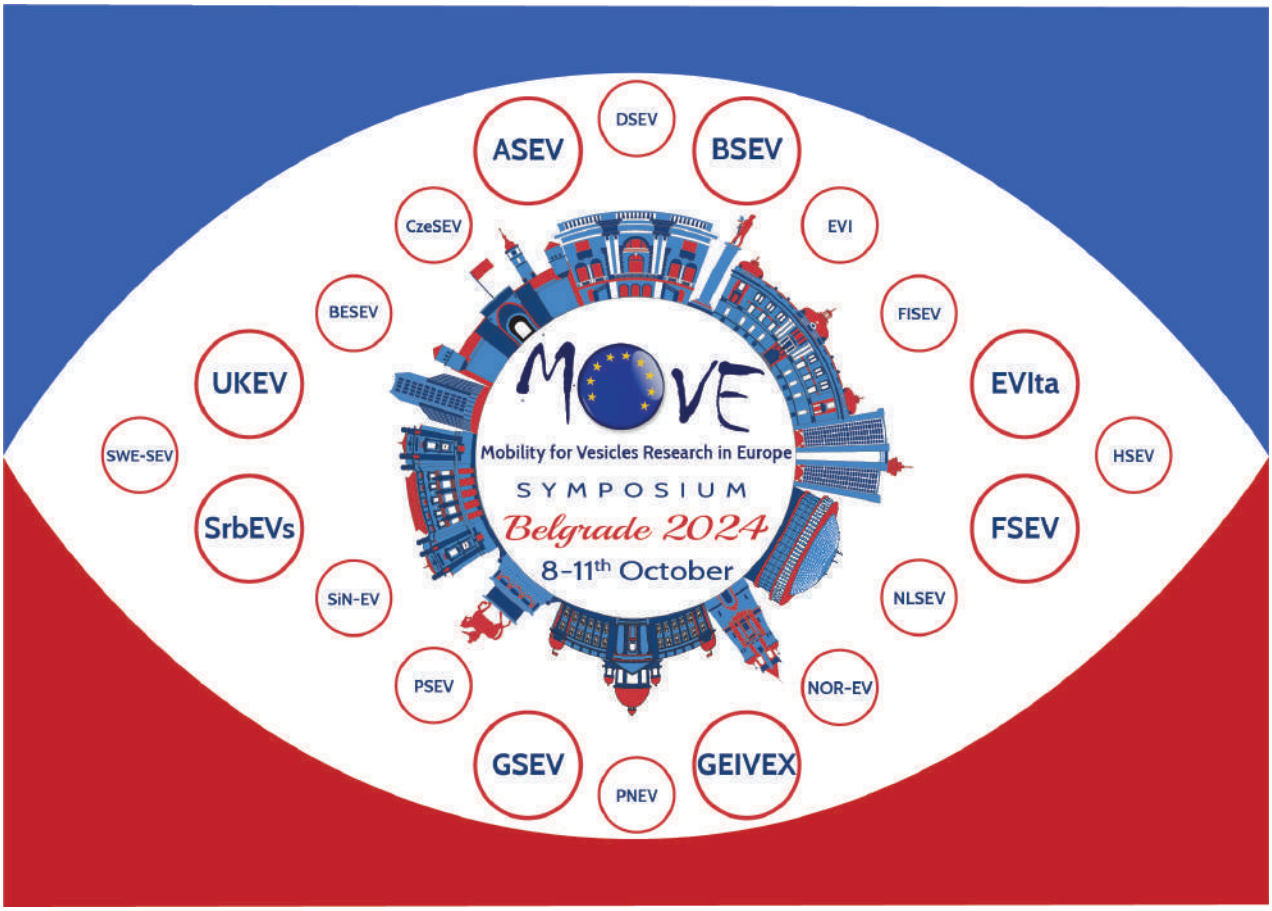
2nd MOVE Symposium



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European National Societies for Extracellular vesicles

Abstract book

2nd MOVE Symposium

8-11 October 2024, Belgrade, Serbia

Organizing Societies

Serbian Society for Extracellular Vesicles, SrbEVs
Austrian Society for Extracellular Vesicles, ASEV
Baltic Society for Extracellular Vesicles, BSEV
Italian Society for Extracellular Vesicles, EVIta
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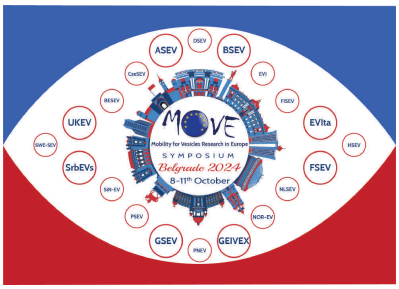

2nd MOVE Symposium

8-11 October 2024, Belgrade, Serbia

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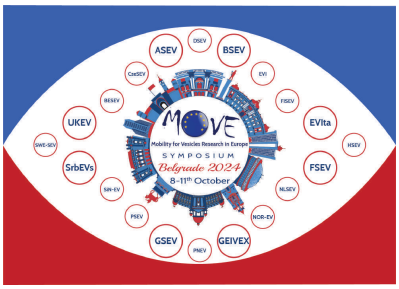

2nd MOVE Symposium

8-11 October 2024, Belgrade, Serbia

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Tobias Tertel
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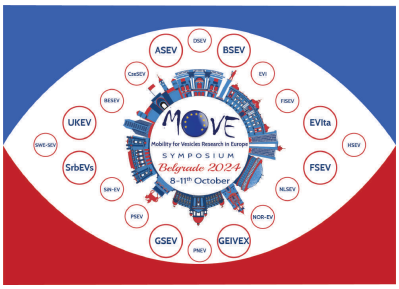
Ludwig-Boltzmann-Institute for Traumatology, Vienna, Austria



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2nd MOVE Symposium

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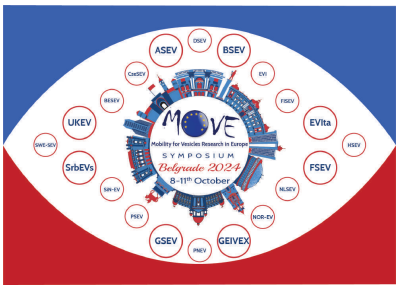

2nd MOVE Symposium

8-11 October 2024, Belgrade, Serbia

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2nd MOVE Symposium

8-11 October 2024, Belgrade, Serbia

Dear participants, colleagues and friends,

It's a true pleasure and honor to welcome you to the 2nd MOVE Symposium and its accompanying Abstract Book, on behalf of the International Organizing Committee.

Over the past 15 years, the field of extracellular vesicles (EVs) research has seen remarkable growth, driven by discoveries of their fundamental roles in physiological processes and their potential as biomarkers and therapeutic tools. This progress has highlighted the need for platforms to connect scientists and facilitate knowledge exchange, leading to the establishment of international and national EV societies.

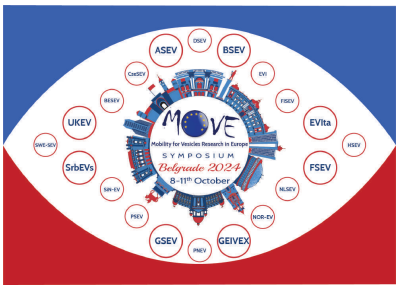
To take the advantage of geographical proximity and expertise, MOVE was formed as an informal consortium of European National EV Societies (NEVS) with the main task to foster MObility for Vesicles research in Europe and encourage communication between the societies aiming to advance and promote EV research and understanding across Europe.

With the same overreaching aim in mind, MOVE expanded its activities to organize 1st MOVE Symposium in Malaga, Spain, in 2023. Four of NEVSs (EV Societies of Spain (GEIVEX), Italy, (EVIta), Germany (GSEV) and United Kingdom (UKEV)), took a lead in organizing this milestone meeting. With more than 350 participants from all over the Europe and topics all across the field of EV research, it was a great success and set the stage for our continued efforts.

This year 8 NEVSs (EV Societies of Serbia (SrbEVs), Austria (ASEV), Baltic countries (BSEV), Italy (EVIta), France (FSEV), Spain (GEIVEX), Germany (GSEV) and United Kingdom (UKEV)), gathered to organize the 2nd MOVE Symposium, in Belgrade, Serbia.

2nd MOVE Symposium featuring 8 keynote lectures from world renown EV scientists, 49 oral presentations and 130 poster presentations across different biological and biomedical disciplines, 10 oral and 17 total presentations of tools for EV research, and over 325 participants from all across the Europe and the world, provides the opportunity to obtain an overview of EV research in Europe and beyond, discover trends and perspectives in EV field and discuss its undiscovered areas and needed research directions.

We hope that 2nd MOVE Symposium will result in formation of fruitful connections between EV enthusiasts and especially provide young scientists with an opportunity to engage with experts and forge lasting relationships that may lead to exciting future projects. As we share a passion for the research of these nano-messengers, fascinating in their complexity, heterogeneity and myriad of roles, we hope this meeting will promote the collaborations and advancements that will help shaping the future of EV research.



2nd MOVE Symposium

8-11 October 2024, Belgrade, Serbia

In the name of the International Organizing Committee, I'd like to thank all our participants for their contributions to this Program and the Abstract Book, and to all keynote speakers for accepting our invitation to share their expertize.

This event could not have been realized without the collective efforts of our 8 sister societies and I extend my deepest gratitude to the presidents of ASEV, BSEV, EVIta, FSEV, GSEV, GEIVEX, and UKEV as well as their dedicated members on the International Organizing Committee and Scientific Committee.

We are also deeply grateful for the financial support provided by the Sponsors and Supporters of this meeting. We acknowledge that we as scientists cannot do our work without appropriate tools, so we consider industry's contributions and collaborations as invaluable for the development of the field.

We thank Ministry of science, technological development and innovations of the Republic of Serbia for their support. Also, we thank Biological faculty of the University of Belgrade for allowing us to use Indico registration website and we are gratefull to the Institute for the application of the nuclear energy, INEP and Chemical faculty of the University of Belgrade for their kind support in organizing this meeting.

Special thank you are due to the Local Organizing Committee, whose hard work has made this symposium in Belgrade a memorable event. Despite being a relatively young society, SrbEVs has taken the lead with exceptional dedication, providing us with a wonderful setting for this gathering. Also, we are very gratefull to all volunteers within the Technical Committee for their valuable help.

Finally, I hope you will enjoy your time in Belgrade and its municipality of Zemun, and will be inspired to visit again to immerse yourself in its rich culture, history and vibrant city life.

