. In parallel, we performed a Genome-Wide Association Study (GWAS) of Osteoporosis (ICD-10 codes: M80, M81 and M82) in 404,215 white-British individuals from UK biobank (UKBB data, application ID 74382). A post-GWAS analysis was conducted to identify gene-related variants with the disease. Finally, a Mendelian Randomization analysis was applied to estimate the causal relationship between urine and blood biomarkers with Osteoporosis. The results obtained from the different experiments (bone signaling, proteomics and miRNA) are being cross-validated with the GWAS results to identify potential candidates that could be specific early biomarkers for osteoporosis.

Development and application of a secretome from mesenchymal stem cells with enhanced osteogenic capacity for the treatment of osteoporosis

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Osteoporosis (OP) is the most prevalent metabolic bone disease. Although effective treatments are currently available, these are severe side effects thus, there is a need to design new clinically safe therapies. Previously, our group has been able to increase bone regeneration in an osteoporotic mouse model by transiently silencing SMURF1, an inhibitor of the bone morphogenetic proteins (BMPs) pathway in endogenous Mesenchymal Stem cells (MSCs). We hypothesize that MSCs primed in this fashion would also produce a pro-osteogenic secretome which could be used as a cell-free therapy, further increasing the safety of the treatment. We assayed the osteogenic potential of the conditioned media (CM) produced by MSCs where SMURF1 has been silenced cells (Sec-SMURF1) both in vitro and in vivo. Murine and human MSCs pre-treated with Sec-SMURF1 show in vitro an over-expression of osteogenic markers as well as a significant increase in the levels of mineralisation and alkaline phosphatase activity. This same effect was observed in MSCs from osteoporotic patients, which have an intrinsically reduced osteogenic potential. Importantly, MSCs pre-treated with Sec-SMURF1 and seeded in scaffolds produced a significantly higher percentage of mature bone matrix in an ectopic murine model also showing a higher presence of osteogenic markers than control scaffolds. The soluble and vesicular fractions of Sec-SMURF were separated, and exosomes successfully purified, as validated by DLS, TEM and the presence of key exosomal markers detected by flow cytometry. Mass spectrometry characterization of the soluble and exosomal fractions from SecSMURF1 show an over-representation of proteins related to Ossification, Skeletal system development, Angiogenesis or Extracellular matrix organization GO terms compared to control samples. Altogether, these results show that SMURF1 silencing stimulates the release of several factors able to induce bone regeneration, setting the basis for engineering MSCs to produce highly pro-osteogenic secretomes which could potentially be used to treat osteoporosis.

Orange-derived extracellular vesicles as viable nanocarriers of bioactive cargoes

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Introduction. The wide-ranging biological activities of microRNAs (miRNAs) promoted research on disease mechanisms and is suggesting appealing therapeutic applications. When unprotected, miRNAs suffer from rapid degradation and appropriate strategies need to be developed to improve their therapeutic potential. As miRNAs can be naturally transported by extracellular vesicles (EVs), the latter have been proposed as specific transport means for drug delivery, conferring stability and increasing resistance against RNase degradation. However, a standard, reproducible and cost-effective protocol for EV isolation as well as a natural source of EV is lacking.

Objective. To study orange-derived EVs as bioactive molecules-carrying vehicles and their ability to boost the delivery of exogenous miRNAs to target cells.

Methods. EVs were isolated from orange, combining ultracentrifugation and size exclusion chromatography. Isolated EVs were 1) loaded with exogenous miRNAs and 2) analyzed for their (poli)phenol contents. Exogenous miRNA uptake by Caco-2 intestinal cell line was examined.

Results. Orange-derived EVs present were successfully loaded with exogenous miRNAs, which were efficiently uptaken by target cells. Orange-derived EVs carry hesperidin.

Conclusion. Intrinsic orange EVs are capable of transporting exogenous miRNAs (and other bioactive molecules (i.e., hesperidin)) and seem to ease their incorporation by target cells.

Milk-derived extracellular vesicles as nanocarriers of dietary phenolic compounds

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The unique features of mammalian extracellular vesicles (EVs), including nanoscale bilayer membrane, natural cell-to-cell communication, biocompatibility, good structural stability, and low toxicity and immunogenicity, have paved the way for delivering bioactives. Dietary (poly)phenols are bioactive molecules, and their consumption is linked to health effects. However, they are extensively metabolized, limiting their biological activity. EVs could protect polyphenols from metabolism. Among various EVs, bovine milk EVs have attracted attention as a scalable source of EVs with the above-mentioned properties. We aim to evaluate whether the polyphenols curcumin (CUR) and resveratrol (RSV) could be efficiently loaded into milk EVs and compare both the delivery to mammary tissue and biological activity in breast cancer cell lines of milk EVs-encapsulated vs. non-encapsulated (free) polyphenols.

Before and after loading with CUR and RSV, EVs were isolated from bovine milk and characterized by Nanoparticle Tracking Analysis, electron microscopy, and Western blot. Loading was assessed by sonication, electroporation, or passive incubation and further purified by size exclusion chromatography. A rat tissue distribution kinetics and biological activity in cell lines were performed.

Non-metabolized CUR and RSV peaked the mammary tissue (aprox. 40 and 300 nM, respectively) 6 min after intravenous administration of Evs-loaded polyphenols but not with non-encapsulated polyphenols. These tissue-occurring concentrations of EVs-loaded, but not free CUR or RSV, exerted a rapid and potent antiproliferative effect on cancer cells but not on non-tumoral cells.

Our results show that milk EVs protected these polyphenols from metabolism and delivered them to the mammary tissue at concentrations compatible with the fast and potent anticancer effects exerted in model cells. This supports EVs as nanocarriers to deliver bioactive molecules in therapy.

Isolation of exovesicles in the host containing pathogen DNA

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Chagas disease, a neglected tropical disease, is now considered a worldwide health concern as a result of migratory movements from Central and South America to other regions that were considered free of the disease, and where the epidemiological risk is limited to transplacental transmission or blood or organ donations from infected persons.

Parasite detection in chronically ill patients is restricted to serological tests that only determine infection by previous infection and not the presence of the parasite, especially in patients undergoing treatment evaluation or in newborns.

We have evaluated the use of nucleic acids from both circulating exovesicles and cell-free DNA (cfDNA) from serum samples from immunologically diagnosed chronic patients. When the nucleic acids thus purified were assayed as a template and amplified the parasite kinetoplastid DNA and parasite nuclear satellite DNA, a 100% positivity rate was obtained for all positive samples assayed with kDNA-specific primers and 96% when SAT primers were used. The results demonstrate that serum exovesicles contain DNA of mitochondrial and nuclear origin, which can be considered a mixed population of exovesicles of parasitic origin.

The results obtained with serum samples prove that both cfDNA and Exovesicle DNA can be used to confirm parasitaemia in chronically ill patients or in samples where it is necessary to demonstrate the active presence of the parasite. The results confirm for the first time the existence of exovesicles of mitochondrial origin of the parasite in the serum of those affected by Chagas disease.

Characterization of ovarian cancer ascites-derived extracellular vesicles for their usage in precision medicine

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Ovarian cancer (OC), considered the most aggressive gynaecological cancer, presents nowadays difficult therapeutic management, due to its late detection, the lack of specific treatments, or the absence of effective biomarkers to predict and assess the therapeutical response, among other reasons. Different strategies are being studied to face this clinical problem, emerging the extracellular vesicles (EVs) as one of the most promising ones. It is well known that cancer cells produce and secrete EVs, which in turn accomplish different functions such as cell communication, chemoresistance mechanisms, or even metastasis favouring. These EVs can be found in different biological samples such as blood or ascitic fluid, which is characteristic in patients with peritoneal carcinomatosis. In this study, we aimed to isolate and characterize OC ascites-derived EVs (OCAEVs) to further determine their interconnectivity with patients' clinical features and possible use in precision medicine. Ascites samples were obtained from the IdISBa Biobank, with the approval of the Ethics Committee (IB1955-12BIO_ref_21-020) after ethical approval of the project by the CEI-IB (IB 4516/21 PI). OCAEVs were isolated by size exclusion chromatography (SEC) and were further characterized through protein content determination by Pierce™ BCA Assay, transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), Western Blot (WB), and MACSPlex Exosome Kit (human) flow cytometry analysis. Preliminary results indicate that OCAEVs present a similar size distribution and protein expression profile between different donors with diverse clinical features. However, differences in protein levels can be further explored to establish clinical markers that correlate with a specific pathophysiological condition or treatment stage. OCAEVs constitute good candidates for their usage in precision medicine. As prospects, these EVs will be compared with EVs derived from other OC models such as patient-derived organoids to establish an interrelation and correlation to improve OC research.

Comparation of platelet-like particles (PLPs) and platelets (PLTs) derived EVs.

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Extracellular vesicles (EVs) are the protagonists of indirect communication between platelets (PLTs) and cancer cells and induce hypercoagulation, tumor progression, angiogenesis and metastasis, reducing patient survival. Our group is focused on the study of pancreatic cancer which is one of the solid tumors with a higher rate of hypercoagulability contributing to its high mortality (survival of less than 10% at 5 years being in Spain the 3rd cause of cancer death). For these reasons, we consider that this mechanism of indirect communication via EVs may be of great importance in the establishment, progression and tumor growth of PDAC. This interaction between PLT and PDAC cells leads to the modification of the content of PLT and therefore also of the content of its released EVs, being this characteristic and reflection of this interaction. Our aim is to generate an in vitro model of megakaryoblast differentiation for the production of Platelet-like particles (PLPs) from MEG-01, in order to study the mechanism of indirect communication that occurs between PDAC and PLTs. In order to validate the usefulness of our model from a descriptive approach, techniques such as NTA, western blot, TEM, zeta potential, conventional and nanoparticle flow cytometry and from a functional approach, co-cultures were performed, comparing with samples derived from PLTs from healthy donors. In a first approximation we have verified that there is a transfer of lipids, RNA and proteins between our model and PDAC, although these preliminary results also point out that there are some differences between the model populations and the physiological sample populations (based on descriptive characterization). We consider that this model may be very interesting because it may have diagnostic and therapeutic applications, which requires further studies to evaluate whether these differences also translate into functional changes.

Exploring the impact of donor pool size on the consistency and features of Platelet Lysate-derived Extracellular Vesicles

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Platelet lysate-derived extracellular vesicles (pEV) have shown significant therapeutic potential in the field of regenerative medicine, having shown in pre-clinical studies to be the more therapeutically potent fraction of platelets secretome. For their obtention, buffy coats from 5 blood donations are used to produce one platelet concentrate (PC). However, it has been reported that PC show high variability in growth factor concentration unless a higher number of donors is pooled. But, as far as we know, variability on pEVs depending on the number of donors has not been reported. Thus, the objective of the present study was to determine the variability, in terms of characteristics and functionality through wound healing assays, between pEV isolated from PC (5 donors) or from MPC (multiple PC, that is 50 donors).

Platelet lysate-derived extracellular vesicles were isolated, under GMP-like conditions in a clean room, using Size Exclusion Chromatography (SEC). The differential characteristics between pEV obtained from PC or from MPC were characterized by means of protein concentration, Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM), and flow cytometry using the MacsPlex™ arrays, that allows surface analysis profiling of EVs. The functionality of the isolated pEV was determined through a wound healing in vitro assay, by the determination of metabolic activity and LDH activity.

A narrower range of coefficent of variation (CV) on the evaluated functional effect (wound closure) was obtained when using pEVs derived from MPC compared to PC. However, the use of a higher number of donors entails higher safety concerns, thus this is an aspect to be discussed with the regulatory authorities for its clinical translation.

Functionalization of MSC-EVs with senolytic drugs and aptamers to target the vasculature

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Introduction: Cellular senescence has been associated with early vascular aging. Senolytic drugs are compounds that induce apoptosis of such senescent cells and two of the most used are quercetin (Q) and dasatinib (D). Several studies have demonstrated that mesenchymal stem cell-derived EVs (MSC-EVs) exert beneficial effects. Thus, the objective of our study is to develop a novel therapy using MSC-EVs as a vehicle for Q and D delivery to the vasculature.

Methods: EVs were isolated from immortalized adipose tissue MSCs by ultrafiltration and size exclusion chromatography (SEC), and characterized by TEM, NTA and ExoView. NTA and light dispersion analyses were used to determine the optimal conditions for Q and D internalisation. Functionalization of the EVs with both senolytics was evaluated by HPLC and MRM mass spectrometry, investigating the utility of several active loading methods. EV's uptake by HUVECs was observed with CFSE staining. MTT assay was used to study the possible cytotoxic effect of EVs. Besides, endothelial-directed aptamers conjugated or not with cholesterol were synthesized in order to compare their incorporation into EVs, with the final goal of specifically delivering the treatment to the vascular tissue.

Results: Optimal concentration of the different agents for EVs functionalization were: DMSO 3%, Q 20 µM and D 4 µM. Efficient functionalization of the senolytics was evidenced. CFSE staining showed that MSC-EVs are internalized by HUVECs and in vitro experiments suggest that EVs, EVs-QC and EVs-DS are not cytotoxic in HUVECs (up to 50.000). Finally, aptamer's modification with cholesterol displayed a better incorporation into EVs and was found to bind to HUVECs.

Conclusion: We have been able to encapsulate quercetin and dasatinib in MSC-EVs without compromising EVs´ properties and the first in vitro experiments showed their uptake by human endothelial cells. Cholesterol modified aptamers were efficiently incorporated into MSC-EVs.

Effect of tumor-derived extracellular vesicles on the differentiation and maturation of monocytes and dendritic cells

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Tumor-derived EVs (TEVs) play a key role in the development and progression of many types of cancer. TEVs interact with, and are taken-up by different target cells, including immune cells, thereby inducing their reprogramming and promoting tumor progression and metastasis.
TEVs are known to exert an immunosuppresive effect by promoting apoptosis and/or suppressing the function of CD8 T lymphocytes, that are the main effectors in the antitumoral response. TEVs also contribute to the emergence of dysfunctional dendritic cells, which have an essential role in the priming and differentiation of CD8 T lymphocytes.
The inhibition of the interaction and uptake of TEVs by immune cells could be an interesting therapeutic approach to counteract their immunosuppressive effect and promote the antitumoral immune response. For this reason, it is important to elucidate the molecules involved in these interactions.
We have described previously that ALCAM/CD166 and ADAM17/TACE are involved in the binding and uptake of TEVs by different target cells and that the tetraspanin CD9 regulates these interactions. Now, we are studying the effect of TEVs and the involvement of these molecules on the differentiation and maturation of monocytes and dendritic cells.