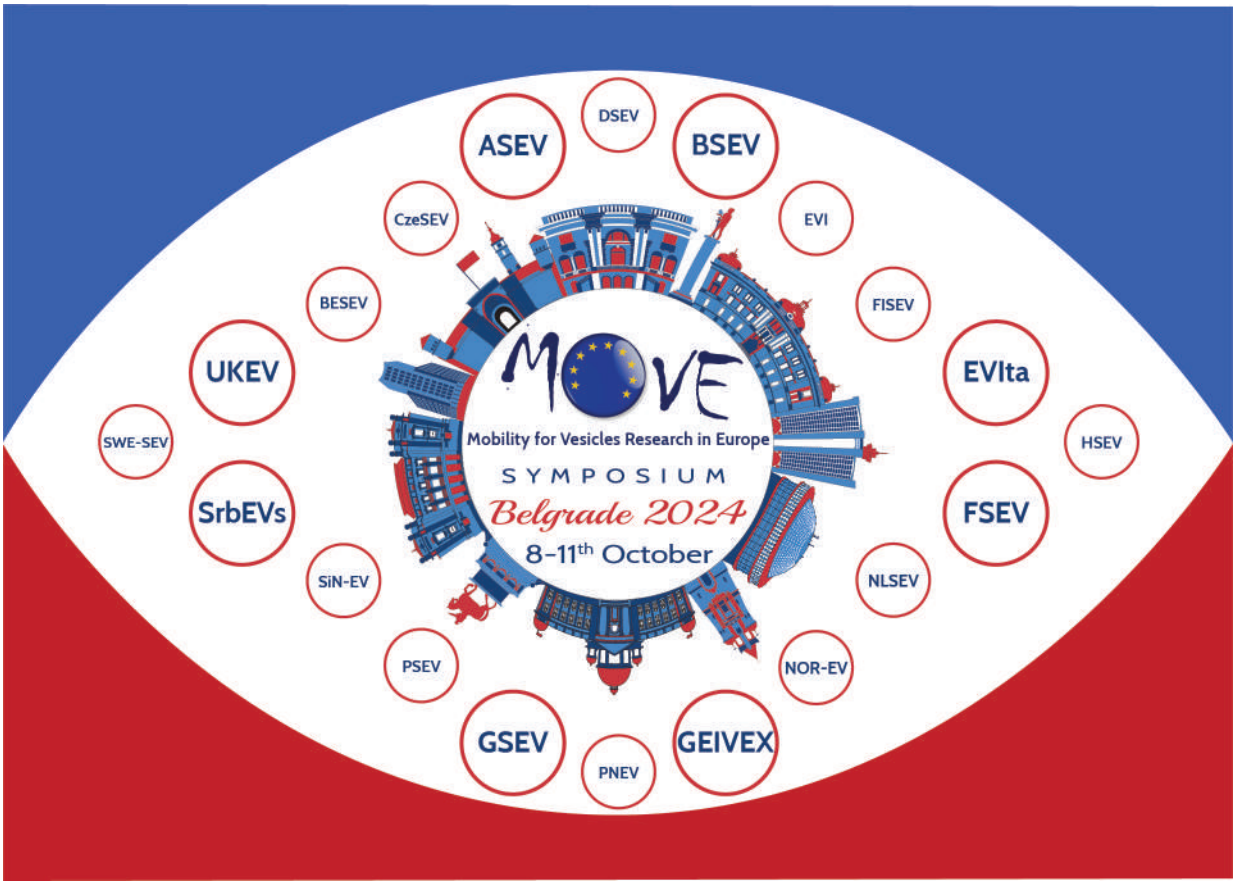
In the name of the International Organizing Committee I wish you all the inspiring and memorable meeting,

Belgrade, October 2024.

Maja Kosanović

President of Serbian Society for Extracellular Vesicles, SrbEVs

PROGRAM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SYMPOSIUM
Belgrade 2024
8-11th October

Tuesday, 8.10.2024. - morning

8:00	Registration (from 8:00)
9:00	**Wellcome notes 15'**

9:15	Keynote	**Frederik Verweij - What goes in, sometimes comes out: developing molecular toolboxes to study the loading, maturation and fate of Multivesicular Bodies** Utrecht University, Div. of Cell Biology, Neurobiology & Biophysics, Utrecht, The Netherlands

9:45	Session 1 B-1	**Basic EV research: Biogenesis/release of EVs and their function in signal transmission 1** Chairs: Jessica Gobbo, Anna-Kristin Ludwig
O-B-1	Unlocking Ovarian Cancer Biomarkers: Insights from Ascitic Extracellular Vesicles	Vendula Pospíchalová
O-B-2	Genome-Wide-CRISPR/Cas9 screening identifies the COMMANDER recycling complex as a key player in EV cargo delivery	María Yañez-Mo
O-B-3	MBLAC2, a new player in the EV field?	Guillaume Médard
O-B-4	Dynamic interactors: Galectins and EVs	Anna Ludwig

10:45	Coffee break 30'

11:15	Session 2 HD-1	**EVs in health and disease 1** Chairs: Moran Yadid, Beate Rinner
O-HD-1	Role of cardiomyocyte-derived extracellular vesicles in post-ischemic cardiac remodeling	Lélia Borowski
O-HD-2	Extracellular Vesicle-Derived miR-375 3p Promotes Fetal Overgrowth in Pregnancies Complicated by Maternal Diabetes	Abigail Byford
O-HD-3	Extracellular vesicles shed by PDAC cells harboring mutant p53 remodel the metastatic niche and promote hematopoietic differentiation	Tomer Cooks
O-HD-4	Developing liver spheroids as a model to investigate the role of colorectal cancer derived small extracellular vesicles in hepatic pre-metastatic niche establishment	Elisa Costanzo

12:15	Lunch break 1h 30'

Tuesday, 8.10.2024. - afternoon

13:45	Keynote	**Tobias Tertel - Extracellular Vesicles as Diagnostic and Therapeutic Biomarkers in Stress, Cancer, and Neurological Disorders** University Hospital Essen, Institute for Transfusion Medicine, Essen, Germany

14:15	Session 3 N-1	**Novel EV preparation/analysis techniques 1** Chairs: Alireza Fazeli, Alicia Llorente	
O-N-1	NANOSPACER: Nanofluidic sizing of extracellular vesicles and biomacromolecules in solution		Oliver Vanderpoorten
O-N-2	Achieving Ultra-Pure EVs: Combining Charge-Based Filtration, Tangential Flow, and Lipoprotein Adsorption		Sehyun Shin
O-N-3	Inline Raman spectroscopy provides versatile molecular monitoring for platelet extracellular vesicle purification		Heikki Saari
O-N-4	Isolation of extracellular vesicles from resistant tumor cells using nanobodies-based immunoaffinity approach		Lidija Filipović

15:15	Coffee break 45'

16:00	Keynote	**Michiel Pegtel - EV-RNA from basic science to diagnostic applications** Amsterdam UMC/Cancer Center Amsterdam, Amsterdam, The Netherlands

16:30	Session 4 BM-1	**EV-based biomarkers - 1** Chairs: Marija Holcar, Tanja Kutzner	
O-BM-1	Predicting Kidney Injury Post-Transplant: The Role of urine EVs and EV-DNA as Biomarkers		Maja Vodušek
O-BM-2	Characterization of circulating extracellular vesicles in patients with major depressive disorder: are them good biomarkers of severity and response to therapy?		Fabiola Stolfi
O-BM-3	Elevated GLAST+ and Proteoglycan-4+ Extracellular Vesicles, with Proteoglycan-4+ Linked to Cognitive Protection in Amyotrophic Lateral Sclerosis		Annalisa Chiocchetti
O-BM-4	Immunodepletion to enrich and select for rare extracellular vesicle populations: pitfalls and suggestions for best practice		Adam Bennett

Tuesday, 8.10.2024. - Poster session

17:30	**Poster session 1 (2h)**

P-B	**Basic EV research: Biogenesis/release of EVs and their function in signal transmission** Chairs: Krizia Sagini, Ishai Luz	
P-B-1	Extracellular Vesicles and MBsomes Intercellular Communication in Skin Wound Healing	Mariane Shouky
P-B-2	Proteomic and lipidomic profiling of extracellular vesicles from tetraspanin-deficient cell lines	Stanislava Sladeček
P-B-3	Extracellular vesicles act faster than you think; Rapid increase in MFGE8 secretion from endometrial cells is an indicator of embryo maternal cross-talk	Subhashini Muhandiram
P-B-4	Physiological vs. Traditional EV Administration: Improved Distribution and Reduced Toxicity with Slow-Release Pumps	Doaa Massad
P-B-5	Role of the different intracellular pathways in the study of the biogenesis of exosomes	Deborah Polignano

P-HD	**EVs in health and disease** Chairs: Marit Inngjerdingen, Dan Lambert	
P-HD-1	Proteomic composition of extracellular vesicles derived from the interaction of Trypanosoma cruzi with myoblasts and intestinal cells	Marcel Ivan Ramirez Araya
P-HD-2	Endothelial progenitor cells-derived extracellular vesicles mitigate the inflammation in septic model	Luigi Menna
P-HD-3	Caveolin-1-overexpression affects extracellular vesicle loading and modulates tumour microenvirnment in a rhabdomyosarcoma model	Rachele Agostini
P-HD-4	Assessing T Cell-Mediated Immunity to SARS-CoV2 infection in extracellular vesicles through affinity capture.	Teresa Valero
P-HD-5	Vesicle-like particles extracted from ginger antagonize staurosporine-induced apoptosis	Delaram Khamari
P-HD-6	The Effect of Extracellular Vesicles Originated from Mesenchymal Cells of Peritoneal Dialysate on the Mechanism of Fibrosis	Péter Bokrossy

Tuesday, 8.10.2024. - Poster session

P-HD-7	Investigating the effects of neutrophil derived extracellular vesicles on lung injury using human precision cut lung slices	Jessica Ghobrial
P-HD-8	CD63+ extracellular vesicles are reduced in hypercholesterolaemic mice and humans	Brachyahu Meir Kestecher
P-HD-9	Synovial Fluid-Derived Extracellular Vesicles: Orchestrators of Endothelial Cell Function and Inflammation	Riham Osman
P-HD-10	The death signals mediated by extracellular vesicles between glioblastoma multiforme (GBM) cells during temozolomide treatment depend on the bioactive cargo specifically loaded by the different GBM lines	Mariana Karimova

P-BM	**EV-based biomarkers** Chairs: Sophie Rome, Milica Božić	
P-BM-1	MARCO as a potential EVs biomarker for Leishmaniasis	Anabela Silva
P-BM-2	Effect of ultramarathon race on Circulating Extracellular Vesicles release and their potential role as exercise biomarkers	Stephanie Fondi
P-BM-3	Flow Cytometric Detection of Extracellular Vesicles Subpopulations in Liquid Biopsies	Sean Patmore
P-BM-4	Mirnomic analysis of urine extracellular vesicles from type 2 diabetic patients under SGLT2 inhibitors treatment: identification of biomarkers for therapeutic response	Beatrice Spokeviciute
P-BM-5	Characterisation of thyroid-originating extracellular vesicles from thyroid cell culture medium and plasma of patients harboring thyroid tumors	Jelena Janković Miljuš
P-BM-6	Modelling the impact of extracellular vesicle cargoes in the diagnosis of CAD	Hargita Hegyesi
P-BM-7	Neutrophil-derived extracellular vesicles serve as potential biomarkers for carbon monoxide poisoning: a pilot study	Sara Nembrini
P-BM-8	Bioinformatic analysis predicts serum EV miRNA hsa-miR-378a-3p as potential downregulator of Ceramide signaling pathway in peripheral blood CD8+ T cells of glioblastoma patients	Milan Stefanović

Tuesday, 8.10.2024. - Poster session

P-I	EVs in interspecies communication Chairs: Hernando del Portillo, Carmen Fernandez-Becerra	
P-I-1	Elucidating the mechanisms underlying the ability of pathogenic Acidovorax temperans and its derived outer membrane vesicles to promote lung cancer progression	Haneen Abu-Freih
P-I-2	Characterization of extracellular vesicles in the interaction between the German cockroach, Blattella germanica, and its symbiotic partners	M. Rosario Gil García
P-I-3	Hepatoprotective effects of nanovesicles derived from lemon: an in vitro and in vivo investigation	Roberta Gasparro
P-I-4	Extracellular Vesicles from Lacticaseibacillus casei Modulate Interspecies Bacterial Interactions	Cecilia D'Antoni

P-N	Novel EV preparation/analysis techniques Chairs: Karin Ekström, Heikki Saari	
P-N-1	Effective enrichment of CD9 positive population of extracellular vesicles from human cerebrospinal fluid in asymmetric flow field flow fractionation and size exclusion chromatography fractions	Hrvoje Križan
P-N-2	Glycan mapping of tumor-derived extracellular vesicles: a multiplex lectin bead approach	Hema Saranya Ilamathi
P-N-3	Development of an analytical reference material for the study of extracellular vesicles	Khirul Islam
P-N-4	Isolation of spontaneously-released brain extracellular vesicles: implications for stress-driven brain pathologies	Ioannis Sotiropoulos
P-N-5	Isolating Outer Membrane Vesicles of Patient-derived Microbiota Pathogens on 3D-printed Ultrafiltration Platforms for Nucleic Acid-based Diagnostics	Nedim Haciosmanoglu
P-N-6	Extracellular Vesicle-Imprinted Optic Biosensors for Breast Cancer Detection	Yeseren Saylan
P-N-7	Microfluidic Chip-Based Systems For Monitoring Cancer Therapy Via Extracellular Vesicles	Eylül Gülşen Yilmaz

Tuesday, 8.10.2024. - Poster session

P-T	**EVs in therapy and regenerative medicine** Chairs: Tobias Tertel, Nuno Santarem	
P-T-1	Exploring Dendritic Cells-Derived Extracellular Vesicles As An Immunotherapeutic Tool In Triple-Negative Breast Cancer	Sofia Quintas
P-T-2	Pooling of umbilical cord mesenchymal stromal cells improves extracellular vesicle yield and therapeutic benefit in inflammatory arthritis	Oksana Kehoe
P-T-3	Inhibitory Effects of Chlorella vulgaris Algasomes on Listeria species Growth and Biofilm Eradication	Dulmini Nanayakkara Sapugahawatte
P-T-4	Autologous plasma-derived EVs for chemotherapy delivery to human tissues	Nick Peake
P-T-5	Local delivery of Dendritic Cell-secreted Extracellular Vesicles using 3D biomaterial scaffolds to promote bone tissue formation and spine fusion	Susana Santos
P-T-6	Bone marrow stromal cell derived extracellular vesicles for bone regeneration	Evangelia Bochti

P-M	**Manufacturing of native and engineered EV products** Chairs: Mandy Peffers, Getnet Midekessa	
P-M-1	Comparison of the effectiveness of anion exchange chromatography, ultracentrifugation, tangential flow filtration and size exclusion chromatography in EV isolation	Reetta Pusa
P-M-2	Membrane vesicles of Lactiplantibacillus plantarum as nanocarriers for synergistic antimicrobial combinations to treat skin wound infections	Liubov Shishaeva
P-M-3	Optimising scalable spheroid-based extracellular vesicle production in stirred-tank bioreactors	Thibaud Dauphin

Wednesday, 9.10.2024. - morning

9:00	Keynote	**Kenneth Witwer** The Johns Hopkins University School of Medicine, Dept. for Molecular and Comparative Pathobiology, Baltimore, USA

9:30	Session 5 HD-2	**EVs in health and disease 2** Chairs: Tomer Cooks, Edit Buzas	
O-HD-5	Effect of Hypercholesterolemia on circulating and cardiomyocyte-derived extracellular vesicles		Csenger Kovácsházi
O-HD-6	Exercise-induced extracellular vesicles in breast cancer		Krizia Sagini
O-HD-7	Horizontal transfer of long non-coding RNA H19 transports splicing factors in recipient cells		Marco Loria
O-HD-8	Extracellular vesicles from a triple negative breast cancer (TNBC) / paclitaxel-resistant cell model carry chemoresistance and inflammation signals.		Marilena Lekka

10:30	Coffee break 30'

11:00	Round table 1 1h

12:00	Lunch break 1h 30'

Wednesday, 9.10.2024. - afternoon

13:30	Session 6 B-2	**Basic EV research: Biogenesis/release of EVs and their function in signal transmission 2** Chairs: Maria-Anthi Kakavoulia, Maria Yanez Mo	
O-B-5	The Role of Extracellular Vesicles in KRAS/STK11 co-mutated NSCLC Immune Escape		Chiswili Yves Chabu
O-B-6	Extracellular vesicles promote migration despite BRAF inhibitor treatment		Afrodité Németh
O-B-7	Neutrophil-derived extracellular vesicles modulate the inflammatory response of monocytes and macrophages		Mátka Nagy
O-B-8	TGF-β induces cholesterol accumulation to regulate the fate of tumor-derived extracellular vesicles		Dorival Mendes Rodrigues Junior

14:30	Coffee break 45'

15:15	Keynote	**Dhanu Gupta - Manufacturing of Engineered EVs for biomedical applications** University of Oxford, Department of Paediatrics Oxford, United Kingdom

15:45	Session 7 N-2	**Novel EV preparation/analysis techniques 2** Chairs: Zoltán Giricz, Rosella Crescitelli	
O-N-5	Plant Extracellular Vesicles as Novel Drug-delivery Systems		Michaela Kocholatá
O-N-6	Time-resolved surface-sensitive waveguide scattering microscopy of single extracellular vesicles reveals content and biomarker heterogeneity		Petteri Parkkila
O-N-7	Isolating and Detecting Extracellular Vesicles on Microfluidic Chips and Metamaterial Sensors		Fatih Inci
O-N-8	Optimization of Extracellular Vesicles Isolation Protocol for Proteomics Analysis from Limited Cell Media Samples		Asia Botto

16:45	Poster session 2 (2h)

19:30	**Networking event**

Wednesday, 9.10.2024. - Poster session

16:45	**Poster session 2 (2h)**

P-B	**Basic EV research: Biogenesis/release of EVs and their function in signal transmission** Chairs: Krizia Sagini, Ishai Luz	
P-B-6	Anti-Inflammatory and Immunomodulatory Strategies as Therapeutic Approaches for Rab27a-Deficient Pancreatic Cancer	Daniel Lopes
P-B-7	EGFR-mutant lung cancer derived EVS induce resistance by activating PI3K/AKT signalling pathway	Dian Salih
P-B-8	Imaging flow cytometry enables studies of the interactions between extracellular vesicles and immune cells	Ulla Impola
P-B-9	Developing microglia cell lines expressing Nef to study the role of extracellular vesicles in HIV neuropathology	Teja Lavrin
P-B-10	Investigating the Influence of Cardiomyocyte-Derived Extracellular Vesicles on Macrophage Activation: A Comparative Analysis between Doxorubicin and DL-11	Tamás Bolyky

P-HD	**EVs in health and disease** Chairs: Marit Inngjerdingen, Dan Lambert	
P-HD-11	Extracellular Vesicle-Encompassed MyomiRs Contribute to Placental Vascular Immaturity in Pregnancies Complicated by Maternal Diabetes	Abigail Byford
P-HD-12	Interaction of cancer-associated exosomes with glyconanotherapeutics and study to cross blood tumor and blood brain barrier as oral or poster presentation.	Olga Janouskova
P-HD-13	Endothelial dysfunction alters extracellular vesicles' character and secretion profile	Binyamin Rosenzweig
P-HD-14	Exploring the connections between tumor cell DNA and EVs	Susana García-Silva
P-HD-15	Targeting pro-tumoral stromal exosomes by disrupting the syntenin-CD138/syndecan pathway as a novel therapy in Multiple Myeloma	Chenggong Tu
P-HD-16	Towards an understanding of melanoma EV heterogeneity, target cell interactions, and molecular functions in the tumor-draining lymph node	Pragati Lodha

Wednesday, 9.10.2024. - Poster session

P-HD-17	Is Placental Extracellular Vesicle Uptake Modulated by Factors Associated with an Obese Diabetic Pregnancy?	Andreea Cristian
P-HD-18	The characterization and variability of blood-derived extracellular vesicles in healthy humans and the influence of recent mild COVID-19	Marija Holcar
P-HD-19	Cerebral ischemia alters the microRNA and protein cargo of brain-derived extracellular vesicles	Olga Neustroeva
P-HD-20	Analysis of the subcellular localization of the transcripts CD81-205 and CD81-215	Dunja Pavlovic

P-BM	**EV-based biomarkers** Chairs: Sophie Rome, Milica Božić	
P-BM-9	Extracellular vesicles in liquid biopsies: do different size exclusion chromatography columns yield different results?	Krisztian Belenyesi
P-BM-10	Neuronal extracellular vesicles as nanotools to study and monitor Angelman Syndrome neurodevelopmental disorder	Chiara De Cesari
P-BM-11	Three methods of isolating EVs from pleural effusion samples of patients with advanced lung adenocarcinoma - potential applications in clinical practice?	Miodrag Vukovic
P-BM-12	Immune checkpoint profiles on circulating extracellular vesicles predict response to immunotherapy in hepatocellular carcinoma	Ramsha Masood
P-BM-13	Exosomal Galectins as Biomarkers and Therapeutic Targets of Glioblastoma	Oksana Batkivska
P-BM-14	Umbilical Cord Blood Extracellular Vesicles (UCBEVs) study in Preterm Infants	Laura Ripoll Seguer
P-BM-15	Utilising extracellular vesicle glycoproteins for non-invasive identification of patients with clinically significant prostate cancer	Demi Pritchard

Wednesday, 9.10.2024. - Poster session

P-I	EVs in interspecies communication Chairs: Hernando del Portillo, Carmen Fernandez-Becerra	
P-I-5	Exploring biomarkers in major depressive disorder: a multi-OMICs approach on stool samples	Chiara Venegoni
P-I-6	Intranasal application of Trichinella spiralis muscle larvae extracellular vesicles alleviate inflammation in mouse model of respiratory allergy	Maja Kosanović
P-I-7	Interactions of Trichinells spiralis muscle larvae extracellular vesicles with target cells and their mechanisms of action	Sofija Glamočlija
P-I-8	Interkingdom communication between oral pathogenic bacteria and Candida species at the level of extracellular vesicles	Zóra Szilovics

P-N	Novel EV preparation/analysis techniques Chairs: Karin Ekström, Heikki Saari	
P-N-8	Development of nanobody-based sandwich ELISA for sensitive detection of HIV-1 protein Nef in extracellular vesicles	Samuel Žvanut
P-N-9	Implementation of Single-domain antibodies-based approach for isolation of extracellular vesicles from human plasma	Marija Tursunović
P-N-10	Isolation and Characterization of Citrus-Derived Nanovesicles Through Three Different Approaches	Vincenza Tinnirello
P-N-11	Optimizing buffer composition for cryoprotection of red blood cell-derived extracellular vesicles	Kinga Ilyés
P-N-12	Evaluating Nano-Flow Cytometry for Detecting HER2-Positive Extracellular Vesicles in Breast Cancer Patients	Karin Ekström
P-N-13	Evaluating the influence of different serum-free culture conditions on the production and function of Natural Killer cell-derived extracellular vesicles	Amanda Sudworth
P-N-14	EV-Imprinted Nanoparticles for Real-Time Detection of Kidney-derived EVs Using Nanoplasmonic Sensor	Beyza Nur Küçük
P-N-15	Double size exclusion chromatography enriches high-quality extracellular vesicles from calf and lamb feces	Chanaka Premathilaka

Wednesday, 9.10.2024. - Poster session

P-T	**EVs in therapy and regenerative medicine** Chairs: Tobias Tertel, Nuno Santarem	
P-T-7	Exploring the anti-inflammatory response of pomegranate-derived extracellular vesicles in an in vitro model of osteoarthritis.	Emily Clarke
P-T-8	Cytokine-primed umbilical cord mesenchymal stem cells enhanced therapeutic effects of extracellular vesicles on osteoarthritic chondrocytes	Thu Huyen Nguyen
P-T-9	Advanced Technologies for Regenerative Medicine: From Tissue Engineering to Extracellular Vesicles	Moran Yadid
P-T-10	The role of extracellular vesicles (EVs) in chronic graft vs. host disease, and the potential function of placental cell-derived EVs as a therapeutic tool	Anat Aharon
P-T-11	Neuropeptide receptor Y as potential target for specific cancer treatment	Katarina Tomic
P-T-12	Advanced 3D In Vitro Cultures as Novel Models for Screening Extracellular Vesicle-based Therapies	Gabriele Vella

P-M	**Manufacturing of native and engineered EV products** Chairs: Mandy Peffers, Getnet Midekessa	
P-M-4	Scalable production of Extracellular Vesicles from Mesenchymal Stem Cells using a Hollow Fiber Bioreactor	Carlos Jesus
P-M-5	Klotho peptide engineered extracellular vesicles prevent epithelial-mesenchymal transition (EMT) and fibrosis in kidney cells via TGF-β inhibition	Tunahan Ergünay
P-M-6	The display of an anti-CS1 nanobody by small extracellular vesicles does not improve disease targeting in multiple myeloma	Michiel De Coster

Thursday, 10.10.2024. - morning

10:20	Keynote	**Bernd Giebel - Clinical Potential of MSC-EVs and Translational Challenges** University Hospital Essen, Institute for Transfusion Medicine Essen, Germany

10:50	Session 8 T	**EVs in therapy and regenerative medicine** Chairs: Susana Santos, Oksana Kehoe	
O-T-1		Extracellular vesicles powered cancer therapy: targeted delivery and enhanced anti-neoplastic effect of adenovirus-based cancer vaccine in humanized melanoma model	Mariangela Garofalo
O-T-2		Extracellular vesicles from clonally expanded immortalized mesenchymal stromal cells protect against ischemia-reperfusion-induced kidney injury	Milica Bozic
O-T-3		Erythrocyte membrane-based vesicles as siRNA carriers for safe and efficient gene silencing therapy	Nina Kostevšek
O-T-4		Effect of blood centrifugation on the platelet, extracellular particle and molecular content of plasma	Veronika Kralj-Iglic
O-T-5		Potential anti-senescence effect of Naringin and Hesperidin-loaded extracellular vesicles in intervertebral disc degeneration	Veronica Tilotta