Methodology:

- TEVs are isolated from the supernatant of the colorectal cancer cell line Colo-320 (either expressing or lacking ALCAM, ADAM17 or CD9) by serial centrifugations, concentration and size exclusion chromatography.
- Monocytes, immature and mature dendritic cells obtained from peripheral blood are incubated with the different TEVs. After six days, we analyze differentiation and maturation markers by flow cytometry.

Results:

Wild type TEVs (ALCAM+, ADAM17+, CD9-) produced by Colo-320 cells promote the differentiation of monocytes into macrophages. In contrast, LPS-induced maturation of dendritic cells was inhibited by wild type TEVs. We have found that the presence of ALCAM, ADAM17 and CD9 on the TEVs surface are relevant for these phenotypical changes.

HEK293T-derived Extracellular Vesicles overexpressing miR219a-5p: a good model for further studies in neurodegenerative diseases

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Nowadays, there is a wide range of methods to isolate Extracellular Vesicles (EVs), but there is no consensus about which one is the optimum. However, the approach used to separate them ought to be determined by the specific scientific question to address.

The aim of this study was to select and characterize the most suitable isolated HEK293 EV-pellet, which overexpresses miR219a-5p, as an efficient delivery system into the central nervous system. In fact, it has been reported that miR219a-5p produce the differentiation of oligodendrocytes precursor cells to myelinating mature oligodendrocytes. To this end, we overexpressed miR219a-5p in HEK293T cells (HEK293T-219) and the same cells with the empty plasmid was used as control (HEK293T). Then, we enriched EVs from conditioned media by centrifugation (20,000xg), ultracentrifugation (100,000xg) and size exclusion chromatography (SEC). Subsequently, we characterized them by NTA, Cryo-EM and qPCR.

We determined that 20,000xg-derived EVs from HEK293T-219 presented a higher level of expression of miR219a-5p. Besides, HEK293T-219 release a significant lower amount of EVs per million cells in comparison with the HEK293T, meaning that the miR219a-5p could have an effect in the production of EVs by the producing cell.

In summary, the optimal approach for isolating EVs for our purpose is a centrifugation at 20,000xg. This simple and fast method has enabled us to work with the resulting pellet in subsequent in vitro studies. In this sense, we have carried out a functional assay to determine how the EVs, in comparison with liposomes, affect primary cortical neurons assessing their electrical activity through the Multielectrode Array Assay (MEA).

These experiments are the first steps before moving to in vivo studies related to EVs-based therapy in neurodegenerative diseases.

Delivery of shRNA minicircles by extracelullar vesicles to halt Parkinson's disease neurodegeneration

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The development of new therapies to slow down or halt Parkinson's disease (PD) progression is a healthcare priority. A key pathological feature of PD is the presence of alpha-synuclein aggregates and transmission of this pathology between neurons plays a central role in disease progression.

Objective: We aimed to evaluate if alpha-synuclein downregulation by shRNA-minicicles (shRNA-MC) delivered by RVG-extracellular vesicles (RVG-EV) administrated after the appearance of the alpha-synuclein pathology halt the neurodegenerative process.

Methods: RVG-EVs were isolated from dendritic cells transfected to express the RVG-peptide and loaded with shRNA-MCs by electroporation. We used a progressive synucleinopathy model based on the intrastriatal injection of alpha-synuclein preformed fibrils (syn PFFs). RVG-EVs containing shRNA-MC were administred intravenously 35 and 80 days after syn PFF injection, a group of mice received only one injection 35 days after syn PFF injection.

Results: The treatment with 2 doses of shRNA-MC RVG-EV decreased significantly alpha-synuclein mRNA and protein levels in the brain. This decrease was reflected in a significant reduction in the number of phospho-alpha-synuclein aggregates, the normalization of the striatal TH staining and the lack of dopaminergic cell death. However, the treatment with only one dose was not sufficient to reduce mRNA or protein levels of alpha-synuclein 105 days after administration.

Conclusions: We demonstrated that the treatment with anti-alpha-synuclein shRNA-MC RVG-EV after the development of pathology is effective to downregulate alpha-synuclein in brain. Moreover, we confirmed that a multidose treatment is necessary to maintain downregulation for long-term treatments.

Plant exosome-like nanoparticles: building up novel animal-free therapeutic agents for drug delivery

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INTRODUCTION

Plant exosome-like nanoparticles (ELNs) have recently been shown to have a very promising therapeutic potential to alleviate inflammation in different pathologies. They have similar properties to extracellular vesicles derived from mammary cells. Hence, they may be a revolutionary therapy because (1) large quantities of EVs are required for in vivo applications, and alternative methods with lower production costs and time are therefore needed, (2) their plant origin facilitates transferring these therapeutic agents into clinical practice and (3) they can be classified as vegan, which may imply a better perception of the final product. Moreover, it is possible to bioengineer them for drug delivery.

METHODS

ELNs' from tea plant, red cabbage, turmeric and ginger roots were obtained. Size exclusion chromatography and ultracentrifugation isolation methods were compared. ELNs were characterized by NTA and TEM. Cell-Mask Deep Red staining was used to test internalization by HUVECs. Flow cytometry analysis of the expression of ICAM-1 and VCAM-1 on inflammed HUVECs treated with ELNs have been performed, as well as cytotoxicity analysis. Proliferation analyses on mesenchymal stem cells treated with ELNs have been carried out. Finally, as a proof-of concept experiment, ELNs have been functionalized with an aptamer and a drug (quercetin), which incorporation has been evaluated by fluorescence/absorbance, HPLC and MRM mass spectrometry.

RESULTS

Efficient methods for ELNs´ isolation have been optimized. Preliminary in vitro experiments evidence internalization of the ELNs in human endothelial cells. Red cabbage ELNs seem to have an anti-inflammatory effect, while turmeric ELNs appear to be pro-proliferative. Cytotoxicity was tested to ensure biosafety of the studied ELNs for future therapeutic use. We were able to efficiently incorporate aptamers (which may allow to target ELNs to specific tissues/cells) and drugs onto tea ELNs.

CONCLUSION

ELNs could be isolated with great yield, are biosafe and constitute novel candidates for drug delivery.

Tetraspanin pattern characterization of extracellular vesicles in multiple sclerosis disease by ExoView R200+ platform

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Multiple sclerosis (MS) is a chronic and demyelinating disease of the central nervous system (CNS). 85% of patients present the relapsing-remitting form (RRMS), and 10-15% present a worsening symptoms from the onset, known as primary progressive form (PPMS). CNS releases extracellular vesicles (EVs) that cross the blood-brain barrier to the circulating blood, showing a high potential as “windows into the brain”.

Up to date, CD63/CD81/CD9 EV-heterogeneity pattern, principal members of the EV-tetraspanin family, in CSF and serum from MS patients has not been reported. The aim of this study was to unravel the EV-tetraspanin heterogeneity of paired CSF- and serum-derived EVs from MS patients.

CD63+-, CD81+-, and CD9+-EVs were measured in unprocessed CSF and serum of RRMS, PPMS and non-MS patients, using single-particle interferometric reflectance imaging sensor (SP-IRIS)-based ExoView R200+ technology. To this end, the human tetraspanin kit was used following the manufacturer's instructions to capture and fluorescently labelled-EVs.

Our results showed a lower presence of EVsCD63+ in CSF and EVsCD81+ in serum samples, without biases associated with a specific EV-purification. Besides, we observed a higher presence of EVsCD9+ in CD81- and CD9-captured EVs in CSF from PPMS with respect to RRMS. Conversely, we observed a higher amount of EVsCD9+ in CD63- and CD9-captured EVs in serum from RRMS with respect to PPMS and non-MS patients. Finally, colocalization percentages of tetraspanin pattern were similar among CSF from RRMS, PPMS and non-MS donors, with a particularly higher percentage of EVsCD81+/CD9+. In serum, the tetraspanin pattern was different when we analyzed CD63-, CD81-, and CD9-captured EVs, but without differences between MS and non-MS patients.

To conclude, the tetraspanin EV-pattern were specific to the biofluid analized and EVsCD9+ could be interesting in MS disease. ExoView technology is a good platform to study a customizable panel of labelled EV-markers to validate new CNS-derived biomarkers.

Implication of secreted CEMIP protein in dermal fibroblast behavior

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CEMIP (Cell migration inducing hyaluronan binding protein) expression has been described in pathological conditions linked to chronic inflammation such as fibrosis and cancer. In addition, CEMIP level in tumor tissue has been correlated with disease progression and survival in various cancer types, and increased CEMIP-content in tumor-derived extracellular vesicles (EVs) has been shown to boost brain metastasis in breast cancer. This work focuses on the implications of secreted CEMIP in dermal fibroblast behavior, one of the main cell populations of the tumor microenvironment in skin cancers such as melanoma.

According to our data, in dermal fibroblast (Detroit 551), cellular CEMIP levels are modulated by histamine and TGF-β1. Alterations on endogenous CEMIP levels modulate secreted amount of this protein, and increased CEMIP secretion upon histamine exposure correlates with enhanced migration capacity of fibroblasts. Moreover, melanoma cells, but not melanocytes, express and secrete CEMIP through small EVs. siRNA mediated downregulation of CEMIP in melanoma (COLO-800) cells is able to decrease both cellular and secreted CEMIP which, in turn, regulates FAK (focal adhesion kinase) activation and cellular migration in dermal fibroblasts. Moreover, melanoma-derived small EVs are incorporated by dermal fibroblasts and stimulate cell motility in a CEMIP-dependent manner.

Enhanced fibroblast motility is often associated with cell activation. In the tumor microenvironment, tumor associated (and activated) fibroblast are characterized by ✔ 𝛂 SMA expression. Incubation of fibroblast with melanoma-derived high and low CEMIP contents did not alter ✔ 𝛂 SMA expression suggesting that they are independent events in our context. We are currently seeking to better characterize the activated fibroblast signature induced by melanoma-secreted CEMIP.

Altogether, these results suggest that the increased expression and secretion of CEMIP observed in melanoma cells could be related to the dermal preconditioning by altering dermal fibroblast behavior in a similar manner as done by acute inflammation inducers like histamine.

Evaluation of microneedle arrays as potential delivery systems for bacterial extracellular vesicles

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Bacterial extracellular vesicles –(B)EVs– have been recently proposed as a potential valuable source of postbiotics: preparations of inanimate microorganisms, and/or their components, that confer a benefit for host's health. However, in order to BEV-based products being usable in clinical applications, suitable formulation and delivery methods that preserve BEV structure and functionality are required. Microneedle arrays (MNAs) are promising systems for transdermal drug delivery, which are being investigated for systemic administration of a number of active compounds, including chemical and biological drugs, vaccines and cells. In this study we evaluated the usefulness of MNAs as a system for BEV administration. BEVs from the Gram-positive probiotic bacteria Bacillus subtilis 168 were isolated from bacterial cultures by ultracentrifugation followed by size-exclusion chromatography, and enclosed in polyvinylpyrrolidone and polyvinyl acetate (PVP/PVA) MNAs. BEVs were then recovered form MNAs by dissolving the latter in sterile PBS, and their structure and active properties compared with those of unprocessed BEVs. Transmission electron microscopy confirmed that BEV structure remained unaltered following inclusion in and subsequent release from MNAs. Moreover, as a proxy to evaluate the impact of microneedle enclosure and release on their biological activity, we assessed the ability of B. subtilis EVs to promote wound healing in cultured immortalized human keratinocytes (i.e., HaCaT cells). Our preliminary data show that both unprocessed and microneedle-derived EVs from B. subtilis 168 accelerate wound healing in vitro, compared to the PBS control. Altogether, our results represent a proof of concept that MNAs may be suitable resources for EV-based pharmaceutical formulations and endorse future studies aimed at evaluating the bioavailability, biodistribution and activity of EVs administered through this system in vivo.

Anti-inflammatory effect of extracellular vesicles from synovial fluid on osteoarthritic synoviocytes

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Introduction. Extracellular vesicles (EV) play various roles in osteoarthritis (OA) as immunomodulators, mesenchymal stem cell recruitment and tissue renewal [1]. In OA patients, there is a low concentration of hyaluronic acid (HA) in synovial fluid (SF), and HA-coated EV have been identified, this suggests that EV may play a role in controlling OA [2,3]. Additionally, MSC and EV have a close relationship and interact to maintain joint homeostasis and participate in cartilage repair and regeneration [4]. The objective of this study is to investigate the impact of SF EV on OA synoviocytes (SOA).

Material and methods. Synoviocytes were isolated from the synovium of OA patients by enzymatic digestion and cultured in 12-well plates (2x105cells/mL/well). After reaching confluence, SOA were stimulated with IL-1β [10 ng/mL] and different concentrations of SF EV [7,20E+08, 7,20E+07, 3,60E+07, 1,80E+07 EV/mL]. Cell viability and proliferation were assessed through MTT and BrdU Proliferation Kit, and the levels of IL-6, HA, and MMP-3 were measured in the cell supernatants using ELISA Kits.

Results. The results showed that cell viability remained unchanged across all conditions. However, SOA proliferation increased significantly in response to IL-1β and EV treatment. IL-1β increased inflammation by significantly increasing IL-6 and MMP-3 production by SOA. This effect was significantly reduced in the presence of SF EV. HA production was not affected by IL-1β or SF EV at any concentration.

Conclusions. The viability of SOA was maintained in all conditions, while their proliferative capacity increased. EV treatment reduced inflammation by lowering IL-6 levels, had no impact on HA concentration, and slowed down matrix degradation by decreasing MMP-3 levels. Further studies are needed to validate these findings and expand our knowledge in this area of research.

IMPLEMENTATION OF SCAFFOLDS EMBEDDED WITH EXOSOMES AS PLATFORMS TO ATTRACT METASTATIC CELLS

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INTRODUCTION: Exosomes, ranging in size from 30 to 120 nm, are small nanovesicles that are actively released by various cells. Their essential role relies in mediating intercellular communication and cargo transfer, making them valuable as non-viral vectors. This inherent capacity enables them to modulate cellular behaviour, serve as diagnostic biomarkers, and actively participate in cancer therapy.

It has been described that some types of exosomes might migrate actively to tumors. As this migration of the exosomes to tumoral cells is well known, we though that the migration could happen also on the contrary way. Therefore, in order to test this hypothesis, we incorporated exosomes into biocompatible scaffolds (exo-scaffolds), designed to retain extracellular vesicles while preserving their structural integrity, with the objective of attracting metastatic cells towards the scaffold. Our aim is to effectively attract and eliminate metastatic tumour cells, thereby offering promising prospects for targeted therapeutic interventions.

OBJECTIVE: To investigate the efficacy of those scaffolds in promoting metastatic cellular migration when attracting by the exosomes bound to them, with the objective of using them in highly metastatic cancer therapies.

RESULTS: The results obtained here provide compelling evidence for the substantial involvement of exosomes in cellular migration. When using transwells and Wound Healing assays, it was observed that exo-scaffolds carried a great proportion of exosomes, and they exhibited a significantly increased concentration of cells attracted when compared to exosome-free scaffolds. These findings hold great promise for future investigations aimed at utilizing exo-scaffolds as a novel cancer treatment.

CONCLUSION: Here we demonstrate that the idea of linking exosomes to scaffolds to attract metastatic cells is possible, and in that way we will stop the advance of the metastatic cells to other niches when the scaffold is inserted on the surgical-space left after the tumor removal.

Omics analysis of prostate cancer-derived extracellular vesicles to unravel their role in tumour generation and proliferation

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The ability of cancer-derived EVs to promote the cancer-associated fibroblasts (CAFs) phenotype has been linked to several types of molecular cargo, including microRNAs (miRNAs), proteins, and to a lesser extent, messenger RNAs and long non-coding RNAs. In the same way, CAFs activation can alter the content of their secreted EVs, which has also been shown to promote tumour progression. In the context of low-grade prostate cancer (PCa), several studies have described the role of some miRNAs and proteins in PCa formation and progression, including both EV and non-EV biomarkers, but none has yet been described as crucial.

Nevertheless, the necessity of minimally invasive biomarkers discovery is manifest, and PCa-derived EVs (PCa-dEVs) have been described as a promising biomarker source. In this context, this study is framed in characterizing five PCa in vitro cell models representing the different PCa biotypes, and their dEVs, to find non-invasive biomarkers that could help to predict tumour initiation and progression from low to high tumour grade. With this aim, transcriptomics and proteomics analysis of PCa cells, PCa-dEVs and CAFs transformed with PCa-dEVs have been performed. Overall, the complete characterization of these specimens and its interpretation inside a clinical context will help to define which molecules are involved in PCa formation and upgrading and, subsequently, could be used inside active surveillance programs after confirming with ex vivo studies.

Use of natural nanotherapies for liver fibrosis based on curcumin encapsulated in milk sEVs

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Introduction: Curcumin is a natural molecule with antioxidant, anti-inflammatory, and hepatoprotective activities, but its high hydrophobicity and low bioavailability limit in vivo applications. Small EVs (sEVs) are emerging as a natural substitute for synthetic nanoplatforms. Their lipidic membrane allows the incorporation of insoluble molecules into their structure, avoiding the limitations associated with hydrophobicity. This work tests the encapsulation of curcumin into milk-sEV (MisEV) structure and further evaluation as a therapeutic nanoplatform. Materials: Curcumin (1000 µg), saponin (2 mg) and milk sEV (200 µg) were mixed at 37ºC for 20' and purified by size exclusion chromatography (MisEVCur). Physicochemical characterization of MisEVCur was performed by NTA, DLS, TEM, nanodrop, nanophotometry and flow cytometry (FC). Cellular assessment of MisEVCur cytotoxicity and uptake in RAW264.7 and HepG2 cells and primary hepatocytes was performed by MTT assay, FC and confocal microscopy. In vivo evaluation was performed in an acute chronic liver disease

model, induced by carbon tetrachloride (CCl4). As treatment, CCl4-mice received 3 injections of 30 µg of sEVCur. Results: Our approach allowed curcumin incorporation into MisEVs structure. No morphological modification was observed by NTA, DLS and TEM (MisEVCur: 120±6.1nm). Optical assessment verified the presence of curcumin (434.5 µg/mL). The controlled release of the curcumin from MisEVCur showed a maximal curcumin delivery of 44.7% after 48h post-incubation. Cytotoxic activity of MisEVCur was confirmed in all cells in a dose- and time-dependent manner, with higher cytotoxicity in macrophages at 48h (%viability: RAW264.7: 3.8%; HepG2: 12.2%; primary hepatocytes: 29.1%). In vivo evaluation confirmed the therapeutic effects, leading to a decrease of serum markers of liver damage (ALT) and a tendency towards decreased liver fibrogenesis and extracellular matrix deposition. Conclusions: We have developed the novel nanoplatform MisEVCur based on encapsulating the therapeutic molecule curcumin into the MisEVs structure and confirmed their therapeutic effects in a preclinical fibrotic model.

Extracellular Vesicles-miRNAs signature in metastatic breast cancer diagnosis

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Extracellular Vesicles (EVs) associated miRNA (EVs-miRNAs) profiling has been shown to be specially altered in breast cancer cells and can be a complementary tool for diagnosis and response´s prediction in localized breast cancer.

Here we present a prospective longitudinal study in patients with breast cancer prior to receiving neoadjuvant chemotherapy in which circulating EVs-miRNAs were obtained from serum of early-stage patients (n=10), metastatic patients (n=6) and healthy donors (n=9), which were subsequently sequenced.

EVs were isolated from serum by serial centrifugation protocol optimized by our laboratory and characterized by western blot and electron microscopy. Then, isolated EVs were lysed and total RNA was extracted with the miRNeasy Kit and sequenced with the TruSeq Small RNA Library Prep Kit. Bioinformatics analysis was performed with the following programs FastQC for quality control, Cutadapt to remove adapters, Bowtie for sequence mapping and miRDeep2 to identify both novel and known miRNAs. The differential expression of the genes was analyzed with EdgeR package of R.

The 25 sequenced samples had an average of 4,883,812 mapped reads, of which a total of 2,656 known miRNAs were mapped and found. The comparison of the expression between groups two by two (Control vs. Early Stage, Control vs. Metastatic, Early Stage vs. Metastatic) showed a total of 12 miRNAs whose expression was differentially significant under the standard FDR < 0.01. Eight of them present differential expression in metastatic stage compared with early stage: miR-183-5p, miR-200a-3p, miR-100-5p, miR-122-5p, miR-192-5p, miR-500a-3p, miR-141-3p (upregulated), let-7f-5p (downregulated) while seven miRNAs were differentially expressed in metastatic stage compared with healthy controls: let-7a-5p, let-7f-5p, miR-144-5p (downregulated) miR-423-5p, miR-141-3p, miR-100-5p and miR-320b (upregulated).

While, these data needs to be validated in an independent cohort, these indicate that this EVs-miRNAs panel could be and excellent tool to distinguish metastatic from both healthy and early breast cancer stages.

Designing a nucleic acid-loaded theragnostic hybrid nanosystem based on extracellular vesicles-coated gold nanoparticles

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Currently, tmany diseases remain with a non-efficacious treatment, which could be attributed to the poor biodistribution of medicines once administered systemically. Although many studies reported the multiple advantages of nucleic acids as the therapeutic biologicals of the near future, their use in clinics is still marginal since many drawbacks are not yet overcome. Mainly, their compromised stability in biological fluids due to the presence of nucleases that promote a premature clearance and the poor biodistribution in the target organs, complicate their clinical translation. In this context, nanomedicine could play a key role, namely in terms of controlling the site and time distribution of nucleic acids. With this general goal in mind, in the current work, we aimed to demonstrate the feasibility of using an hybrid gene delivery system composed of gold nanoparticles, carrying nucleic acid and coated with extracellular membranes. Beyond the use of a reporter nucleic acid, as a proof-of-concept study, gold nanoparticles were selected for the simultaneous possibility of tracking the nanosystem inside the body, while extracellular vesicles coating was used to take advantage of their tropism to the same cell lineage they come from, thus avoiding the addition of usually expensive targeting moieties. We were able to formulate, for the first time, this complex nanosystem, demonstrating at in vitro level, selective cell uptake of all the nanosytem components. In conclusion, we have designed a valuable tool that can be applied for the theragnosis of a variety of diseases requiring the delivery of active nucleic acids.

Combination of cation-exchange chromatography and filtration for lipoprotein depletion and plasma RNA detection

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Introduction: Lung cancer (LC) patients presenting chromosomal aberrations, can benefit from targeted drugs. ALK inhibitors improve quality of life and survival of ALK fusion-positive patients. Because tumor tissue availability in LC patients is often compromised, ALK rearrangement testing using liquid biopsy is a promising approach. Extracellular vesicles (EVs) could provide a diagnostic advantage compared to free nucleic acids, in particular RNA fragments. However, in plasma, the presence of lipoproteins (LPs) that share similar biophysical properties with EVs, and the relatively low abundance of tumor derived-EVs make its implementation in clinical practice a very challenging task. The main purpose of this study is the characterization of EVs containing ALK fusion RNA in LC patients and the development of easily automated methods for clinical diagnosis.
Methods: Enrichment of EVs and partial depletion of LPs from pools of either healthy donor (HD) or ALK-negative LC patient plasma was performed by cation-exchange chromatography (CEC). EV-enriched samples were then concentrated using a 300kDa bio-inert filters, and CD63, CD9 and ApoB markers were analyzed by western blot. Finally, total RNA from EV-enriched samples was isolated and the amount of RNA was estimated by analyzing the housekeeping genes GAPDH and β-actin by qPCR. LC markers, like ALK-translocation RNA was also tested by dPCR.
Results: Western blot results demonstrated an enrichment of CD9 and CD63 EVs and a partial depletion of ApoB-LPs (LDL/VLDL) in moderately acidified plasma samples after CEC followed by 300 KDa filter concentration, compared to unprocessed plasma samples. In this regard, EV-enriched samples obtained after 300kDa concentration showed higher expression of housekeeping genes compared to unprocessed plasma samples, both in HD and LC samples.
Conclusion: Our results show that CEC combined with 300KDa filter concentration enhances EV-RNA enrichment, making it a promising approach for the detection of biomarker, such as ALK fusions, in LC patients.

Production and isolation of F7 peptide tagged extracellular vesicles loaded with α-galactosidase A to improve cell internalization and in vitro efficacy

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Recombinant α-galactosidase A (GLA) enzyme is currently used for treating Fabry disease patients. Previous studies in our team have shown that encapsulation of the GLA in extracellular vesicles (EV) preserves GLA's enzymatic activity and improves its efficacy, compared to the free GLA protein. In an effort to increase cell-internalization capacity and efficacy of these therapeutic EVs , we have now produced EVs loaded with GLA (EV-GLA) and tagged with F7, a cell-penetrating peptide derived from the extracellular domain of CD300f.

Two different methods have been used to incorporate F7 peptide on the EV membrane: 1) EDC-mediated chemical conjugation of the synthetic peptide in previously isolated EV-GLA and 2) simultaneous recombinant expression of Lamp2A-fused F7 with GLA. In both cases EVs were obtained from HEK293F cells, diafiltered and concentrated using Tangential Flow Filtration (TFF) and subsequently purified by Size Exclusion Chromatography (SEC). EVs from no-transfected HEK293F cells were obtained as a control. Composition of EVs was characterized by nanoparticle tracking analysis (NTA) and Western blot in all four type of EVs, control EVs, non-tagged EV-GLA, chemically tagged F7-EV-GLA (cF7-EV-GLA) and recombinantly tagged ones (rF7-EV-GLA). Functional activity of GLA was verified by crude enzymatic activity assays. In vitro assays included cell internalization of EVs stained with DiR by flow cytometry and demonstrated that EV cell internalization is favoured in the presence of F7 peptide. Moreover, ongoing in vitro efficacy assays using endothelial cell cultures derived from GLA KO mice will demonstrate whether the inclusion of this peptide by chemical conjugation or recombinant expression improves also the efficacy of non-tagged EV-GLA.

"Cancer Stem Cell secreted Extracellular Vesicles lead tumor plasticity regulation and stroma activation in TNBC models"

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Introduction: Triple Negative Breast Cancer (TNBC) tumors are particularly aggressive due to inter and intratumor heterogeneity. Within, a small cell fraction of non-specialized cancer cells with stem cell-like features, Cancer Stem Cells (CSC), have been widely recognized as responsible for tumor resistance, metastasis and recurrence events. Moreover, CSC homeostasis in TNBC has been proven a highly plastic phenomenon largely affected by tumor microenvironment (TME) signaling. In this scenario, intercellular communication mediated by extracellular vesicles (EVs) secretion is paramount to provide TNBC adaptive potential. Particularly, tumor-derived EVs (tEVs) have been shown to impact tumor cells and their TME e.g. conferring drug resistance or transforming stroma cells towards phenotypes fostering tumor growth and distal spread. However, the specific contribution of the distinct tEV subsets have been poorly studied.

Methods: Here, CSC fluorescently tagged by stable expression of an ALDH1A1:tdTomato cassette, were sorted according to their fluorescence and expanded in cell culture. EVs were harvested from conditioned mediums by a combination of differential centrifugation, diafiltration and precipitation. Impact of tEVsCSC on cancer (MDA-MB-231) and stroma fibroblast models (CAFs IDC and CCD19-Lu), were tested in vitro in terms of 3D ECM remodeling, gene expression and cytokine secretion and in vivo, assessing their capability to prepare the premetastatic niche for metastatic outgrowth.