11:50	Coffee break 30'

12:20	ST1	**Sponsor's Talks 1**	
ST-1	Beckman Life Sciences	The path for a successful flow cytometry analysis of extracellular particles	Anis Larbi
ST-2	Unchained Labs	Solving the EV characterization riddle	Alex Shephard
ST-3	FiberCell Systems	Clinical Scale Production of Extracellular Vesicles in a Hollow Fiber Bioreactor	John Cadwell
ST-4	Izon	Eyes on Izon – Throughput and Volume Scale Up Solutions	Joshua Karam
ST-5	Myriade	Videodrop : a Companion Tools for Repeatable & Reproducible Results in Extracellular Vesicle Research	Philippe Garabedian

Thursday, 10.10.2024. - afternoon

13:20	Lunch break 1h 30'

14:50	Keynote	**Antonio Marcilla - Extracellular vesicles in interspecies communications: from helminths to plants** University of Valencia, Faculty of Pharmacy, Dep. for Pharmacy and Pharmaceutical Technology and Parasitology, Valencia, Spain

15:20	Session 9 I	**EVs in interspecies communication** Chairs: Irma Schabussova, Carmen Fernández-Becerra	
O-I-1	Host Immune Cell Membrane Deformability Governs the Uptake Route of Malaria-Derived Extracellular Vesicles		Daniel Alfandari
O-I-2	Therapeutic potential of extracellular vesicles of E. coli O83 in immunomodulation and allergic airway inflammation		Irma Schabussova
O-I-3	Functional effects of cows' milk derived particles on human inflammatory responses in vitro		Charlotte Lawson
O-I-4	Exploring Extracellular Vesicle-Mediated Intercellular Communication in Malaria Cryptic Infections Using Organs-on-a-Chip		Nuria Sima Teruel

16:20	Coffee break 10'

16:30	Round table 2 1h

Thursday, 10.10.2024. - Poster session

17:10	**Poster session 3 (2h)**

P-B	**Basic EV research: Biogenesis/release of EVs and their function in signal transmission** Chairs: Krizia Sagini, Ishai Luz	
P-B-11	Exploring the Biodistribution of p53-Containing EVs for Targeted Cancer Therapy	Ishai Luz
P-B-12	Unraveling the Role of Galectins in Mesenchymal Stromal/Stem Cell-Mediated Immunomodulation through Extracellular Vesicles	Tanja Kutzner
P-B-13	Characterisation of mast cell- derived extracellular vesicles during degranulation	Kelsey Fletcher
P-B-14	EVs biogenesis and secretion machinery in distinct Alzheimer's disease mimicking models	Margarida Vaz
P-B-15	TGF-β induces cholesterol accumulation to regulate the fate of tumor-derived extracellular vesicles	Dorival Mendes Rodrigues Junior

P-HD	**EVs in health and disease** Chairs: Marit Inngjerdingen, Dan Lambert	
P-HD-21	Role of Extracellular Vesicles in Parkinsonian disorders	Cristiano Lucci
P-HD-22	Comparative analysis of surface glycosylation of prostasomes from human seminal plasma of normozoospermic and asthenozoospermic men	Ninoslav Mitic
P-HD-23	Extracellular release of a disintegrin and metalloproteinase correlates with periodontal disease severity	Ahmad Aljohmani
P-HD-24	Phenotypic Alterations in Tissue Extracellular Vesicles from Renal Cell Carcinoma Under Mechanical Stress	Gaia Bianchi
P-HD-25	Extracellular vesicles from saliva of Rheumatoid Arthritis patients have a distinct profile compared to healthy controls	Marko Prokic
P-HD-26	Cargo of circulating extracellular vesicles regulates CDKN1A gene expression according to the MS disease severity	Ivan Jovanović

Thursday, 10.10.2024. - Poster session

P-HD-27	The Role of Extracellular Vesicles in Renal Cell Carcinoma Progression	Aline Seiko Carvalho Tahyra
P-HD-28	Extracellular Vesicles in Amyotrophic Lateral Sclerosis: Identification of Hub Regulatory Genes and Their Role in Disease Development Mechanisms	Katarina Živančević
P-HD-29	Exosome processing in the context of Alzheimer´s disease	Petra Riegerová

P-BM	**EV-based biomarkers** Chairs: Sophie Rome, Milica Božić	
P-BM-16	Milk extracellular vesicles decipher the secrets of metabolic health status of lactating cowss	Madhusha Prasadani Yasalath gamage
P-BM-17	Spectral signature of plasma-derived sEVs supports the classification of cancer patients	Tímea Böröczky
P-BM-18	Utilising machine learning for development of an EV-RNA assay for early detection of aggressive prostate cancer	Haiyan An
P-BM-19	Distinguishing Primary and Metastatic Clear Cell Sarcoma via Protein Signatures in Extracellular Vesicles	Djenana Vejzovic
P-BM-20	Changes in the miRNA cargo of EV-mediated feto-maternal communication following light treatment	Bence Nagy
P-BM-21	Comparative Proteomics analysis of EVs Isolated by different methods from T. cruzi-Infected Cardiac Cells and Their Transcriptional Effects	Berta Barnadas
P-BM-22	Comprehensive Profiling of Endometriosis-Derived Extracellular Vesicles Unveils Novel Potential Biomarkers for Endometriosis	Karolina Soroczyńska

Thursday, 10.10.2024. - Poster session

P-I	EVs in interspecies communication Chairs: Hernando del Portillo, Carmen Fernandez-Becerra	
P-I-9	The role of extracellular vesicles in oral squamous cell carcinoma-Candida interaction	Éva Veres
P-I-10	Probiotic EVs binding to mucin and an in vitro model of intestinal epithelial barrier	Cecilia D'Antoni
P-I-11	Nanovesicles from frozen homogenized Fasciola hepatica adults exhibit similar properties to extracellular vesicles from parasitic cultures	Christian Miquel Sánchez-López
P-I-12	Establishing an extracellular vesicle isolation protocol for a beneficial plant fungus	Loukia Kellari
P-I-13	Arabidopsis thaliana root cells interact with outer membrane vesicles (OMVs) produced by plant beneficial bacterial strain Paraburkholderia phytofirmans PsJN	Dragana Nikolic

P-N	Novel EV preparation/analysis techniques Chairs: Karin Ekström, Heikki Saari	
P-N-16	Detection of organelle-specific dyes labelled extracellular vesicles with colocalization-fluorescence Nanoparticle Tracking Analysis	Getnet Midekessa
P-N-17	Multi-Parametric Surface Plasmon Resonance - powerful new technology to measure small EV size and concentration and more.	Abhishek Sharma
P-N-18	Specific extracellular vesicle detection and isolation in complex samples	Kris Ver Donck
P-N-19	Quantification of EV surface proteins with fluorescent labelling and single EV detection	Freya Leech
P-N-20	Size-based extracellular particle sorting: methods and media effects on breast cancer cells	Barbora Popova

Thursday, 10.10.2024. - Poster session

P-T	**EVs in therapy and regenerative medicine** Chairs: Tobias Tertel, Nuno Santarem	
P-T-13	Influence of Trophoblast-Derived Extracellular Vesicles on Atopic Dermatitis-like Keratinocyte Phenotype	Mirjana Nacka-Aleksić
P-T-14	Extracellular Vesicles from Extravillous Trophoblast Modulate D-galactosis-Induced Keratinocyte Senescence	Milica Jovanovic Krivokuca
P-T-15	The Effects of Extravillous Trophoblast Cell-Derived Extracellular Vesicles on Cell Viability and Cisplatin Response in Choriocarcinoma Cells	Andrea Pirkovic
P-T-16	Collagen Hydrogel Embedded SHED-EVs for Enhanced Osteogenesis	Marija Milivojevic
P-T-17	Extracellular Vesicles as Therapeutic Agents for Inflammation Resolution and Tissue Regeneration	Ivana Milic
P-T-18	The Mechanisms and Effects of Autologous Extracellular Vesicles on Joints and the Nervous System	Samira Ali

P-M	**Manufacturing of native and engineered EV products** Chairs: Mandy Peffers, Getnet Midekessa	
P-M-7	Effects of industrial processing on the functional properties and uptake efficiency of nanovesicles derived from pomegranate juice	Christian Miquel Sánchez-López
P-M-8	Functionalization of Extracellular Vesicles Surface with Hyaluronic Acid Derivatives: Comparison of Different Approaches	Giulia Duca
P-M-9	The Importance of Established and Optimized Analytical Capabilities to Develop End to End and Scalable Therapeutic Extracellular Vesicles Processing	Aslan (Mehdi) Dehghani

Friday, 11.10.2024.

9:00	Keynote	**Rossella Crescitelli - Exploring Tissue-Derived Extracellular Vesicles: Cancer Biomarkers and Immunotherapeutic Potential** University of Gothenburg, Sahlgrenska Center for Cancer Research, Göteborg, Sweden

9:30	Session 10 M	**Manufacturing of native and engineered EV products** Chairs: Charlotte Lawson, Michele Guescini	
O-M-1		Targeting the tumor microenvironment: a click chemistry-based surface-functionalization method and a therapeutic-loading strategy for artificially produced erythrocyte-derived extracellular vesicles	Maria Chiara Ciferri
O-M-2		Gla-Rich Protein-loaded EVs for cardiovascular research: Production via baculovirus-insect and human cell systems	Joana Carreira
O-M-3		Comprehensive Classification of Antimicrobial and Cell-Penetrating Peptides in Vesicle Interactions	Tasvilla Sonallya

10:30	Coffee break 15'

10:45	ST2	**Sponsor's Talks 2**	
ST-6	Eppendorf	Evolution with Ultracentrifugation	Robert Skraban
ST-7	Particle Metrix	Colocalization of Biomarkers using Nanoparticle Tracking Analysis	Christina Klasen
ST-8	EXODUS	EXODUS, High-Efficiency Isolation of Exosomes and Its Application in Diagnosis and Treatment	Zoey Wang
ST-9	Fox Biosystems	Use cases on quantitative affinity isolation of Extracellular Vesicles for downstream assays	Kris Ver Donck
ST-10	Cytek	Small Particle Detection Using Flow Cytometry: Full Spectral and Imaging Flow Cytometry	Erwin Swart

11:35	Coffee break 30'

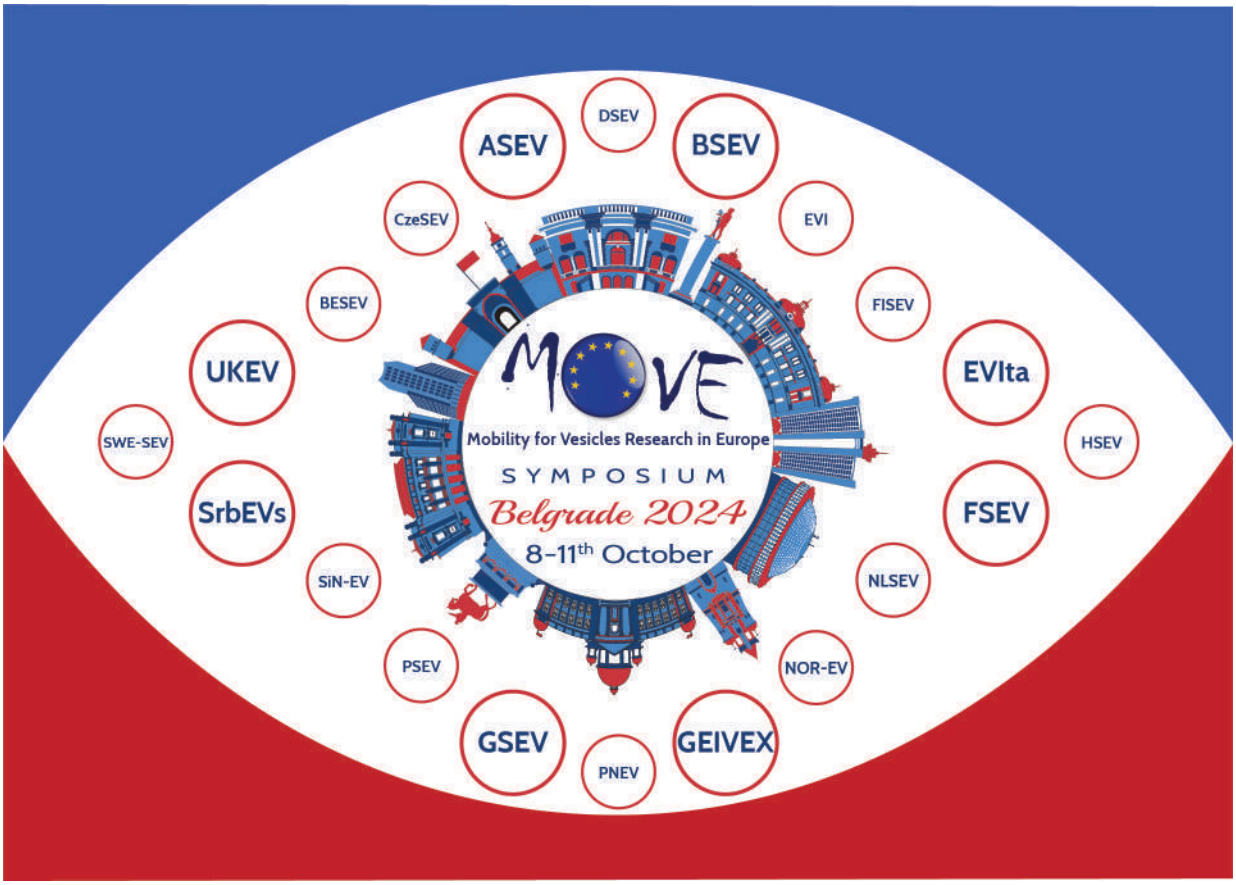
Friday, 11.10.2024.

12:05	Session 11 BM-2	**EV-based biomarkers 2** Chairs: Jason Webber, Sanja Goč	
O-BM-5	miR-146a-5p and miR-21-5p from extracellular vesicles as potential biomarkers in gestational diabetes		Zorana Dobrijević
O-BM-6	Brain-derived blood extracellular vesicles as potential biomarkers in multiple sclerosis (MS): Pilot results from relapsing MS patients receiving anti-CD20 therapy		Shamundeeswari Anandar
O-BM-7	Tumour-Derived Extracellular Vesicles Enriched in CAIX Indicate Hypoxia in Drug-Sensitive and Drug-Resistant Breast Cancer		Rawan Almasri

12:50	Session 12 HD-3	**EVs in health and disease 3** Chairs: Saara Laitinen, Vendula Pospichalova	
O-HD-9	Influence of platelet extracellular vesicles on T cell function and endothelial integrity in allergic inflammation		Elena Izquierdo
O-HD-10	Mechanisms of extracellular vesicle uptake in G. intestinalis and host cell interactions: role of clathrin and caveolin-mediated endocytosis		Marcel Ivan Ramirez Araya
O-HD-11	Endothelial cell derived extracellular vesicles in chemotherapy		Giulia Artemi

13:35	**Awards and Closing remarks**

Keynote lectures



Antonio Marcilla, PhD, Full Professor of Parasitology, Universitat de València, and Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics, Hospital Universitario y Politécnico “La Fe”-Universitat de Valencia, Spain.

His research deal with the isolation and characterization of extracellular vesicles from different sources, and their role in interkingdom communications, mainly in parasites and plants, and their usefulness in controlling different pathologies. His group described for the first time the isolation and characterization of extracellular vesicles in helminths, as well as the identification of microRNAs. His team has been also pioneered exploring the use of helminth EVs in vaccination and immune regulation. Supervisor of 11 PhDs and more than 20 Master's Theses, he has published more than 120 articles in high-impact scientific journals, with more than 22,600 citations (h-index: 45).

Founding member of the Spanish Group on Extracellular Vesicles (GEIVEX), where he has been treasurer and responsible for education activities. Member of the International Society of Extracellular Vesicles (ISEV), participating in the organization of the ISEV 2018 annual meeting in Barcelona, as well as in numerous activities and workshops. He has promoted EVs among a wide research community, organizing workshops like Valensicles, and courses funded by COST, Universidad Internacional Menéndez Pelayo and Fundación Premios Jaume I, among others. Member of the editorial board of several journals in the field of Molecular Biology, Parasitology and Extracellular Vesicles. He is Member of the Royal National Academy of Pharmacy (Spain).

Extracellular vesicles in interspecies communications: from helminths to plants

Antonio Marcilla[1,2]

[1]Àrea de Parasitologia, Departament de Farmacia i Tecnologia Farmacèutica i Parasitologia, Universitat de València, Burjassot (Valencia) Spain; [2]Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics, Health Research IIS La Fe-Universitat de València, Valencia, Spain.

Extracellular vesicles (EVs) have been described as natural nanoparticles carrying bioactive molecules and released by almost all kingdoms of life, constituting an evolutionary conserved mechanism of intercellular and interspecies communication. Many studies have reported their presence and different roles in bacteria, fungi, plants, protozoans, helminths and mammals. Our group first described the release of EVs by helminths as well as their proteomic and transcriptomic characterization (1, 2). We and others have reported the role of helminth EVs in host-parasite communications and their usefulness in controlling parasitic disease since they provide a novel source of biomarkers, treatment targets as well as potential vaccination agents (3-8). Along with studies on helminths, data will be also presented regarding the characterization of plant derived EVs (PDEVs) and their potential as a sustainable source for delivery agents for specific (parasite) molecules (9, 10). Different PDEVs have been characterized, some of them exhibiting biological activities (e.g., modulating the immune system, either by in vitro assays using cell cultures, or by in vivo studies using available rodent models).

The potential roles of EVs in the interaction between plants and insects will be also addressed, as well as preliminary data on the insect-endosymbiont interaction.

1. Marcilla et al., 2012. https://doi.org/10.1371/journal.pone.0045974
2. Bernal et al., 2014. https://doi.org/10.1016/j.jprot.2014.02.012
3. Montaner et al., 2014. https://doi.org/10.3389/fimmu.2014.00433
4. Coakley et al., 2016. https://doi.org/10.1016/j.molbiopara.2016.06.003
5. Trelis et al., 2016. https://doi.org/10.1016/j.ijpara.2016.07.003
6. Roig et al., 2018. https://doi.org/10.3389/fmicb.2018.01036
7. Sánchez-López et al., 2021. https://doi.org/10.1016/j.molimm.2021.03.020
8. Sánchez-López et al., 2023. https://doi.org/10.1002/jev2.12317
9. Pérez-Bermúdez et al., 2017. https://doi.org/10.1016/j.ejps.2016.09.022
10. Sánchez-López et al., 2022. https://doi.org/10.1039/d2fo01806c

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Bernd Giebel studied biology in Cologne and received his PhD in 1996. In 1999. he moved to the Heinrich-Heine-University of Düsseldorf, to work with human hematopoietic stem and progenitor cells. In 2008 he switched to the University Hospital Essen, continued his studies on human somatic stem cells and started to work with EVs in 2009.

Setting a focus on mesenchymal stem/stromal cell-derived EVs (MSC-EVs), together with collaboration partners his group demonstrated the therapeutic potential of MSC-EVs in a human GvHD-patient and in different animal models. Coupled to his international success, in 2023, he was appointed as full professor for Translational Extracellular Vesicle Research at the University Hospital Essen.

Research wise, it is his goal to efficiently translate MSC-EVs into the clinics and to set up appropriate quality control platforms. Furthermore, it is his mission to promote national and international EV research. In this context, BG acted as the founding president of the German Society of Extracellular Vesicles (GSEV) from 2017 to 2023. Since 2018, he is co-chairing the exosome working group of the International Society of Gene and Cell Therapy (ISCT) and is part of the scientific advisory board of four SME companies, Innovex Therapeutics, Mursla LTD, PL BioScience and ReNeuron. Furthermore, he is a consultant of FUJIFILM Wako Chemicals Europe GmbH and founding director of Exosla LTD.

Clinical Potential of MSC-EVs and Translational Challenges

Bernd Giebel

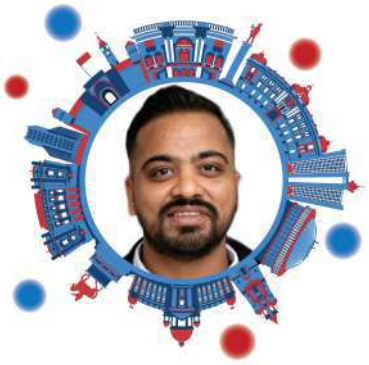
Institute for Transfusion Medicine, University Hospital Essen, University Duisburg-Essen

Human mesenchymal stromal cells (MSCs) are a therapeutically relevant, heterogenous cell entity with immunomodulatory and pro-regenerative potentials. Apparently, MSCs mediate a huge proportion of their therapeutic effects via extracellular vesicles (EVs). Connected to several advantages in using cell-free products for the therapeutic setting, MSC-EVs emerged as promising novel therapeutic agent for various diseases, including graft-versus-host disease (GvHD), ischemic stroke, COVID-19 and sepsis.

It is our current mission to optimize the MSC-EV production strategy in a scaled, GMP compliant manner, and to set up an appropriate quality control platform to translate MSC-EVs into the clinics. One of the challenging aspects in this context is inherited from the MSC field, i.e. contradictory reports on the efficacy of MSC therapies. Apparently, not all MSC products mediate therapeutic effects when applied into patients. Similarly, we observe functional differences among independent MSC-EV preparations; even when same MSC stocks were used as starting material. Thus, to avoid draw backs as they occurred in the MSC field by failing to show efficacy in a phase III clinical trial for GvHD treatment, it is one of our most important missions to address and appropriately handle the heterogeneity aspect.

To this end, we have set up a lentiviral, hTERT-based immortalisation strategy and raised MSC lines at the clonal level. EVs released by these clonally expanded immortalized MSCs (ciMSCs) reveal immunomodulatory activities and confer therapeutic activities in vivo. According to our understanding, we thus have fulfilled an essential milestone towards scaled and standardized production of MSC-EV-based therapeutics.

Still, a number of additional steps need to be fulfilled to translate MSC-EV products into the clinics, e.g. improving up- and downstream processing procedures and the functional testing of obtained MSC-EV products.



Dhanu Gupta is presently a Senior Postdoctoral Researcher and a Fellow of the Swedish Brain Foundation at the University of Oxford.

In his role, dr Gupta leads a dynamic research team focused on various aspects of gene therapy, particularly for the central nervous system (CNS).

His current projects involve the development of both viral and non-viral vectors, such as engineered extracellular vesicles, through the application of directed evolution screens and machine learning. These efforts are aimed at advancing genome editing modalities and nucleic acid drug development for pediatric neurological disorders within the scope of precision medicine.

Manufacturing of Engineered EVs for biomedical applications

Dhanu Gupta[1,2]

[1]Department of Laboratory Medicine, Karolinska Institutet, Huddinge, Sweden

[2]Institute of Developmental & Regenerative Medicine, University of Oxford, Oxford, United Kingdom

Engineered extracellular vesicles (EVs) present a promising avenue for the targeted delivery of genome editors, such as CRISPR-Cas systems, and biotherapeutic drugs, revolutionizing precision medicine. However, challenges remain in the large-scale production, efficient cargo loading, and specific targeting of EVs to particular cells or tissues. This abstract focuses on emerging strategies that leverage endogenous engineering approaches to enhance the loading and functionalization of EVs. By harnessing the cell's natural EV biogenesis pathways, these methods enable efficient packaging of therapeutic cargo without relying on conventional techniques like electroporation or transfection. Advances in genetic and protein engineering of donor cells are being explored to direct the secretion of EVs loaded with genome editors or therapeutic agents, as well as modifying their surface proteins for improved targeting specificity. These endogenous approaches offer a more streamlined and scalable solution to producing therapeutic EVs, addressing critical challenges in manufacturing, while ensuring high efficiency, biocompatibility, and safety for clinical applications.