Results:In TNBC monocultures unbalancing EVs status quo towards tEVCSC enriched environments resulted in cancer cell differentiation and less malignant cancer phenotypes. Interestingly in multicellular models tEVsCSC favored myofibroblastic CAF activation boosting spheroids invasive potential in comparison to EVbulk, derived from non CSC. In vivo, animals pre-treated with repeated doses of tEVCSC displayed significantly higher metastatic burdens than EVbulk accompanied with a broader detection of alphaSMA+ fibroblast in the lungs.

Plasma-derived extracellular vesicles from ovarian cancer patients and their impact on healthy ovarian epithelial cells

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Small extracellular vesicles (sEVs) are membrane-enclosed structures (50-150 nm) that contain several molecules such as ncRNAs, mRNAs, DNA fragments, and proteins. The molecular cargo of sEVs can regulate mRNA expression in target cells affecting cell signalling and playing a crucial role in tumour progression. Thus, the study of sEVs produced by tumour cells could help to identify new biomarkers for early detection, diagnosis, prognosis, and treatment evaluation in cancer research. Recent investigations are exploring sEVs' biological functions and understanding their role in cancer. In this study, we examined the impact of plasma-derived sEVs from ovarian cancer (OC) patients on healthy ovarian epithelial cells. We aimed to investigate whether these vesicles may play a role in the development and progression of OC. We first collected plasma samples from a group of OC patients (n=12) from FIGO phases (III-IV) and a control group (n=12), and subsequently isolated sEVs by different techniques (i.e., differential centrifugation and tangential-flow-filtration). Characterization of sEVs preparations by nano-flow cytometry and immunoblot was also performed. Next, healthy ovarian epithelial cells (IOSE-80T) were cultivated with plasma-derived sEVs from different groups, and their effects were monitored. Our results showed that OC preparations were able to induce changes in gene expression (i.e., MUC-1 and BRCA) and modify the cellular behaviour in the healthy ovarian epithelial cells in contrast to control samples, indicating that circulating sEVs could play a role in the development of a pro-tumorigenic phenotype. Future experiments aim to better describe the protein and nucleic acid cargo of sEVs to identify potential

effector molecules. Our findings may have significant implications for the early detection and treatment of OC and suggest that plasma-derived sEVs may serve as useful biomarkers and novel targets for this disease.

IL-8/CXCR1 axis induces release of small extracellular vesicles from A2780 ovarian cancer cells with a pro-angiogenic profile

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Introduction: Small extracellular vesicles (sEVs) play an important role in tumor progression, and sEV of cancer stem-like cells (CSCs) can contribute to metastasis. In several tumors CSCs express high levels of CXCR1, but its contribution in the biogenesis of metastatic sEV remains unclear. We hypothesize that IL-8/CXCR1 activation could modify the production sEVs with a pro-tumorigenic activity. Methods: Ovarian cancer cells A2780 which overexpress CXCR1 were stimulated with IL-8, and proteins involved in sEVs biogenesis (Alix, HRS, TSG101 and CD9) were evaluated by western blot. sEV were characterized measuring proteins enriched in sEVs by western blot, and their size and concentration were determined by Nanotracking Analysis. The effect of sEVs in recipient cells was performed in endothelial (HUVEC) cells and tube formation, permeability and cell adhesion experimets were performed in vitro, and angiogenesis was evaluated in vivo using a CAM model. The protein content of sEVs was evaluated by Mass-Spectrometry. Results: We observed that activation of IL-8/CXCR1 in A2780 cells increased the level of proteins related to sEVs biogenesis accompanied with an increase in the sEVs release. Also, transfer of sEVs released in response to IL-8/CXCR1 activation induced tube formation, cell adhesion and permeability of HUVEC cells, and angiogenesis in vivo. Proteins involved in angiogenic properties were enriched in this sEV and could be responsible to induce pro-tumorigenic properties. Summary: Our findings suggest that the IL-8/CXCR1 axis could modulate the biogenesis and release of sEVs with pro-tumorigenic activity, suggesting this axis as a new possible target to block tumor progression mediated by sEVs.

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EV-encapsulated Pt nanoparticles as radiosensitizers for antitumoral therapy

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Cancer has lately become a highly relevant health issue. In 2020, almost 20 million new cases were diagnosed. This scenario demands the implementation of new therapeutic strategies for such a group of heterogeneous diseases. In this context, nanomaterials present a broad range of features that allow addressing this kind of issues from a different point of view, such as unique fluorescent or optical properties, or high surface-to-volume ratio.

Taking benefit of these properties, nanomaterial-based approaches raise the possibility of improving traditional therapies to overcome the limitations that they present. Regarding to this point, nanomaterials with the capacity of enhancing the radiotherapeutic effect are arising, based on the capacity of high Z atoms such as platinum to transfer excess of energy to surrounding particles for their ionization.

The combination of these materials with target-specific systems, such as extracellular vesicles, can give rise to widely improved therapies, allowing those promising NPs to reach tumoral areas. Our group has a wide experience on carrying NPs using EVs as transporters with great success.

The strategy here presented focuses on a Pt-based ultra-small nanomaterial, which combines the above-mentioned feature with oxygen generation by catalase-like activity, producing O2-based ROS thus creating more stable DNA strand breaks. This nanomaterial, when internalized in cells, demonstrates high biocompatibility if solely applied, and presents significant cytotoxic effects when combined with X-ray irradiation, which was deeply studied in vitro and also in vivo to assess its functionality.

However, since the main objective is improving the nanomaterial delivery to the tumoral areas, Pt NPs have been successfully associated with exosomes and transferred to the cells with the objective of studying the in vivo radiosensitization. Thus, we developed a target-specific therapy that allows nanoparticles to arrive more efficiently to the tumour, taking advantage of the affinity that exosomes present for their parental cells.

EXOGAG, the new method for the isolation of Extracellular Vesicles and Glycoproteins, unmasks biomarkers and new molecular mechanism in kidney disease.

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Glycosaminoglycans (GAGs) are large polysaccharides that interact through glycosidic bonds with proteins and lipids, forming the extracellular matrix; or with secreted proteins, such as uromodulin. Glycosylation is altered in pathologies, as cancer or kidney diseases.

GAGs are also present in extracellular vesicles (VEs), nanometric structures delimited by a lipid bilayer that cells release and whose charge (RNA / miRNA, DNA and proteins) is essential in intercellular communication.

Our group has developed a method for GAG, glycoproteins and VEs isolation in any biological sample, called EXOGAG (commercialized by Nasas Biotech), which led us to identify and characterize new signalling mechanisms, and identify new prognostic/diagnosis biomarkers, for example, in polycystic kidney disease (PKD).

Urine samples have been collected from patients genetically diagnosed with type I and II PKD at different stages of the disease. Using EXOGAG, GAG-glycoprotein-VEs complex has been isolated and characterized by different proteomic techniques (Western Blot, mass spectrometry), image characterization (electron microscopy, immunofluorescence) and vesicular component analysis (ExoView® or NanoTracking Analysis®).

EXOGAG has allowed us to identify new biomarkers in urine (in protection) in PKD patients, which are altered in disease progression, even anticipating currently used kidney damage markers. The characterization of these complexes has led us to discover signalling mechanisms between the different segments of the nephron, and whose function is altered in different pathologies, including polycystic kidney disease.

This new method for isolating the fraction associated with GAG in urine samples has allowed us to identify prognostic/diagnostic biomarkers of kidney diseases, based on glycoprotein and vesicular profile. Likewise, it has led us to identify new signalling mechanisms of the nephron, which opens a new field for a better

understanding of renal pathophysiology. These results uncovered the potential as a method of EVs isolation for its use in the research of new cellular communication pathways or cellular mechanisms.

STUDY AND VALIDATION OF THE METABOLIC SIGNATURE OF EXTRACELLULAR VESICLES AS A PROGNOSTIC AND PREDICTIVE MARKER IN PANCREATIC CANCER

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Pancreatic ductal adenocarcinoma (PDAC) is an extremely lethal disease caused by late diagnosis, aggressiveness and lack of effective therapies. Based on its intrinsic heterogeneity, patient stratification models have been correlated according to molecular and metabolic subtypes: the lipogenic subtype is related with a classical molecular signature, whereas glycolytic is associated with an aggressive basal profile. This suggests associations between tumour genetic signature, metabolic profile and aggressiveness. Indeed, based on a pre-defined metabolic PDAC signature, we were able to classify both patient-derived pancreatic xenografts (PDXs) and established cell lines into glycolytic and lipogenic subgroups. The properties of extracellular vesicles (EVs; release into biological fluids, cell-specific content of origin) let us hypothesise that the identification of its metabolic signature in liquid biopsies can be used for the proper stratification of PDAC patients into metabolic subtypes. We have compared different EV isolation techniques corresponding to glycolytic and lipogenic metabolic profiles, to subsequently characterize enzyme and metabolite content. We have succeeded in obtaining sufficient EVs by ultracentrifugation, the combination of ultrafiltration and immunoprecipitation, CD9 affinity column, and SEC from both supernatants of tumor cells in culture and blood extracted from mice with orthotopic pancreatic tumors. Moreover, we have classified exosome isolation techniques according to their specificity, quantity, phenotype and grouping of EVs obtained and downstream applications, based on our own results. Furthermore, we have studied the uptake and internalization of EVs in pancreatic cancer cell lines and in cells from the tumor microenvironment to confirm their role in communication. Finally, we have showed that enzymes and metabolites useful for the proper classification can be detected in EVs. We have additionally performed metabolomic analyses of cell lines and EVs to study the correspondence of both subtypes. While the results are preliminary, they open the possibility of achieving a better clinical management of this deadly disease.

Unveiling the Potential of Tumour-Derived Exosomes in Ewing Sarcoma: Implications for Dynamic Cell-Cell Communication and Tumour Spreading

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Ewing sarcoma is a rare, highly aggressive cancer primarily affecting osseous tissue. It is distinguished by the presence of chimeric transcription factors, notably EWSR1::FLI1, which assume a pivotal role in tumorigenesis. Perturbations in EWSR1::FLI1 expression levels have been proposed to modulate the cellular phenotype. EWS::FLI1high cells exhibit a more proliferative profile, while EWS::FLI1low cells display enhanced metastatic properties.

Tumour-derived exosomes (TDEs) possess the capacity to transport and deliver various biomolecules to recipient cells, thereby exerting an influence on their behaviour. These TDEs not only impact the tumorigenic cells themselves, contributing to the establishment of a highly proliferative and metastatic tumour but also exert an influence on non-tumorigenic cells within the microenvironment and the pre-metastatic niche. We hypothesized that each EWS::FLI1 profile, EWS::FLI1high/low, will be characterized by a distinct TDE secretion pattern. Our work aims to identify which are the key molecules that could govern these dynamic processes to

better understand how the tumour behaves.

To characterize the composition of these TDEs, we employed the A673/TR/shEF cell model of Ewing sarcoma, wherein EWSR1::FLI1 expression can be down-regulated upon doxycycline stimulation. Proteomic and miRNA-seq analysis were conducted on exosomes isolated from EWSR1::FLI1high and EWS::FLI1low A673/TR/shEF cells. We have also integrated mRNA-seq and miRNA-seq data from cells to search for target genes of the detected miRNAs, calculating the anti-correlation and overrepresentation in miRNA-target databases.
Our findings reveal a marked presence of proteins associated with integrin-mediated cell surface interactions and degradation of the extracellular matrix pathways in the EWS::FLI1low phenotype. Furthermore, 24 differentially expressed miRNAs linked to cell-substrate adhesion, angiogenesis, and osteoblast differentiation were identified between the two conditions.
The comprehensive characterization of TDE content will provide valuable insights into tumour dissemination, thereby facilitating the identification of novel therapeutic targets.

Exploring a multiplexed bead-based flow cytometry assay designed for profiling of CNS-derived extracellular vesicles

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Extracellular vesicles (EVs) originating from the CNS can enter the blood stream and carry molecules characteristic of disease states. Therefore, circulating CNS-derived EVs have the potential to serve as liquid-biopsy markers for early diagnosis and follow-up of neurodegenerative diseases and brain tumors. Monitoring and profiling of CNS-derived EVs using multiparametric analysis would be a major advance for biomarker research. Here, we explored the performance of a multiplex bead-based flow-cytometry assay (EV Neuro) for semi-quantitative detection of CNS-derived EVs in body fluids.

EVs were separated from culture of glioblastoma cell lines (LN18, LN229, NCH82) and primary human astrocytes and measured at different input amounts in the EV Neuro assay. In addition, EVs were separated from blood samples of small cohorts of glioblastoma (GB), multiple sclerosis (MS) and Alzheimer's disease patients as well

as healthy controls (HC) and subjected to the EV Neuro assay. Glioblastoma cell lines and primary human astrocytes showed characteristic EV profiles with enhanced signal intensities at increased EV input. Blood-derived EV marker profiles in patients and healthy controls were largely similar. Notably, data normalization revealed individual EV populations that were significantly increased in disease, such as CD36+EVs in glioblastoma and GALC+EVs in multiple sclerosis. tSNE and heatmap clustering analysis separated GB patients from HC, but not MS patients from HC. Correlation analysis revealed a potential association of CD107a+EVs with neurofilament levels in blood of MS patients and HC.

In conclusion, the EV Neuro assay can be used for semi-quantitative profiling and phenotyping of EVs in complex sample material. High effect sizes and large sample cohorts are required to obtain reliable statistical results in cross-sectional biomarker studies. Nevertheless, the study shows that initial EV-associated biomarker discovery and monitoring of circulating EV profiles in CNS disease states are feasible using the EV Neuro assay.

Challenges of scalable production and effective purification of mesenchymal stromal/stem cell derived extracellular vesicles for clinical application

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The clinical potential of extracellular vesicles (EVs) derived from mesenchymal stromal/stem cells (MSCs) necessitates overcoming formidable production and purification obstacles. Ensuring a smooth transition from research to clinical implementation entails standardized production and optimization, as well as specific medium requirements. Our aim is to identify a low protein content MSC medium that is animal-component-free and lacks primary human components, but still preserves the therapeutic function of obtained EV products.

The establishment of clonally immortalized MSCs (ciMSCs) represents a significant advancement that opens up new avenues for standardized processes and products. In terms of identifying a suitable MSC medium, the growth and EV secretion performance of ciMSCs was analyzed in different cell culture media, including DMEM media supplemented with differing concentrations of human platelet lysate (hPL) as well as commercially serum-/xeno-free or chemically defined media. Expansion in hPL-free and chemically defined media turned out to require higher seeding densities, particular detachment agents, and plastic wares with specialized surfaces.

ciMSCs could be expanded in DMEM supplemented with hPL down to 1 %. However, compared to DMEM supplemented with 2.5% hPL, the population doubling time was clearly increased. The ciMSCs also exhibited consistent expandability in serum-/xeno-free and in chemically defined media. However, preliminary results imply that in hPL-reduced/-free media significantly less EVs are secreted than in regular media.

Despite the reduced ciMSC-EV secretion, the therapeutic potency of resulting EVs remains to be investigated. After defining an appropriate MSC medium, future work will focus on refining downstream processing procedures for MSC-EV production, aligning with Good Manufacturing Practice standards, to facilitate the clinical application of MSC-EVs.

EVs as Biomarkers for Monitoring Response to Radiotherapy

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Each year, 600,000 people are diagnosed with squamous cell carcinoma of the head and neck (HNSCC). Although radiotherapy is a promising treatment option for HNSCC, patients can develop radiation resistance, resulting in a poor prognosis. Therefore, biomarkers to monitor radio-response obtained through non-invasive liquid biopsy would allow for the identification of the appropriate patient group that would benefit the most from this therapeutic approach, personalizing the radiotherapy while minimizing its side effects.

Extracellular vesicles (EVs) such as exosomes recently emerged as a promising candidates for this application since an increasing number of studies have shown that radiotherapy can increase exosome secretion and alter exosome cargo. Exosomes also play a role in immunomodulation and hypoxia-induced radioresistance development, as well as cell-to-cell communication and the activation of numerous signaling pathways. Taken together, exosomes and their content hold great promise for clinical application for radiotherapy response.

In the current project, we aim to isolate and characterize EVs from a cohort of HNSCC patients as well as cell lines to identify novel markers of radiotherapy response and better understand the role if EVs in radiotherapy. Here we will present preliminary results whereby we have established methods of isolation and characterization of EVs. Currently, we aim to assess their protein and microRNA content to identify novel biomarkers of radiotherapy response in a longitudinal manner. In parallel, we aim to assess the effects of radiotherapy on EV content in vitro and how radiotherapy derived EVs can interact with other cell types such as non-irradiated cancer cells, healthy and immune cells through co-culturing and immunofluorescence.

Taken together, we aim to both identify novel markers which have both clinical and biological relevance in HNSCC.

Exploration of novel EV-associated transcriptional biomarker candidates for early chemotherapy response prediction in cancer cells

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Cancer patients ineligible for surgery are typically treated with systemic chemotherapy or targeted therapy. However, there is a substantial portion of patients for each therapy who do not respond to it. Since the therapeutic success is generally monitored via imaging techniques, it takes time before non-responders are identified and the tumor can grow further or even metastasize before a different drug is chosen. Therefore, we aimed to identify a candidate biomarker signature of extracellular transcripts in vitro, which could enable rapid assessment of success or failure of therapy from liquid biopsy samples. Several publications have described tremendous changes in cellular gene expression upon exposure of breast cancer cell lines to chemotherapeuticals (Izbicka et al., Cancer Genomics & Proteomics 2005; Los et al., Cytometry 2002). However, to our best knowledge no study assessed whether those changes can also be measured in circulating extracellular RNA.

We treated two different breast cancer cell lines (MCF-7: luminal-A; BT-474: luminal-B) in triplicates with three doses (0 to 1000 nM) of two commonly prescribed therapeutics. Previous studies, including the PRISM drug repurposing resource (https://depmap.org/repurposing), indicate that the two cell lines are susceptible to paclitaxel and resistant to fulvestrant. After assessing cellular viability as a measure for treatment response, we analyzed the expression change in both cellular and cell free RNA using RNA-Seq and RT-qPCR. Furthermore, we compared two different methods (ultracentrifugation and PEG precipitation) for cell-free RNA isolation from the supernatant, and characterized the size, number, distribution, and protein markers of extracellular vesicles in the output of both methods.

Through evaluation of the dose-dependent RNA-Expression compared to a non-treatment control, we identified a specific EV-associated transcriptional biomarker signature for each cell line that significantly predicted the response to each therapy in vitro, making this study an important step towards assessing chemotherapeutic success in liquid biopsies.

Small Extracellular Vesicles as Biomarker for Myocardial Infarction with Non-Obstructive Coronary Arteries (MINOCA)

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Patients with a myocardial infarction with non-obstructive coronary arteries (MINOCA) suffer from many small and scattered microinfarctions that cause severe cardiac damage due to omitted oxygen supply. These occlusions are not visible by routine cardiac catheterization examination. Instead, time-consuming imaging procedures are necessary, which delay the appropriate therapeutic treatment. As a result, MINOCA is associated with a high mortality rate and a high risk for serious complications. The urgent need for innovative diagnostic tools is the motivation of the current study that aims to evaluate the potential of small extracellular vesicles (EVs) to identify MINOCA at an early stage. Therefore, we used a novel animal model that mimics all clinically relevant aspects of MINOCA by autologous micro-thrombo-embolization. Additionally, an animal control group was included that mirrors a classical myocardial infarction (MI) by balloon occlusion of the coronary arteries. EVs were isolated by ultracentrifugation from serum collected before (baseline), 120 and 360 minutes after induction of MINOCA or MI, respectively. Our preliminary results indicate that protein concentrations of EVs are enhanced upon induction of MINOCA as well as MI as compared to their respective baseline levels. We were also able to observe in all groups the spheroid-like shape of EVs by transmission electron microscopy. In future, we plan to examine the obtained EVs in further detail for their concentration, size, and transported cargo by methods like nanoparticle tracking analysis, flow cytometry, or mass spectrometry. Hopefully, certain molecules, so-called biomarkers, will be identified that enables an early and non-invasive diagnosis of MINOCA.

Forming of a Protein Corona on Extracellular Vesicles increases Uptake into Immune Cells

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The influence of a protein corona on the uptake of nanoparticles in cells has been demonstrated in various publications over the last years. Extracellular vesicles (EVs), which are natural nanoparticles, attract growing attention as therapeutically effective nanoparticles and drug carriers for different diseases. Similar to nanoparticles, EVs acquire a protein corona upon contact with biological fluids that influences their uptake by target cells. For nanoparticles and EVs, the protein corona has a crucial impact on drug delivery and therapeutic efficacy. Here, we compare the influence of a protein corona on EVs to engineered liposomes as a synthetic nanoparticle analogue often applied as drug carrier. Therefore, we use a proteomic approach in order to analyze the protein composition of the EVs themselves and the protein composition of a human blood plasma protein corona around EVs. Moreover, we analyze the influence of the protein corona on EV uptake into human monocytes and compare it with the influence on the uptake of engineered liposomes. We show that the adsorption of a protein corona increases the uptake of EVs in human monocytes. This can be attributed to the presence of complement system proteins in the protein corona. Our results demonstrate the relevance of the protein corona for EV uptake, which will aid their use in therapeutic applications.

A comparative analysis of circulating tumor DNA in extracellular vesicles and circulating cell-free DNA in non-small cell lung cancer

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Liquid biopsy has emerged as a promising approach for detecting genetic alterations in cancer patients and guiding treatment decisions. Although conventionally considered as a single analytic compartment, circulating tumour DNA (ctDNA) has been detected both as cell-free DNA (cfDNA) and as cargo of extracellular vesicles (EVs). However, the clinical value of both sources for ctDNA detection is still debated. This study aimed to compare the performance of EV-DNA and cfDNA in detecting tumour-derived KRAS mutations in plasma of non-small cell lung cancer (NSCLC) patients.

Small and large (sEV, lEV) EVs were isolated from NSCLC cell lines and patient plasma using differential ultracentrifugation. Both EV subpopulations were characterized using electron microscopy, nanoparticle tracking analysis, and immunoblotting and differed in size and cargo. To isolate EV-DNA, two DNA isolation kits were compared and showed differences in DNA yield. Based on the results, we developed a protocol to isolate EV-DNA and cfDNA from a single sample. A comparison between EV subpopulations demonstrated higher DNA amounts in lEVs. Treatment with proteinase K and DNase I prior to DNA isolation resulted in a significant reduction in lEV-DNA yield, suggesting that the EV-DNA is at least partly associated with protein complexes on the vesicle surface. To determine the minimum number of tumour EVs required for detection of the KRAS-G12C mutation, a spike-in of healthy plasma samples with different concentrations of EVs from KRAS wild-type or mutant NSCLC cell lines was conducted and KRAS was detected by digital PCR. The variant allele fraction of mutKRAS ctDNA was determined in paired EV-DNA and cfDNA samples from NSCLC patients.

This study revealed that comparable amounts of EV-DNA and cfDNA with varying fragment length are present in patient plasma. Further analyses will compare EVs and cfDNA for liquid biopsy-based ctDNA detection with the aim to improve molecular testing in NSCLC.

EV miRNA and protein composition of primary glioblastoma cells changes in 3D organoid models

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Introduction

2D in vitro cultures only insufficiently depict the heterogeneous tumor events in vivo. Tumor organoid models could therefore represent a promising new cell system to study cell processes in the three-dimensional tumor complex. Here, extracellular vesicles (EVs) as an intercellular communication system play a crucial role regarding tumor progression and invasiveness. In our study, we compared the EV miRNA and protein composition of 2D and 3D models of glioblastoma multiforme (GBM), the most common and aggressive brain cancer in adults.

Material & Methods

EVs were obtained from the supernatant of six 2D and 3D primary GBM models by immunoaffinity (IA) or precipitation (PP). EV characterization and quantification was performed by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). EV (CD9, CD63, CD81, TSG101)-, apoptotic body (CYTOC)- and glioblastoma related (CD44, SOX2, C1QA) protein markers were determined by flow cytometry (FCM). EV miRNA expression profiles were analysed via RNA Seq in terms of expression differences, mRNA targets and respective pathways. Moreover, proteomics (LC-MS/MS) of the tumor cell supernatant was performed.

Results

Nine downregulated and three upregulated EV miRNAs in 3D culture compared to 2D culture were found.

Overrepresentation analysis showed that miRNA targets are preliminarily involved in immune/ stress response and cell proliferation regulation. A higher EV concentration released by 3D models of glioblastoma patients compared to 2D models was measured. Proteomics of 3D culture showed various altered proteins, mainly related to the extracellular matrix organization and tumor progression.

Discussion

Our comparison study of 2D versus 3D primary glioblastoma models revealed differences in EV quantity, miRNA-profile and proteome composition. Interestingly, cells of the same patient cultured in the 3D model apparently shed more EVs. Moreover, pathway analysis indicate that particularly immune and extracellular matrix relevant signaling are altered in 3D models, potentially crucial factors for tumor progression in vivo.

Bacterial membrane vesicles as potential biomarkers for bacteremia diagnostics

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Bacteria inhabiting the in- and outside of the human body can either play an innoxious, beneficial as probiotics, or even pathogenic role, e.g. during a sepsis. Recent studies have shown that bacteria can secrete membrane vesicles (bMVs), quite similar to extracellular vesicles (EVs) and might be crucial in microbiome-host interactions. Thus, bMVs can be potentially used as biomarkers for bacteremia diagnostics.

We investigated the composition and functionality of bMVs of two different bacterial species from different culture media (lysogeny broth, tryptic soy broth and RPMI 1640) throughout the different phases of growth (lag-, log- and stationary-phase). The bMVs were isolated by ultracentrifugation and analyzed using nanoparticle tracking analysis, EV flow-cytometry, cryogenic electron microscopy, transmission electron microscopy and mass spectrometry-based proteomics. Moreover, we examined pro-inflammatory cytokines IL-1 and IL-8 in the human monocyte cell line THP-1 by treatment with bMVs.

In both species we observed an increase of particle numbers with longer incubation periods while the morphologies in both transmission and cryogenic electron microscopy were similar at each time point and condition. With EV flow-cytometry similarities of the common bMV markers OmpA+ GroEL- and OmpA- GroEL+ could be observed and we found both consistently expressed as well as unique proteins throughout the treatments. More than 100 bMV related proteins could be identified and could serve as potential new biomarkers, amongst them ribosomal proteins, GroEL, proteins with catalytic functions in e.g. glycolysis or pyruvate conversion and autonomous glycyl radical cofactor grcA.

The choice of medium and incubation time influences bMV protein composition. However, we could identify several consistently expressed proteins which have relevance as potential biomarkers for bacteremia

diagnostics. Moreover, our flow-cytometry results indicate that different bMV subpopulations may be shed and our functional assays imply that bMVs isolated from the different groups retained their functions and lead to comparable cytokine induction.

Cre-loxP reporter tumor model to study EV uptake and its effects

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Introduction: Pancreatic cancer (PC) poses significant challenges due to its elusive symptoms, late detection, and limited treatment options. Extracellular vesicles (EVs) released by pancreatic cancer cells influence the tumor microenvironment (TME) by transferring their cargo to recipient cells. Bcl2-associated anthogene 6 (BAG6) plays a crucial role in determining the characteristics of EVs and their impact on tumor progression. To study the effects of EVs on specific cells in vivo, we utilized a Cre-loxP reporter mouse model of PC.
Material & Methods: Using Bag6-positive and Bag6-deficient tumor cells, we investigated the impact of Bag6 on tumor growth and aggressiveness in a preclinical mouse model of PC. Tumor cells released EVs containing cre mRNA and were transplanted into floxed dtomato reporter mice. By employing immunofluorescence staining and single-cell RNA sequencing, we tracked the uptake of EVs at the individual cell level.
Results: Genetic depletion of Bag6 resulted in accelerated tumor growth and significant alterations in the cellular composition of the TME. We studied the impact of EVs by performing immunofluorescence staining of GFP-positive cells and single-cell transcriptome sequencing. The identification of distinct clusters/populations of GFP-positive cells in both wild-type (WT) and Bag6 knockout (KO) injected animals revealed the influence of Bag6 on the immune cells dynamics and functions within the TME.
Discussion: Our findings provide valuable insights into the role of Bag6 in determining the immune phenotype in PC. Notably, the absence of Bag6 was associated with increased uptake of vesicles and initiation of tumor development. Utilizing both Cre-loxP approach and single-cell sequencing, we elucidated the in vivo transfer of tumor-derived EVs and identified the recipient cells involved. This integrated approach enhances our understanding of the phenotypic features and functional implications induced by EVs in PC.