Frederik Verweij started his PhD in 2009 in the lab of Dr. Pegtel in Amsterdam (NL) where he studied the sorting of a viral oncoprotein into exosomes. During his PhD, he also pioneered the visualization of exosome secretion from living cells by a TIRF live-imaging approach.

In 2014, he started as a PostDoc at the Curie Institute in Paris (FR) in the labs of Dr. Raposo an Dr. Van Niel to study the physiology of endogenous exosomes in vivo by developing a novel zebrafish model system to study endogenous EVs at high spatio-temporal resolution.

Since September 2021, he started as group leader in the division of Cell Biology, Neurobiology and Biophysics at Utrecht University. His group focusses on continuing the development and application of smart molecular tools to study EV biology, shedding new light on EV cargo loading, biogenesis, trafficking and function.

What goes in, sometimes comes out: developing molecular toolboxes to study the loading, maturation and fate of Multivesicular Bodies

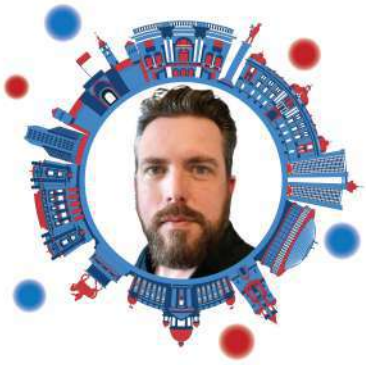
Anna E. George[1,2], Misko Bobeldijk[1,2], Jelle van den Bor[1,2], Maarten P. Bebelman[1,3,4], D. Michiel Pegtel[3], Guillaume van Niel[5,6], Frederik J. Verweij[1,2]

[1]Department of Cell Biology, Neurobiology and Biophysics, Utrecht University, Utrecht, The Netherlands; [2]Centre for Living Technologies, Alliance Eindhoven University of Technology, Wageningen University & Research, Utrecht University, University Medical Center Utrecht, The Netherlands; [3]Department of Pathology, Cancer Center Amsterdam, Amsterdam University Medical Center, Amsterdam, The Netherlands; [4]Division of Medicinal Chemistry, Amsterdam Institute for Molecules Medicines and Systems, VU University, Amsterdam, The Netherlands; [5]Institute for Psychiatry and Neurosciences of Paris, Hopital Saint-Anne, Université de Paris, Institut national de la santé et de la recherche médicale, U1266, Paris, France; [6]CRCI2NA, Nantes Université, Inserm UMR1307, CNRS UMR6075, Université d'Angers, Nantes, France

Exosomes are lipid bound extra-cellular vesicles that are formed within so called Multi Vesicular Bodies (MVBs) by protein- and lipid cargo driven inward budding of the limiting membrane. These MVBs undergo endosomal maturation and release exosomes when they MVBs fuse with the Plasma Membrane (PM) instead of being targeted for lysosomal degradation. The exact mechanism by which endosomes are formed, and in particular 1) loaded with cargo, and are 2) either rendered competent to fuse with the PM to release their exosomal cargo or undergo degradation by fusion with lysosomes is still unclear. We develop and apply molecular tools to decipher these processes by live microscopy.

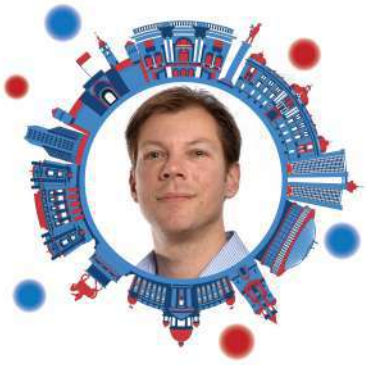
Here we combine a previously developed CD63-based pH-sensitive optical EV reporter with opto- and chemogenetic approaches, and a novel EV-cargo reporter to study EV-cargo loading and MVB maturation and fate. To study this, we combine traditional and advanced microscopy methods, including spinning-disk, lattice-light sheet and dual-color TIRF-microscopy.

We identify exosome secretion as a multi-step process, where MVBs travel to the peri-nuclear area first, before they move towards the cell periphery. This step-wise maturation can render MVBs fusion competent with the PM. During this process, EV-cargo loading with newly synthesized proteins appears to occur via both direct and indirect pathways. Interestingly, MVBs that do not fuse with the PM, also reside in the perinuclear area, where instead, they are marked for degradation. As proposed and strongly suggested by previous results, degradation and secretion appear to be mutually exclusive fates that balance each other out.



Kenneth Witwer is an associate professor of molecular and comparative pathobiology and neurology at the Johns Hopkins University School of Medicine. His research focuses on extracellular vesicles, RNA-mediated regulation, biomarker discovery, and therapeutic modulation of innate and intrinsic defenses. His group is particularly interested in neurodegenerative diseases including the HIV-associated neurocognitive disorders, Alzheimer's, and Parkinson's.

Witwer has served as Secretary General and Executive Chair of Science and Meetings for the International Society for Extracellular Vesicles (ISEV), the leading scientific organization in his field. He has been a scientific advisor to the US National Institutes of Health (Extracellular RNA Communication Consortium, Stage 1) and the US Environmental Protection Agency (FIFRA SAP). He is an associate editor of the Journal of Extracellular Vesicles.



Michiel Pegtel, Amsterdam UMC/Cancer Center Amsterdam, Amsterdam, The Netherlands

The "Exosomes Research Group" headed by Michiel Pegtel at the Amsterdam University Medical center/Cancer Center Amsterdam study i) the molecular dynamics of exosome biogenesis and release ii) small RNA sorting into exosomes with the purpose of developing liquid biopsy strategies and iii) exploiting exosomes and EVs for therapeutic delivery. Intrigued by the discovery of Valadi & Lotvall (Validi et al., Nat Cell Biol, 2007) demonstrating that small extracellular vesicles (EVs) enclose mature miRNAs and can transport functional messenger RNA, Michiel and sunsequently the group of Ochiya were first to demonstrate evidence of functional transfer of miRNAs to recipient cells via exosomes (Pegtel et al., PNAS 2010, Kosaka et al., JBC 2010). In his lecture Michiel will briefly discuss this early work and place this into context of his more recent studies and unpublished ERG findings on miRNA and tRNA sorting into EVs, the latter being a surprisingly highly abundant RNA class in EVs (Scheepbouwer et al., BioRxiv 2023). Apart from basic RNA biology, the ERG aims to translate the knowledge of EV-RNA into clinical applications with a focus on minimally invasive diagnostics for patients with hemato-oncological neoplasms (Drees et al., JEV 2021, Drees et al., J Ex Biol 2024). A recent unpublished study on plasma EV-miRNA sequencing as diagnostic method will be discussed.

EV-RNA from basic science to diagnostic applications

C. Gómez-Martín[1,2*], E.E.E. Drees[1,2*], M.A.J. van Eijndhoven[1,2], N.J. Groenewegen[1,2,9], S. Wang[1,2], S.A.W.M Verkuijlen[1,2], J.R.T. van Weering[3], E. Aparicio-Puerta[4-7], L. Bosch[1,2], K.A. Frerichs2,10, C.P.M. Verkleij[2,10], M.J. Kersten[2,10], J.M. Zijlstra[2,10], D. de Jong[1,2], C.G.M. Groothuis-Oudshoorn12, M. Hackenberg[4-7,9], J.R de Rooij[9], N.W.C.J. van de Donk[2,10], D.M. Pegtel[1,2,9#]

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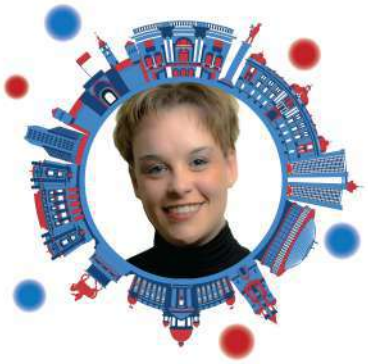
Multiple Myeloma (MM) is an incurable plasma cell neoplasm of the bone marrow with inter- and intra-patient clonal heterogeneity that leads to irregularity of therapeutic responses. Minimally-invasive biomarkers predicting response may help to personalize treatment regimens. We developed a single-nucleotide resolution small RNA sequencing method called IsoSeek (van Eijndhoven et al., Star protocols 2023) to profile thousands of miRNAs and their single-nucleotide variants (isomiRs) in circulating extracellular vesicles (EVs). IsoSeek is currently the most accurate small RNAseq method when compared to 9 other protocols for isomiR detection (Gomez-Martin et al Cell Reports Methods 2023). In this we analyzed >100 plasma samples from 59 MM patients treated with daratumumab-containing regimens and 44 age-gender matched controls. With machine learning, EV-miRNA/isomiR models (signatures) were rigorously trained and tested to predict active disease in relapsed/refractory (RR) MM patients undergoing treatment (AUC EV-isomiRs:0.98,CI:0.94-1.00). We trained another model to forecast response to treatment (AUC EV-isomiRs:0.84,CI:0.66–1.00) in RR MM patients. This model was strongly prognostic for progression-free survival (HR:6.36,CI:2.24-18.07,p<0.0001). Comprehensive targetome and network analysis of isomiR signatures identified MYC and BCL2 as targets including plasma cell expressed miRNAs. We propose plasma EV-isomiR sequencing as tumor-naïve liquid biopsy alternative for invasive bone-marrow biopsies to asses and predict response to treatment and survival outcome for MM patients (Gomez-Martin et al., unpublished). The advantages and disadvantages of EVs as biomarker source will be highlighted.



Rossella Crescitelli is Associate Professor at University of Gothenburg, Sahlgrenska Center for Cancer Research, Sweden. She has fourtheen years of experience in the field of extracellular vesicles. Her research work is mainly focused on the development of methods to isolate subpopulations of extracellular vesicles, particularly those originating from tissues.

Moreover, she has long experience in analysis of extracellular vesicles and tissues by transmission electron microscopy. After completing her Degree in Medical Biotechnologies (2005 – 2010) at University of Naples “Federico II”, she moved in North of Italy for Doctoral studies in Biotechnologies for Human Health, University of Eastern Piedmont “Amedeo Avogadro” (2010-2014). During Doctoral studies, she has been invited by Prof. Jan Lötvall to work at KreDing Research Centre, University of Gothenburg (2011-2013) where she conEnued to work as post doc fellow (2014- 2019). From 2020 she is employed at Sahlgrenska Center for Cancer Research. She opEmized a protocol to isolate subpopulaEons of extracellular vesicles from tumor Essues. The protocol was published in Nature Protocols in January 2021. She has recently received a 4-year grant from the Swedish Research Council to establish her own research group. The focus of her research will be to shed light of the role of extracellular vesicles in the tumor microenvironment.

Exploring Tissue-Derived Extracellular Vesicles: Cancer Biomarkers and Immunotherapeutic Potential

Rossella Crescitelli[1], Kyong-Su Park[2], Su Chul Jang[2], Cecilia Lässer[2], Jan Lötvall[2], Roger Olofsson Bagge[1,3]

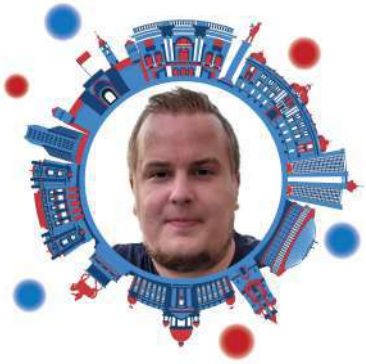
[1]Sahlgrenska Center for Cancer Research and Wallenberg Centre for Molecular and Translational Medicine, Department of Surgery, Institute of Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; [2]Krefting Research Centre, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine at Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; [3]Department of Surgery, Sahlgrenska University Hospital, Region Västra Götaland, Gothenburg, Sweden

Extracellular vesicles (EVs) are nanoscale lipid bilayer paticles crucial for intercellular communication. Their pivotal role within the cancer microenvironment has been explored across various tumor models, elucidating their involvement in cancer cell migration, angiogenesis, and cell survival. Notably, EVs derived from diverse cancer types exhibit promising diagnostic and prognostic utility, holding considerable potential for advancing cancer care.