A Comparative Engineering Study Distinguishing Breast Cancer Exosomes from Normal Exosomes using their Property of Thermal Stability

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Conventional screening methods such as mammogram, usually, fail to detect breast cancer until a late stage. This major problem has pushed scientists to look for other methods such EVs liquid biopsies for the early stage detection of breast cancer.

The presented experimental work succeeded to differentiate between cancer and normal-like exosomes secreted by female breast cancer cells (MCF-7) and normal-like cells (MCF-10A). Using a new approach named Time-Temperature Method (TTM), we demonstrated that distinguishing MCF-7 and MCF-10A exosomes using their property of thermal stability is very possible with a P-value less-than 0.001. Our experimental design and hypothesis testing subjected both exosomes (MCF-7 and MCF-10A) under an extreme temperature condition of 57°C for 24 hours. Unexpectedly, the lab results showed that the smaller size cancer exosomes (MCF-7) exhibited a higher thermal stability (less degradation level) than the larger size normal-like exosomes (MCF-10A). Moreover, our engineering thermal design revealed a new principle called “exosome thermal-enrichment”, where a doped sample (1:1 ratio of MCF-7 and MCF-10A exosomes) can be enriched with MCF-7 exosomes by thermal degradation of the less thermally stable exosomes (MCF-10A) in the mixture. The exact mechanism of exosome thermal degradation is still unknown, however, the study hypothesizes a relationship between the sizes of exosomes and their membrane macromolecules. TTM proved that different populations of exosomes have a distinct thermal stability. The proposed experimental work can be expanded by characterization of heterogeneous circulating exosomes isolated from blood samples of normal and patients with different stages of breast cancer.

By January 2022, there are more than 3.8 million women with a history of breast cancer in the U.S. This recent statistic by the American Cancer Society alerts scientists to work together towards developing a simple, fast, and reliable technology for the early stage detection of breast cancer.

Characterizing extracellular vesicles derived from tumor-associated macrophages in the ovarian cancer microenvironment

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In ovarian cancer (OC), tumor progression and metastasis are exacerbated by ascites, which centrally contributes to the tumor microenvironment (TME). Tumor-associated macrophages (TAMs) represent a prominent immune cell type in ascites and promote cancer progression. Beyond tumor cells and TAMs, ascites fluid has also been found highly enriched in soluble factors, as well as extracellular vesicles (EVs). To what extend TAMs contribute to the EV component of the TME, remains, however, unclear.

To tackle this question, monocyte-derived macrophages (MDMs) from human peripheral blood were polarized in vitro into classically (M1-like) and alternatively activated (M2-like) MDMs, as well as into TAM-like cells, using incubation in ascites. In parallel, ex vivo TAMs isolated from OC patient ascites were also analyzed. For all cell types, EVs were isolated from conditioned media (serum-free; 24h) and characterized by state-of-the art methods including nano flow cytometry (nFCM) and mass spectrometry-based proteomics.

The analyses revealed particle release rate and median EV size as comparable between TAM-like and M1-like samples, which was in marked contrast to M2-like cells, which were unexpectedly found to prolifically release EVs. Additionally, single-EV fluorescence analyses identified a higher frequency of CD63-positive EVs from M2-like, as compared to M1- and TAM-like preparations. Moreover, TAMs presented with a decreased

N-glycosylation level in CD63 protein, concomitant with a decrease in EV release, diverging EV biogenesis markers, as well as EV-associated proteome.

Taken together, our data suggest that despite the immunosuppressive cellular phenotype displayed by TAMs and their pro-inflammatory secretory profile supporting tumor progression, EVs released by them display traits setting them apart from those classical macrophage subtypes. Our study thus provides new insight into EV biogenesis in TAMs in the context of OC and the ambiguous contribution of these cells to the TME.

EphrinB3 acts as a by-pass driver in mutated epidermal growth receptor-driven Non-small cell lung cancer treatments and is loaded in extracellular vesicles.

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Background: Extracellular vesicles (EVs) are released by all cell types, have been associated with several cancer hallmarks and may provide information about tumor progression or response to therapy. In this work, we explored EVs and their protein cargo released from mutated epidermal growth factor receptor (EGFR)-driven non-small cell lung cancer (NSCLC) cells prior and post EGFR-tyrosine kinase inhibitors (TKI) erlotinib or osimertinib. We also evaluated the EphrinB3/EphA2 signaling cascade as a bypass resistance driver in this context. Methods: EphrinB3 silencing in EGFR mutant cell line H1975 and its effect on EGFR-TKIs sensitivity were studied in clonogenic assays. EVs were isolated from the H1975 cell line and an osimertinib resistant subline H1975/OR in response to osimertinib using size exclusion chromatography (SEC). EVs characterization

was performed by western blot and Nano Tracking Analysis (NTA). Proteomic profiling was made using Proximity Extension Assays (PEA) and Mass Spectrometry (MS). Results: We found that H1975 cells were sensitized to erlotinib and osimertinib when EphrinB3 was silenced. Both EphrinB3 and EphA2 were confirmed in EVs post TKI treatment. Protein profile by PEA analysis showed differentially expressed proteins when comparing H1975 cell line as a baseline against osimertinib and H1975/OR with or without osimertinib. HGF and FGF-BP1 were upregulated in EVs from cell line H1975/OR and H1975/OR with osimertinib treatment. These are currently validated and explored in context of EphrinB3 and EphA2 signaling. Summary: Our data suggest that the EphrinB3/EphA2 axis could act as a pro-survival signaling cascade. EphrinB3 and EphA2 are loaded in the EVs, suggesting a possible effect on recipient cells which needs further verification. In response to osimertinib, H1975 and H1975/OR showed an increase in EVs release which have different protein profile expression which could be further explored as treatment monitoring biomarkers.

‘Sweet EV’: Tracing different glucose carbon isotopic labeling patterns in EVs versus source breast cancer cells

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Metabolic rewiring is an important hallmark of cancer. In addition to supplying building blocks for biomass and cell growth, metabolites can also act as signaling molecules and thereby affecting gene and protein expression or influence the surrounding cells in the tumor microenvironment. Extracellular vesicles (EVs) are key mediators of intercellular communication at both local and distal proximity. EVs elicit their versatile biological roles by trafficking complex molecules, including DNA, RNA, proteins, lipids and metabolites. It has been recognized that tumor derived-EVs play an important role in in tumor progression, and it is likely that metabolic rewiring of tumor cells results in different EV metabolite cargo. Previously, proteomic profiles of EVs were reported to differentiate breast cancer (BC) subtypes, indicating that EV-associated cargos could have biomarker potential. Yet, which metabolic features are present and how they are incorporated in BC-derived EVs remain unanswered. Here, we investigated the metabolic contents of EVs secreted by different BC cell lines. Using stable isotope 13C6-glucose tracer, we compared metabolic pathway activity of EV-parental cells and EV metabolite cargo. As expected, metabolic flux analyses revealed that the different BC cell lines have unique metabolic rewiring patterns in multiple metabolic pathways, including amino acid, nucleotide, glycolysis, TCA cycle and lipids. Moreover, this metabolic rewiring in BC cell lines resulted in different metabolic compositions in EV’s derived from these BC cell lines. More important, dissimilar isotopic enrichment patterns were evident within EVs and their parental BC cell line, suggesting a selective loading of metabolites. Collectively, our results revealed unique glucose downstream metabolic cascade in different BC subtypes, and their diverging shuttling into EVs. This work provides novel insights into metabolic features in EVs and their producer cells, and highlights the prospect of EV fluxomics as footprints for fundamental cancer-EV metabolism research.

Analysis of plasma-isolated extracellular vesicles – a way to predict treatment response in immune checkpoint inhibitor treated metastatic non-small cell lung cancer patients?

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Background: Liquid biopsies for non-invasive analysis of biomarkers (BMs) are highly needed for monitoring treatment response in metastatic non-small cell lung cancer (NSCLC) patients. One way is to analyse small extra cellular vesicles (sEVs) released into blood prior and post therapy. sEVs contain RNA, miRNA, DNA and proteins

partly reflecting their cell of origin and are important communicators for tumors with their surroundings. We have isolated sEVs prior immune checkpoint inhibitor (ICI) treatment. Our aims were to identify protein BM signatures related to treatment response and to PD1 or PD-L1 expression in sEVs.
Material and Methods: sEVs were isolated from 0.5-1 ml EDTA plasma of NSCLC patients (n~80) prior treatment with ICI, alone or combined with chemotherapy. The Izon's qEVoriginal gen 2, 70nm columns were used for EV isolation and particle concentration and size examined by Nanoparticle Tracking Analysis (NTA). Proteins were profiled using proximity extension assay (PEA), on the Immuno-Oncology® and Oncology II® panels. Qlucore® Omics Explorer was used for data analysis and visualization. Characterization of sEV markers and validation of putative protein BM profiles was done by Western blotting and ELISA.

Results: The median size of sEVs was around 100 nm with concentrations from 1.5x 1010 to 3.6x1011 particles/ml plasma. The sEVs expressed the exosome markers CD9 and TSG101. PEA protein profiling showed that both oncogenic- and immune signaling proteins were present with heterogenous expression in individual patient samples. Protein signatures that correlated to genomic alterations of the tumor, PD-L1 expression level, survival- and treatment response were evaluated. We also explored the PD1 and PD-L1-associated protein cargo.

Conclusion: We found that sEVs isolated from small amounts of EDTA-plasma are possible sources of BMs. Multiple protein signatures rather than individual protein profiling may allow for non-invasive BM profiling of NSCLC patients when given ICI alone or combined with chemotherapy.

Exploring the role of Rab3 in exosome release from prostate cancer cells

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Introduction

Exosomes are extracellular vesicles (EVs) released by cells upon fusion of multivesicular bodies with the plasma membrane. However, the mechanisms underlying the secretion of exosomes are still unclear. Rab proteins play important roles in vesicular membrane trafficking along the endocytic and the secretory pathway. In particular, Rab3 has been involved in cellular secretion, and specifically Rab3D and Rab3B have been found to be enriched in urinary exosomes from prostate cancer patients compared to healthy males. In this study we investigated the role of Rab3 isoforms in exosome release in prostate cancer cells.

Methods

The role of Rab3A, Rab3B and Rab3D, in exosome release was investigated by siRNA depletion from the highly metastatic cancer cell line PC-3. Knockdown efficiency was verified by western blot (WB) and specificity by qPCR. Small extracellular vesicles (sEVs) were then isolated by differential centrifugation from cell media and analyzed by nanoparticle tracking analysis (NTA) and WB. Moreover, confocal microscopy was used to investigate potential alteration of the endo-lysosomal system in depleted cells. Alterations in other cellular pathways was investigated by following the trafficking of the toxin ricin.

Results

Our results indicate that depletion of Rab3D or Rab3B, but not Rab3A, in PC-3 cells increase the release of sEVs enriched in exosomal markers such as Alix and Syntenin. Moreover, Rab3 depletion did not affect sEV size distribution, as shown by NTA. No significant changes in the transport of externally added ricin to the Golgi apparatus and cytosol were shown, suggesting that Rab3 is mainly associated with secretory processes.

Summary/Conclusion

This study reveals that Rab3D and RabB depletion in prostate cancer cells may play a role in exosome secretion, but further experiments are required to verify the endosomal origin of these sEVs.

THE USE OF EV MEMBRANE PROTEINS AS TARGETS FOR CAPTURING URINARY EVs IN NOVEL THERMAL LATERAL FLOW IMMUNOSENSORS

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According to the global cancer observatory, approximately 1.4 million new cases of prostate cancer (PCa) and 375.000 related-deaths were registered worldwide in 2020. Due to this, the development of sensitive, versatile and robust biosensors for differentiating between different stages of PCa represents an urgent need in the management of this disease. The fact that extracellular vesicles (EVs) are a molecular fingerprint of the cells of origin and that are present in biofluids like urine, makes EVs a very interesting source of biomarkers for PCa diagnosis and follow-up.

Currently, working with EVs involves in many cases their purification and characterization through time consuming and relatively inefficient techniques. A relatively unexplored method for testing EVs is the lateral flow immunoassay (LFIA), which is particularly useful as a point-of-care diagnostic tool. In this work, we propose

the development of an ultrasensitive LFIA biosensor based on the use of gold nanoprisms (AuNPrs) as thermal labels. This method takes advantage of the interesting optical property of AuNPrs that allows them to transform light into thermal energy when they are irradiated with a light source.

For the development of the thermal LFIA, pooled EVs purified from healthy donor's urine were used. The biosensor was optimized using anti-CD63 immobilized on a nitrocellulose strip as capture antibody and anti-CD9 coated AuNPrs as the detection system. Then, anti-CD9 coated AuNPrs were replaced with anti-PSMA coated AuNPrs to detect EVs isolated from urine samples of PCa patients. By irradiating the AuNPrs with a laser, we have been able to detect amounts of EVs from PCa patients lower than 10e7 EVs/ml, thus showing than thermal LFIA have a higher sensitivity compared to classical LFIA. Finally, we were able to differentiate between pools of urinary EVs derived from healthy donors and PCa patients using this assay.

Isolation and Analysis of Extracellular Vesicles for the Evaluation of Microsatellite Instability in Endometrial Cancer

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As the most common gynecological cancer, and with an estimated incidence rise of >50% worldwide by 2040, endometrial cancer (EC) is in dire need of better platforms for inexpensive, early-stage diagnosis and therapy selection [1]. Immunotherapy has impacted EC showing an increase in survival rate, but only in a small percentage of treated patients [2]. The selection of this sub-group of responders is complicated due to the lack of reliable biomarkers. However, microsatellite instability (MSI) has been shown to be a good candidate for immunotherapy selection [3]. MSI is typically characterized by accumulated deletions in short, repetitive sequences of the DNA. Since evaluation of MSI requires cancer-originated genetic material, extracellular vesicles (EVs) shed by the tumor can be used to extract this valuable genetic cargo in a minimally-invasive way [4]. EVs are a diverse group of membrane-bound phospholipid vesicles used for intercellular communication, and can be found in most body fluids, including liquid biopsies. In this work, we explore a glycosaminoglycan-focused chemical assay (ExoGAG, Nasasbiotech S.L.) to collect EVs from plasma, as the complex formed between EV-ExoGAG allows for a simple centrifugation step to enrich for all particles. A microfluidic concentrator is also implemented to improve enrichment and collect EVs for further analysis. Such analysis will be performed using a biosensor based on surface-enhanced Raman scattering (SERS), a single-molecule sensitive technique, to perform MSI assessment from EV-derived DNA.

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Enhancing Brain Organoid Growth through Electrical Stimulation-Induced Amplification of Extracellular Vesicles

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Traditionally, electrical properties of the cells were only considered in the context of electroactive cells and action potential. However, recent works show the significant role of low intensity ionic currents and plasma membrane voltage in governing vital biological functions of cells. Previously we showed the role of exogenous electrical stimulation (ES) in triggering regenerative responses in mesenchymal stem cells, such as proliferation, differentiation and migration. The effects of ES on cell behavior have links to alteration of Vmem, calcium influx and lipid rafts, which are also key players in the regulation of cell secretions and biogenesis of extracellular vesicles (EVs).

In this study, for the first time we demonstrated that low intensity electrical pulses in frequency range of 1-50 Hz and intensity of 25 mV/mm, significantly increases the number of EVs secreted from human adipose tissue derived mesenchymal stem cells (ASCs). The morphology and size of EVs produced by ES remain unchanged. We demonstrated that the number of particles secreted has a relation with ES parameters. We tested the effect of EVs from electrically stimulated ASCs (ES-EVs) on acceleration of brain organoid growth and compared the results with EVs from non-stimulated cells (CTRL-EVs). We found that human brain organoids treated with ES-EVs demonstrate ~1.5 times larger area compared to those treated with CTRL-EVs after 1 week. Moreover, the proliferation marker, Ki76, and PHH3, the mitosis marker, are significantly more expressed in organoids treated by ES-EVs compared to CTRL-EVs.

Our results suggest that ES could be a promising novel tool for EVs engineering to increase the yield of production and control the cargo of therapeutic EVs. ES can be incorporated in bioreactors for boosting EVs production and fine-tuned for boosting desired therapeutic effects. In addition, ES-EVs can be a relevant method for accelerating the growth and/or maturation of brain organoids.

The proteome of extracellular vesicles in DLBCL plasma patients is associated to the immune response and signaling transduction

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Diffuse Large B Cell Lymphoma prognosis in the clinic relies on immunohistochemistry and attempts to develop multiple immunohistochemistry-based algorithms suffered from lower than desired agreement rates with gene expression profiling classifications. Diffuse large B cell lymphoma (DLBCL) is an aggressive B cell lymphoma characterized by a heterogeneous behavior. Extracellular vesicles are secreted by all cell types and are currently established to some extent as representatives of the cell of origin. Extracellular vesicles in blood circulation have been demonstrated as proxy of an individual health status. The present study characterized and evaluated the diagnostic and prognostic potential of plasma extracellular vesicles (EVs) proteome in DLBCL by using state-of-the-art mass spectrometry. The EV proteome is strongly affected by DLBCL status, with multiple proteins uniquely identified in the plasma of DLBCL. A proof-of-concept classifier resulted in highly accurate classification with a sensitivity and specificity of 1 when tested on the holdout test data set. On the other hand, EV proteome differences from non-germinal center B-cell like (non-GCB) and GCB subtypes was more subtle. Functional analysis suggested that antigen binding is regulated when comparing non-GCB and GCB. Survival analysis based on protein quantitative values and clinical parameters identified multiple EV proteins as significantly correlated to survival, such as, COROA1 and PSMB2.

Lipidomic analysis of hypoxic small extracellular vesicles from head and neck squamous cell carcinoma cells revealed upregulation of glycerophospholipids

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Solid tumors, including head and neck squamous cell carcinomas (HNSCCs), characterize areas of hypoxia. Our previous study demonstrated that HNSCC cell lines exposed to hypoxia released increased number of small extracellular vesicles (sEVs) with altered proteomic cargo. Here, we compared the lipidomic profile of sEVs released from cells cultures in normoxic and hypoxic conditions.

We collected sEVs from supernatants of HNSCC cells (PCI-30) and normal control cells (HaCaT keratinocytes) exposed to 21 % (normoxia) and 1 % (hypoxia) oxygen supply. sEVs were isolated using size exclusion chromatography (SEC) and characterized by nanoparticle tracking analysis, electron microscopy, immunoblotting, high-resolution mass spectrometry. Expression profiles of lipidomic gene signatures were compared between a total of 522 cases of primary HNSCC and 44 normal control samples obtained from the Cancer Genome Atlas (TCGA).

Analyzed sEVs had 125–135 nm of diameter averagely, carried CD63, CD9 but not Grp94. 815 and 735 lipids were significantly dysregulated in hypoxic tumor-derived sEVs (TsEVs) in compared to normal counterparts exposed to hypoxia, and to normoxic TsEVs, respectively. 236 lipids, common for both datasets, and thus sophisticated for hypoxic TsEVs, were represented mostly by glycerophospholipids (GPs). Hypoxia determined distribution of 281 unique lipids in TsEVs in compared to normal, including 104 differed also from normoxic TsEVs lipidome. Adaptation of HNSCC cells to hypoxia was associated mostly with enrichment in number and intensity of GPs, including unique, and with upregulation of phosphatidic acid. Transcriptomic validation revealed, that expression levels of genes involved in the catabolism of GPs significantly correlated with hypoxia-, angiogenesis- and sEV secretion-related genes in the TCGA HNSCC cohort, as well as with poorer survival of HNSCC patients.

Adaptation to hypoxia revealed plasticity of lipid profile in HNSCC-derived sEVs and thus, sEV-associated lipids may emerge as clinical biomarkers for tumor progression or signature of tissue hypoxia in HNSCC.

Immortalized and primary adipose tissue- derived mesenchymal stem/ stromal cells as a source of extracellular vesicles for tissue repair – comparative study

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Background: Mesenchymal stem/ stromal cells (MSCs) represent one of the most commonly used cells for therapeutic applications. Extracellular vesicles (EVs) released by stem cells including MSCs are considered as cell-free therapy products in medical applications, e.g., in cardiovascular diseases currently representing a leading cause of death worldwide. However, progressive senescence of human primary MSCs (priMSCs) is one of the major obstacles in harvesting sufficient amounts of their EVs for therapeutic applications. Thus, the aim of the study was to examine immortalised MSCs (imMSCs) as a potential alternative source of clinically relevant EVs (MSCs-EVs) for future applications.

Methods: Comparative assessment of basic biological properties: morphology, viability, proliferation rate, antigenic profile, and trilineage differentiation capacity, was performed on priMSCs and imMSCs in vitro. Comparative analyses of basic properties of EVs secreted by those two types of cells were also performed.

Results: Our results show that imMSCs exhibit similar biological properties such as morphology and trilineage differentiation capacity as priMSCs. However, imMSCs possess higher proliferation rate and negligible senescence during prolonged culture, when compared to priMSCs, which allows for their long-term expansion required for abundant EV collection. Our also data demonstrated that MSC-EVs secreted by both types of cells have similar properties. However, imMSCs secrete significantly higher amount of EVs compared to priMSCs.

Conclusion: Our data show that imMSCs retain their mesenchymal identity compared to priMSCs. However, imMSCs exhibit higher proliferation rate, lack of senescence, and importantly secrete higher amount of EVs comparing to priMSCs, which suggest that imMSCs may be a promising source of EVs for future biomedical applications.

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Actin rearrangement reduces CD20 and ICAM1 antigens on tumor-derived EVs and improves lymphoma immunotherapy.

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We discovered that salinomycin (SAL), previously shown to eradicate breast cancer stem cells, strongly upregulates the surface levels of CD20 antigen on B-cell-derived malignancies, including a variety of Burkitt's lymphoma and diffuse large B-cell lymphoma (DLBCL) cell lines as well as chronic lymphocytic leukemia (CLL) patients-derived primary malignant cells.

We, therefore, tested the hypothesis that SAL can be particularly beneficial when combined with anti-CD20 monoclonal antibodies for the improvement of the B-cell non-Hodgkin lymphoma therapy. Indeed, the efficacy of the therapeutic anti-CD20 antibody, rituximab, was significantly increased by SAL, both in vitro and in a xenograft mouse model.

The extracellular vesicles (EVs) deriving from the tumor cells of B-cell origin carry the CD20 antigen and therefore limit the efficacy of rituximab toward malignant cells. Surprisingly, the EVs deriving from SAL-treated tumor cells carried reduced levels of CD20 antigen and exhibited a less negative effect on rituximab efficacy. Additionally, on such EVs, the level of ICAM1 (antigen required for NK cell adhesion to tumor cells) was also decreased.

To understand the molecular mechanisms, we performed a proteomic analysis of EVs deriving from SAL-treated tumor cells. The analysis revealed a dramatic drop in the levels of actin and proteins involved in actin nucleation and reorganization, such as WAVE2, cofilin, ezrin, and moesin. The drop in levels of b-actin and cofilin was detected only in EVs but not in total cell extracts and correlated well with the reduction in levels of CD20 and ICAM1 antigens.

In light of our discoveries, it becomes reasonable to consider salinomycin as a promising drug candidate to combine with therapeutic anti-CD20 antibodies in the future treatment of B-cell non-Hodgkin lymphoma.

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Inactivation of EWSR1-FLI1 oncogene characteristic of Ewing sarcoma using Therapeutic Extracellular Vesicles (TEVs).

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Ewing sarcoma is a rare and aggressive bone cancer affecting children and young adults. These tumours are characterised by chromosomal translocations resulting in chimeric transcription factors (i.e. EWSR1-FLI1) that govern the tumorigenesis process. Previous studies carried out in our group have demonstrated that EWSR1-FLI1 gene inactivation using CRISPR-Cas9 tools produced cell growth arrest and senescence. However, the delivery of Cas9 machinery to target cells in vivo is a challenge and new approaches are necessary. Extracellular vesicles for Cas9 ribonucleoprotein delivery have emerged as an interesting tool due to their low immunogenicity and versatility. We have generated therapeutic extracellular vesicles (TEVs) containing Cas9 and specific sgRNAs designed to inactive EWSR1-FLI1 using a combination of molecular strategies that include the use of RNA binding proteins, ribozymes and protein dimerization domains. TEVs can be efficiently isolated by filtration and centrifugation from the culture medium of HEK293T cells transfected with the appropriate plasmids. The size and concentration of TEVs were determined by NTA and the amount of Cas9 protein contained in TEVs was quantified by western blot using a Cas9 recombinant protein standard curve. In vitro assays showed that the addition of these TEVs to Ewing sarcoma cell lines (A673, MHHES1 and A4573) produced around 50% gene editing (that is, gene inactivation) at EWSR1-FLI1 gene after 72h demonstrating the effectiveness of this approach. We are improving this delivery system to increase its specificity to target Ewing sarcoma cells for its in vivo application. In conclusion, the use of TEVs to induce gene editing at least in vitro is efficient and would be readily applicable to other pathologies in which gene editing-based therapies could represent a therapeutic option.

Effects of Trophoblast Extracellular Vesicles on D-Galactose Induced Premature Aging of Keratinocytes

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Premature aging of keratinocytes, the predominant cells in the epidermis, is associated with various skin disorders and contributes to the overall aging process. D-galactose-induced aging has been widely used to model premature aging in vitro. Due to the abundance of amino acids, peptides, vitamins, trace elements and growth factors, nutrients and biological active components known to modulate key pathways involved in cellular aging and rejuvenation, placental extracts have a rich, historical precedent in the field of anti-aging skin care and therapy strategies.

Trophoblast extracellular vesicles have demonstrated regenerative and protective properties in various cell types. However, the potential effects of trophoblast extracellular vesicles on D-galactose-induced premature aging of keratinocytes remain unclear.

In this study, HaCaT cells were treated with extracellular vesicles isolated from HTR-8/SVneo cell-conditioned medium, followed by the administration of D-galactose to induce premature aging. Cell morphology and viability, cellular senescence markers, reactive oxygen species production, and migratory properties were evaluated to assess the impact of trophoblast extracellular vesicles on premature keratinocyte senescence.

The results demonstrated that trophoblast extracellular vesicles effectively attenuated D-galactose-induced premature aging in keratinocytes. They also modulated the expression of senescence-associated markers in keratinocytes. Additionally, trophoblast extracellular vesicles exhibited antioxidant and anti-inflammatory activities, promoting cellular resilience against oxidative stress and inflammation associated with premature aging.

In conclusion, this study highlights the potential of trophoblast extracellular vesicles to attenuate keratinocyte senescence and modulate senescence-associated gene expression. The regenerative and protective properties of trophoblast extracellular vesicles make them a promising candidate for developing novel strategies in managing aging-related skin disorders. Further research is warranted to elucidate the specific mechanisms underlying the effects of trophoblast extracellular vesicles on keratinocyte senescence and optimize their therapeutic potential.