Current insights into EVs in vivo are limited, relying on cell line models that offer only a partial understanding of their true nature within living organisms. Our study aimed to address this gap by investigating EVs in their native environment. Using advanced transmission electron tomography, we analyzed metastatic and normal liver tissues, revealing distinct spherical structures (30–200 nm) between cells, providing a first 3D visualization of tissue EVs.

We developed a tissue EV isolation protocol and conducted mass spectrometry analysis, uncovering enrichment of mitochondrial membrane proteins in melanoma-derived EVs compared to non-melanoma cells. Notably, mitochondrial inner membrane proteins, MT-CO2 and COX6c, were elevated in in the plasma of melanoma, ovarian, and breast cancer patients. Furthermore, DNA analysis of melanoma-derived EVs showed that the mutant allele frequency was higher in DNA from tumor-derived EVs compared to total DNA isolated from plasma patients. These results underline the potential use of tumor-derived EVs as cancer biomarkers.

Expanding our investigation, we explored the therapeutic potential of melanoma-derived EVs in cancer immunotherapy. Through immunization of mice with synthetic bacterial vesicles (SyBV- bacterial-derived EVs modified to not induce a severe toxic response) and melanomaderived EVs, we observed significant tumor regression in melanoma-bearing mice. This therapeutic effect was mediated by Th-1 type T cell immunity. In conclusion, our study pioneers 3D visualization of tissue EVs and establishes a robust tissue EV isolation protocol. We demonstrate the promising utility of cancer-derived EVs as biomarkers and immunotherapeutic agents, advancing cancer management.



Tobis Tertel studied biochemistry at the University of Bielefeld, earning his degree before embarking on a PhD at the Institute of Transfusion Medicine at the University Hospital Essen. His research focuses on mesenchymal stromal cell-derived extracellular vesicles, aiming to harness their therapeutic properties, particularly for conditions like graft-versus-host disease. Additionally, Tertel is pioneering the use of EVs as diagnostic tools in various diseases.

In 2023, he was appointed to the Executive Board of the German Society for Extracellular Vesicles, underscoring his dedication to advancing EV research both nationally and internationally. His innovative approach combines clinical parameters, cytokine profiling, and cell analysis with EVs to enhance disease diagnosis.

Tertel also actively contributes to the global research community through his involvement in the International Society of Gene and Cell Therapy, where he is an early stage professional on the Exosome Committee, and the International Society of Extracellular Vesicles. His ultimate goal is to combine traditional medical approaches with EV technology to create robust diagnostic and therapeutic strategies for clinical practice, ensuring efficacy and safety through established quality control platforms.

Extracellular Vesicles as Diagnostic and Therapeutic Biomarkers in Stress, Cancer, and Neurological Disorders

Tobias Tertel

Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

Introduction: Extracellular vesicles (EVs) serve as promising non-invasive biomarkers with potential to enhance diagnostics and therapeutic monitoring across diverse diseases, including cancer, neurological disorders, and stress-related conditions. EVs reflect dynamic cellular changes by transporting molecular cargo, offering insights that go beyond traditional diagnostics. However, inconsistencies in sample handling, processing, and isolation introduce pre-analytical variability, which limits the reliability of EV-based diagnostics. In this study, we directly address these challenges and advance the use of EVs as precision biomarkers across multiple disease contexts.

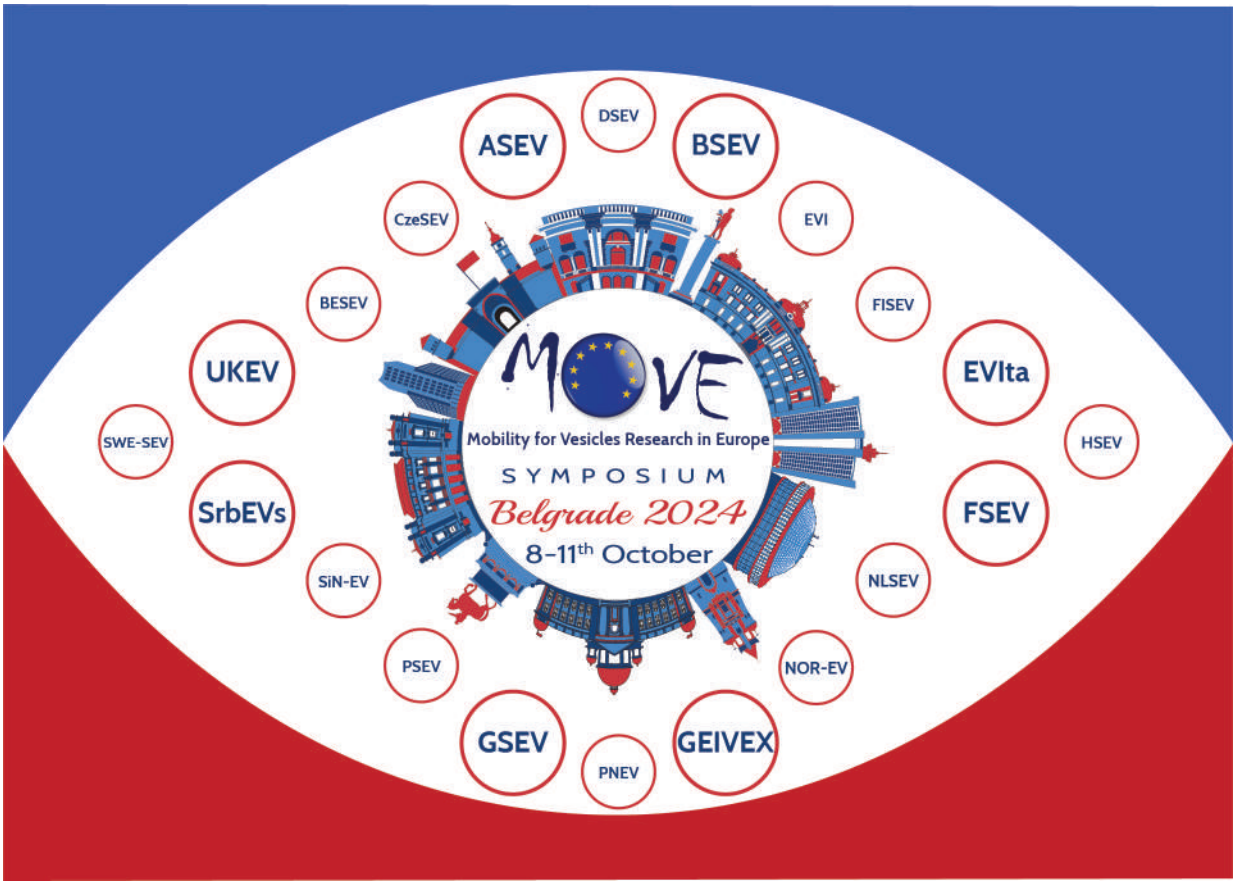
Methods: We collected and analyzed over 3,000 biofluid samples, including plasma, serum, cerebrospinal fluid, and urine, from patients with HER2-positive breast cancer, meningeosis, lung and head and neck cancer, stroke, multiple sclerosis, and individuals exposed to psychosocial or physical stress. We optimized pre-analytical protocols by standardizing centrifugation and anticoagulant parameters to ensure consistency in EV isolation. We used advanced imaging flow cytometry to characterize EV subpopulations and assess their diagnostic relevance across these disease conditions.

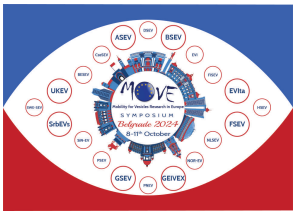
Results: By optimizing pre-analytical conditions, we significantly improved the reproducibility and accuracy of EV profiling across all biofluids. In HER2-positive breast cancer, HER2+ EVs strongly correlated with disease progression and treatment response, demonstrating their predictive value. Similarly, we identified distinct EV profiles in stroke and multiple sclerosis that correlated with disease severity, positioning EVs as reliable biomarkers for neurological conditions. In stress-related conditions, EVs exhibited unique patterns that correlated with the intensity and type of stress exposure, reinforcing their role as dynamic, real-time physiological markers. These findings suggest that EVs can serve as universal biomarkers to enhance diagnostic precision across multiple medical fields.

Conclusion: This study demonstrates the transformative potential of EVs in diagnostics and therapeutic monitoring. By addressing pre-analytical variability and employing advanced imaging technologies, we show that EVs not only complement but also extend the capabilities of traditional diagnostic methods. These findings highlight the ability of EVs to provide disease-specific insights that could revolutionize personalized medicine. We recommend further research to integrate EV-based diagnostics into clinical practice, refining and expanding their utility across broader medical contexts.

Basic EV research: Biogenesis/release of EVs and their function in signal transmission

- oral presentations -





O-B-1

Unlocking Ovarian Cancer Biomarkers: Insights from Ascitic Extracellular Vesicles

Pospíchalová V.[1] Vyhlídalová Kotrbová A.[1], Gömöryová K.[1], Mikulová A.[1], Kravec M.[1], Potěšil D.[2], Zdráhal Z.[2], Kotouček J.[3], Bednaříková M.[4], Hausnerová J.[4], Weinberger V.[4], Blériot C.[5], Ginhoux F.[5], Bryja V.[1]

[1]Faculty of Science, Masaryk University, Brno, Czech Republic; [2]Central European Institute of Technology, Masaryk University, Brno, Czech Republic; [3]Veterinary Research Institute, Brno, Czech Republic; [4]University Hospital Brno and Medical Faculty, Masaryk University, Brno, Czech Republic; [5]Institut Gustave Roussy, Villejuif, France

Introduction: High-grade serous carcinoma (HGSC) of the ovary, fallopian tube, and peritoneum is the most common and lethal subtype of ovarian cancer. Despite advancements, effective biomarkers for managing HGSC remain limited, hindering early detection and personalized treatment. Many HGSC patients develop ascites, a fluid buildup in the peritoneal cavity, creating a complex tumor microenvironment (TME) rich in extracellular vesicles (EVs), which may serve as potential biomarkers.

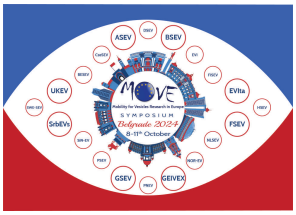
Methods: We isolated EVs from the ascites of HGSC patients using orthogonal methods and analyzed their protein content using mass spectrometry. Single-cell RNA sequencing was integrated to trace the cellular origin of these EVs, with flow cytometry used for profiling of ascitic cells.

Results: Contrary to expectations, the majority of EVs in HGSC ascites were derived from non-malignant cells, such as macrophages and fibroblasts, rather than tumor cells. Moreover, cell type-specific EV markers provided more accurate prognostic insights than ascitic cells.

Conclusion: Our findings highlight the potential of proteomic analysis of ascitic EVs to effectively complement cellular analysis in delineating the full composition of the HGSC TME. These insights offer new avenues for improved patient stratification based on the TME and the development of diagnostic and therapeutic strategies in HGSC by leveraging EV-associated biomarkers.

Funding information: This work was supported by the project National Institute for Cancer Research (Programme EXCELES, ID Project No. LX22NPO5102) – funded by the European Union – Next Generation EU.

CIISB, Instruct-CZ Centre of Instruct-ERIC EU consortium, funded by MEYS CR infrastructure project LM2023042 and European Regional Development Fund-Project "UP CIISB" (No. CZ.02.1.01/0.0/0.0/18_046/0015974), is gratefully acknowledged for the financial support of the measurements at the CEITEC Proteomics Core Facility and Cryo-electron microscopy and tomography core facility CEITEC MU.



O-B-2

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

Miguel Palma-Cobo[1], Victor Toribio[1], Carlos Enrich Bastús[2], Albert Lu[2], **María Yáñez-Mó[1].**

[1]Dept Biología Molecular, Universidad Autónoma de Madrid, IUBM, Centro de Biología Molecular Severo Ochoa, IIS-IP, Madrid, Spain: [2] Universidad de Barcelona, Facultad de Medicina, IDIBAPS, Barcelona, Spain

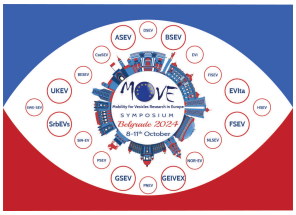
Introduction: The outstanding potential of Extracellular Vesicles (EVs) in medicine, deserves a detailed study of the molecular aspects regulating their incorporation into target cells. We have performed a Genome Wide CRISPR (GWC) screen by flow cytometry sorting, to identify possible molecular candidates that may regulate EV uptake.