Characterisation of extracellular vesicles from mesenchymal stem/stromal cells as new delivery system for functionalized gold nanoparticles

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Functionalized-gold nanoparticles (fGNPs) gained a lot of interest as perspective theranostics agents due to their stability, high loading capacity, oxidation resistance, surface plasmon resonance in NIR-spectra and scaled-up production. However, their pathology-targeting remain challenging, leading to off-target accumulation, lower therapeutic efficacy and potential systemic toxicity. The use of extracellular vesicles (EVs) could overcome these limitations by sequesteration of fGNPs from undesired interactions and improving targeting capabilities. The aim of this study is to characterize EVs from human mesenchymal stem/stromal cells (MSCs) loaded with neutravidin-coated GNPs, as a first step in the development of a novel theranostics system.

MSCs were treated with fGNPs for 24h before rinsing and cultivation in EVs-free medium for 24h. Interleukin (IL)-6 and IL-8 levels were measured in media by ELISA before EVs separation by differential centrifugation. EVs count and size were determined by NTA. EVs markers were assessed by Western blot. Identification of EVs loaded with fGNPs was assessed by transmission electron microscopy (TEM).

MSCs internalized fGNPs and the secretion of IL-6 and IL-8 was altered compared to the untreated MSCs. The concentrations of EVs from control and fGNPs-treated MSCs had average concentration of 7.5x1010 ± 2.1x109 p/mL and 5.5x1010 ± 5.9x109 p/mL and the average sizes were 163.4±1.4 nm and 166.1±06 nm, respectively. Both preparations were positive for CD63 and CD81 markers, with similar Western blot bands. TEM analysis suggested that MSCs are indeed able to release fGNPs within EVs, but also free fGNPs and the ratio between fGNPs-loaded EVs and unloaded-EVs was relatively low.

In conclusion, MSCs are potential producers of fGNPs-loaded EVs. Therefore, in the next step, loading of EVs with GNPs will be further optimized and their functional properties will be assessed to fully exploit a combination of beneficial properties of specific therapeutics, GNPs and MSC EVs for designing novel theranostics tools.

Developing an Extracellular Vesicle RNA Test for Early Detection of Patients at Risk of Aggressive Prostate Cancer

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Prostate cancer (PCa) is one of the most common cancers, accounting for over 23% of cancer in men throughout Europe. While many PCa patients experience indolent, slow growing, tumours some patients have more aggressive tumours that progress rapidly and are therefore clinically significant. Unfortunately, current diagnosis methods mean it is challenging to accurately diagnose patients without using biopsy. There remains an unmet need for the development of non-invasive tests to identify patients at risk of aggressive PCa.

Activation of the prostate stroma to a tumour reactive state is a common feature typically associated with aggressive PCa. But analysis of stromal cell phenotype is not possible without biopsy. We recently reported the utility of extracellular vesicles(EVs), present within patient serum, as a surrogate indicator of the histological nature of the PCa. Here we have built upon our previous work and have further explored the utility of prostate stroma-derived EVs for early detection of aggressive PCa.

PCa-associated stromal cultures were derived from radical prostatectomy tissue specimens obtained from 15 patients. EVs were isolated from cell conditioned media and their RNA cargo was profiled using RNA sequencing. The most abundant EV-associated transcripts were subsequently measured in patients' serum using qPCR to evaluate their diagnostic capability. We identified an RNA panel capable of discriminating patients with high-risk cancer (Gleason score=4+3 and ≥8) from patients with low-risk cancer (Gleason score=6 and 3+4). Furthermore, a selected set of RNA biomarkers were able to predict disease outcome.

In conclusion, our study highlights the utility of serum-derived EVs, and RNA contained within, as a companion diagnostic test, with prognostic capability, for PCa. Such an assay has the potential to limit the need for biopsy therefore improving the lives of men with PCa.

Using extracellular vesicle glycoproteins for early detection of aggressive prostate cancer

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Prostate cancer (PCa) accounts for more than one in four male cancers, killing ~12,000 men per year in the UK. Many patients have slow growing, low-risk tumours. Some patients, however, only present symptoms when the disease is at an aggressive, high-risk, stage. Despite recent advancements in imaging technologies, a lack of diagnostic information, means there remains a need for biopsy. This is invasive, with associated risks, and even when combined with the standard blood test (PSA) still lacks absolute reliability for detection of high-risk disease. Better blood-based tests are therefore required to facilitate early detection of aggressive PCa.

The aim of this project is to develop an assay for detection of extracellular vesicles, present within patient serum, capable of distinguishing between aggressive high-risk PCa and low-risk disease, therefore limiting the need for biopsy. We have previously shown that glycoproteins, present on the EV surface, promote tumour growth in vivo. But the potential of EV-proteoglycans as PCa biomarkers remains unclear.

Here we have tested a panel of 20 lectins for detection of glycoproteins present on either non-cancerous prostate (PNT2 and BPH) or PCa (LNCaP, Du145 and PC3) cell derived EVs. From this, we streamlined our lectin panel to create a multiplex plate-based assay for detection of EVs in patient serum. We have tested our EV assay using archived serum samples (n=150) from patients with low, intermediate, or high-risk localised PCa.

We hope that this approach can, eventually, be translated into the clinic to provide a non-invasive, reliable, blood test for early identification of patients with aggressive PCa. Such a test will provide additional key information during PCa diagnosis, allowing for a more informed management of disease. Furthermore, this assay has the potential to limit the need for invasive biopsy, therefore improving the lives of men with PCa.

Novel EV quantification methods and their use in ageing biomarker discovery

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An ageing population brings many challenges, with the increase in complex multimorbidities being chief among them. While medicine has made considerable advances in the last 50 years in tackling acute illnesses, research is desperately needed to tackle the vast number of chronic conditions such as arthritis, diabetes, and Alzheimer's disease.

The build-up of senescent cells is primarily associated with ageing. The presence of a large percentage of senescent cells and their secretions contributes to many conditions associated with ageing, including arthritis, diabetes, and Alzheimer's disease. While there are methods for the detection and quantification of the percentage of senescent cells in a patient's body, these methods are expensive, time-consuming and error-prone. As such, there is a desperate need for novel biomarkers of senescence, as well as novel detection methods for these biomarkers.

Due to their presence in blood and saliva, EVs are particularly promising for developing novel assays for various pathological conditions, including senescence. Their presence in bodily fluids would allow for easy and relatively non-invasive quantification of diseases, without the need for invasive and expensive biopsies. This would facilitate the clinical translation of therapies targeting senescent cells, and ultimately allow the development of technology to screen for senescence, ensuring that candidates suitable for treatment are identified as soon as possible.

Novel EV quantification methods, including zeta potential analysis and Quartz Crystal Microbalance technology, have proven to be robust EV characterisation methods and have enormous potential for the high throughput trialling of novel EV biomarkers.

Here, we propose to develop novel nanoplasmonic sensors to detect extracellular vesicle-associated biomarkers of senescence in saliva. This technology could be used for drug development and for screening entire populations for the pathogenic accumulation of senescent cells in their body, facilitating the move away from reactive medicine towards beginning interventions before a patient becomes ill.

Transcriptomic analysis of astrocyte origin enriched plasma EVs in Alzheimer's Disease

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Introduction: Dementia is a neurodegenerative brain disorder characterized by cognitive decline. Accurate diagnosis of the various forms of dementia is as important as effective treatments as misdiagnosis can cause problems for individuals with symptoms of psychosis and parkinsonism. Accurate subtyping is therefore essential for safe clinical management. Extracellular vesicles (EVs) are proposed as potential biomarkers for forms of dementia. Because of their potential for transferring biological information, and their ability to act as carriers of biomarkers for specific diseases, EVs are currently a novel avenue to explore for the diagnosis or treatment of dementias.

Method: We isolated RNA from EVs from total plasma and astrocyte derived plasma EVs (GLAST) and neuronal derived EVs in individuals with Alzheimer's disease and performed RNA-sequencing. We identified differentially expressed genes (DEG) and performed functional enrichment analysis. We compared expression in EVs in total plasma EVs versus GLAST astrocyte derived and healthy controls (HC) and AD cases. We also used cryo-TEM imaging for the characterisation of EV in human plasma samples.

Results: Enrichment analysis for AD vs HC total EV group, showed enrichment for GABAergic neuron activity. The AD vs HC Glast EV group showed 5 differentially expressed mitochondrial genes all of which were downregulated. AD Glast EVs and HC Glast EVs also were associated with mitochondria related functions. The AD and HC total EV vs Glast EV group showed enriched genes which are downregulated and relate to microtubule polymerization.

Discussion: Mitochondrial dysfunction has been associated with Alzheimer's disease previously and Aβ accumulation within the mitochondria has been observed within AD brain. Astrocytes convert excessive glutamate via TCA cycle of mitochondria to preserve the neural environment. Therefore, the mitochondria related functions associated with differentially expressed Glast EVs is biologically relevant and is a promising avenue in the development of biomarkers for dementias.

Developing a device for the isolation of ovarian cancer exosomes using nanoporous filteration

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Ovarian cancer is the deadliest gynaecological cancer in women. It is known as the silent killer as ambiguous symptoms delay prompt diagnosis, affecting survival chances. In fact, most cases are diagnosed in advanced stages where a 5-year survival rate is around only 20%. However, early-stage diagnosis can offer over 90% 5-year survival prospect.

The lack of specific and sensitive ovarian cancer molecular markers significantly contributes to late diagnosis.

Cancer-derived exosomes are promising targets for cancer diagnostics. Exosomes (~30-150 nm in diameter) are an extracellular vesicle sub-type released by cells as a form of intercellular communication. Their rich molecular cargo containing proteins, lipids and genetic material stimulate cellular activities within target cells that in turn, contribute to biological processes related to multiple conditions including, cancer. Exosomes are observed to be secreted in higher quantities by tumour cells and the bioactive molecules they carry are specific to the parent cancer cells. This means that, cancer-derived exosomes are readily available targets and accurate to identify novel cancer screening biomarkers. Furthermore, exosomes are released into bodily fluids thus, they are accessible through minimally invasive methods to determine a diagnosis.

A key challenge is towards the isolation of exosomes, which is mostly by conventional techniques for example, ultracentrifugation. These are time-consuming and laborious procedures. Here, we present an approach using a nanoporous membrane in a microfluidic device to isolate exosomes from ovarian cancer cell line OVCAR-3.

The manufacture of a microfluidic device able to harvest exosomes from patient samples is an attractive idea for clinical applications as time and costs for exosome sample preparation would significantly reduce. Additionally, filtered samples can be screened for ovarian cancer exosomes based on their specific biomarkers. As an ovarian cancer screening tool, this device would offer more prompt patient care and improve survival probabilities.

Identification of Pro-resolutive ω3 Oxylipins in Human Milk Extracellular Vesicles for Potential Therapeutical Application in Inflammatory Bowel Diseases

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Human milk (HM) is the most important nutrient for the feeding of premature infants (PIs) and has been shown to prevent common severe diseases associated with prematurity including necrotizing enterocolitis (NEC). Recent works reported the relevance of human milk derived extracellular vesicles (HM-EVs) in the oral treatment of different inflammatory bowel disease (IBD). Most of these studies have focused on the capacity of EVs to transport proteins and miRNAs known for their pro-resolutive effects, facilitating their delivery through the intestinal tract. However, the content of lipid mediators is less well studied. Oxylipins and Specialized Pro-Resolving Mediators (SPMs) enhances the host defense, by resolving the inflammation and tissue repair, in particular concerning the derivatives of the omega (ω)-3 docosahexaenoic acid (DHA). In this work we wanted to study the presence of oxylipins in HM-EVs and to explore their therapeutic effects in intestinal disorders. We detected higher amount of several oxylipins within the HM-EVs than in the HM matrix, namely 14-HDHA, 17-HDHA and 19,20-DiHDPA (ω3 oxylipins). Through in vitro experiments, we demonstrated that a cocktail of these three molecules was as effective as EVs conferring protection to intestinal Caco-2 cells against inflammatory and hypoxic damage. ω3 oxylipins treatment increased viability of epithelial cells and the integrity, preserving junctional molecules while reducing cytotoxicity and oxidative stress. In addition, oral administration of ω3 oxylipins in a mice model of IBD prevented colon shortening, reduced intestinal epithelium disruption, inhibited infiltration of inflammatory cells and tissue fibrosis, and increased integrity of the mucus layer. Our results provide evidence of the pro-resolutive role of oxylipins derived from HM-EVs in the context of intestinal diseases and support their use in fortification formulas for feeding PIs.

The interplay between metalloprotease cleavage and extracellular vesicle secretion of immune ligands affects tumour recognition

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Immune recognition of tumours strongly relies on the expression of immune activating ligands at the surface of cancer cells. Several tumour-expressed immune ligands, such as NKG2D-ligands (MICA/B and ULBPs) or DNAM-1-ligands (CD155) lead to the activation of immune effector cells for cancer elimination. However, different cell trafficking events can modulate their expression. In fact, release of immune ligands as soluble proteins, some after the cleavage by matrix metalloproteases (MMPs), other associated to extracellular vesicles (EVs), constitute immune evasion mechanisms resulting in tumour progression. Thus, MMPs could play a critical role regulating tumour immune response. MMPs commonly undergo dysregulation during cancer progression, and MMP inhibitors (MPIs) were tested as anti-cancer drugs, but their effects were not as promising as expected. Here, we studied the role of MPIs in the regulation of the expression of several immune ligands that can also be released in EVs.

Using both CHO (Chinese hamster ovary) transfectant cell lines and cells endogenously expressing different MICA alleles and CD155, we demonstrate that these molecules were abundantly released in EVs when metalloprotease cleavage was inhibited in vitro. Functionally, EV-MICA induced downmodulation of the activating receptor NKG2D on NK cells. After 24-h cell exposure to MPIs, increased values of soluble MICA were detected in supernatants by ELISA, compared to 2-6 h treatments, probably reflecting the longer time required for recruitment into EVs. The molecular mechanism by which this type of regulation affects different immune ligands is now under investigation. Our data suggest that MMP inhibition can deeply affect the anti-tumour response by regulating several immune targets, including certain checkpoint molecules. We are investigating whether a more general cell biology pathway could be involved in these observations.