Methods: 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 $500x10^6$ cells and $3.6x10^{12}$ 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.

Results: 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

Conclusion: Our data clearly supports the involvement of the COMMANDER complex in EV uptake and cargo delivery.

Funding information: This work has been supported by grants PID2020- 119627GB-I00 and DTS21/00134 from Ministerio Español de Ciencia e Innovación (Spain) from to MY-M.



O-B-3

MBLAC2, a new player in the EV field?

Guillaume Médard[1,2]

[1]Technical University of Munich, Germany; [2]National and Kapodistrian University of Athens, Greece.

Introduction: HDAC6 inhibitor tubacin has been shown to produce a strong vesicle phenotype that cannot be attributed to HDAC6 inhibition. An off-target protein is therefore likely at play.

Methods: We developed a chemoproteomics assay for drug profiling, which features an affinity matrix made out of three hydroxamate molecules immobilized on microbeads. We then profiled 53 drugs, including tubacin, using a dose-dependent competition assay against this affinity matrix, followed by mass-spectrometry based bottom-up proteomics readout.

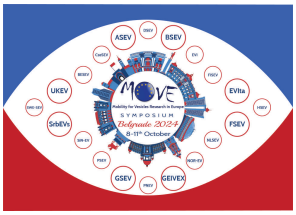
Additionally, the supernatant of HEK293 cells treated with controls or MBLAC2 inhibitors or siRNA pools where processed by qEV size exclusion chromatography and nanoparticle tracking analysis.

Results: We found that 24 hydroxamate HDAC drugs are also low nanomolar metallo-β-lactamase domain-containing protein 2 (MBLAC2) inhibitors, including tubacin. Importantly ACY-738 was found to be HDAC6-selective, while its structural analogue ACY-775 was a dual HDAC6/MBLAC2 inhibitor, making them an ideal set to study the role of poorly studied MBLAC2. We discovered that MBLAC2 inhibition or knockdown leads to the accumulation of extracellular vesicles in HEK293 cell culture.

Conclusion: The surprising identification of MBLAC2 as a frequent HDAC drug off-target led to its contextualization in extracellular vesicles biology. MBLAC2 inhibition effect on vesicles levels might be synergistic with HDAC inhibition in certain pathologies and might alone be a therapeutic target.

Reference: Lechner, S., Malgapo, M.I.P., Grätz, C. *et al.* Target deconvolution of HDAC pharmacopoeia reveals MBLAC2 as common off-target. *Nat Chem Biol* **18**, 812–820 (2022).

Funding information: Deutsche Forschungsgemeinschaft (DFG), SFB 1309, projects 401883058 & 407391208.



Dynamic interactors: Galectins and EVs

Anna-Kristin Ludwig[1], Maria-Anthi Kakavoulia[1], Peter Spies[2], Tanja-Jasmin Kutzner[3], Bernd Giebel[3], Tobias Tertel[3], Hadi Karimzadeh[1], Xavier Blanchet[4], Sophie Franzmeier[5], Bernd Giebel[3], Herbert Kaltner[1]

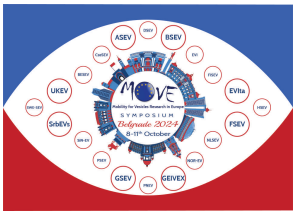
[1]Physiological Chemistry, Department of Veterinary Sciences, Faculty of Veterinary Medicine, Ludwig-Maximilians-Universität Munich, Germany; [2]University of Applied Sciences, School of Life Sciences, Muttenz, Switzerland; [3]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Germany; [4]Institute for Cardiovascular Prevention (IPEK), Ludwig-Maximilians-Universität Munich, Germany; [5]Department of Neuropathology, Institute of Pathology, Technical University Munich, Munich, Germany

Introduction: Mesenchymal stem cells (MSCs) revealed promising therapeutic effects in various clinical applications, primarily through paracrine signaling, through their extracellular vesicles (EVs), serving as effective mediators of cell-free therapy, circumventing safety concerns. Galectins are carbohydrate-binding proteins with promising immunomodulatory properties. Here, we investigate the relationship between MSC-EVs and galectins caused by the findings of their co-existence in EV preparations.

Methods: We used different methods (ELISA and Flow cytometry) to detect the presence and binding of Galectin-1 and -3 to MSC EVs. To provide insights into the strength of interactions between EVs and the molecules of interest, such as galectins and the CD63 antibody, we used Flow- Induced Dispersion Analysis (FIDA).

Results: Flow cytometric analysis showed double positive populations for galectin- and tetraspanin-positive EVs, as well as single galectin positive particles. The effective binding number of EVs (EC50) with FIDA could be determined. Different concentrations of the CD63 antibody showed that low concentrations lead to a more effective binding and that it depends on the interaction time. For galectin-1 and -3, we detected weak binding to MSC EVs because we were working with particle numbers under the EC50. We could detect clear binding for galectin-1 and -3 for standard EVs from Serum.

Conclusion: Our results confirm that galectins are present in EV preparations and further interact directly with each other by active binding. These findings suggest that EVs and galectins are natural dynamic interactors in vitro and ex vivo.

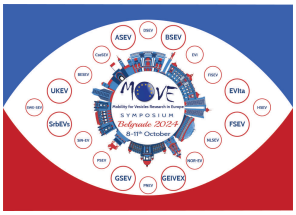


The role of extracellular vesicles in KRAS/STK11 co-mutated NSCLC immune escape

Gangadhara Prasad, Durai Muniswami, Vakil Ahmad, **Chiswili Yves Chabu**

Biological Sciences, University of Missouri, Columbia, MO, USA

Oncogenic KRAS signaling cooperate with STK11 loss of function mutations to give rise to aggressive lung cancers that are resistant to immunotherapy. The underlying mechanisms are not fully understood. We found that oncogenic KRAS and STK11 loss of function mutations cooperatively stimulate the small G-protein ARF6. In turn, ARF6 activation triggers the release of immunosuppressive extracellular vesicles (EV) that potently promote tumor immune evasion in a mouse model of KRAS/STK11 co-mutated Non-Small Cell Lung Cancer (KS-NSCLC). Inhibition of ARF6, reprograms the tumor immune microenvironment into an active state and correspondingly suppresses tumor size. We will describe the immuno-modulating effects of ARF6-dependent EV in the setting of KS-NSCLC. Our findings suggest that targeting ARF6 will sensitize KS-NSCLC to immune surveillance and immunotherapy.



O-B-6

Extracellular vesicles promote migration despite BRAF inhibitor treatment

Afrodité Németh[1], Gréta L. Bányai[1], Nikolett K. Dobos[1], Tamás Kós[1], Anikó Gaál[2], Zoltán Varga[2], Edit I. Buzás[3,4,5], Delaram Khamari[3], Magdolna Dank[6], István Takács[6], A. Marcell Szász[6], Tamás Garay[1,6]

[1]Péter Catholic University, Faculty of Information Technology and Bionics, Budapest, Hungary; [2]Research Centre for Natural Sciences, Institute of Materials and Environmental Chemistry, Biological Nano-chemistry Research Group, Budapest, Hungary; [3]Department of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary; [4]ELKH-SE Translational Extracellular Vesicle Research Group, Budapest, Hungary; [5]HCEMM-SE Extracellular Vesicle Research Group, Budapest, Hungary; [6]Semmelweis University, Department of Internal Medicine and Oncology, Division of Oncology, Budapest, Hungary

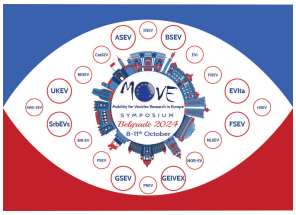
Introduction: Malignant melanoma (MM), one of the deadliest skin cancers, is characterized by high metastatic potential and frequent resistance to therapies. Approximately half of melanoma patients harbor BRAF mutations, highlighting the importance of BRAF-targeted therapies, such as vemurafenib and dabrafenib, used as single treatment or in combination with the MEK inhibitor trametinib. Extracellular vesicles (EVs) play a critical role in metastasis formation and the development of resistance in cancer. Therefore, we aimed to investigate EVs' contribution to cancer formation by evaluating the impact of EVs on cell proliferation, sphere growth and cancer cell migration (i.e. a crucial early step in metastasis formation). Moreover, EVs' involvement in drug resistance was examined under vemurafenib, dabrafenib, trametinib and dabrafenib-trametinib combined therapies.

Methods: The impact of EVs, isolated from the supernatants of a pair of syngeneic melanoma cell lines, was investigated on the respective cell lines' proliferation and migration. Primary tumor growth was examined through cell viability and spheroid growth assays, while migration was analyzed determining mean squared displacement (MSD) and total traveled distance (TTD) using video microscopy and single-cell tracking. Additionally, the ability of EVs to mediate resistance to vemurafenib, dabrafenib, trametinib, and the combination of dabrafenib and trametinib was assessed using video microscopy and single-cell tracking.

Results: Our findings indicate that EV treatments had a more substantial effect on cell migration as compared to cell proliferation and spheroid growth. Notably, EVs were able to mitigate the inhibitory effects of BRAF inhibitors, but they were ineffective against the MEK inhibitor and the combination of BRAF/MEK inhibitors.

Conclusion: In summary, our study enhances the understanding of the complex role of EVs in tumor progression, metastasis, and drug resistance in MM.

Funding information: This research was supported by the National Research, Development and Innovation Office through the grant TKP2021-EGA-42.



O-B-7

Neutrophil-derived extracellular vesicles modulate the inflammatory response of monocytes and macrophages

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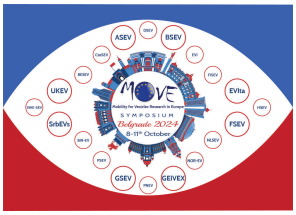
Introduction: Extracellular vesicles (EVs) are released by every known cell type, their ability to transferbioactive molecules to recipient cells make them crucial mediators of intercellular communication. Our laboratory formerly characterized three different types of neutrophil- derived (PMN) extracellular vesicles: released spontaneously, generated upon opsonized zymosan treatment and produced by apoptotic cells. Our previous experiments showed that these PMN-EVs exert either anti- or proinflammatory effects on neutrophils. The aim of our current work to investigate the effect of PMN-EVs on partner immune cells viability and effector functions.

Methods: Neutrophils and monocytes were isolated from human blood, macrophages were differentiated via M-CSF. Spontaneously (spEV), upon opsonized particle stimulation (oZ-EV) and during apoptosis (apoEV) generated EVs were isolated by two-step centrifugation and filtration. EVs effect on viability was observed by flow cytometry (FC) and LDH cytotoxicity assay. Monocytes ROS production was monitored by lucigenin assay, cytokine production was measured by ELISA and CD11b expression were determined by FC. The macrophage differentiation was investigated by FC and fluorescent microscopy, the migration was measured by scratch assay.

Results: Monocyte viability was significantly improved by apoEV. SpEV significantly delayed monocytes' ROS production and reduced LPS-induced IL-8 production. A significant CD11b expression level decrease was occurred on spEV-treated monocytes, which can explain the observed effects. SpEV and apoEV significantly increased the number of differentiated macrophages and apoEV treatment led to an increase in migration capacity.

Conclusion: Neutrophils are key players in inflammation. Pro-inflammatory PMN-EVs regulate processes in the early phase of inflammation. During resolution phase, PMNs interact with monocytes and macrophages to promote tissue homeostasis by releasing anti-inflammatory EVs.

Funding information: EFOP-3.6.3-VEKOP-16-2017-00009; NKFIH FK137770; TKP2021-EGA-24; János Bolyai Research Scholarship of the Hungarian Academy of Sciences



TGF-β induces cholesterol accumulation to regulate the fate of tumor-derived extracellular vesicles

Dorival Mendes Rodrigues-Junior[1]*, Chrysoula Tsirigoti[1,2] ,Konstantina Psatha[3], Dimitris Kletsas[4] ,Michalis Aivaliotis[3], Carl-Henrik Heldin[1], Aristidis Moustakas[1]*

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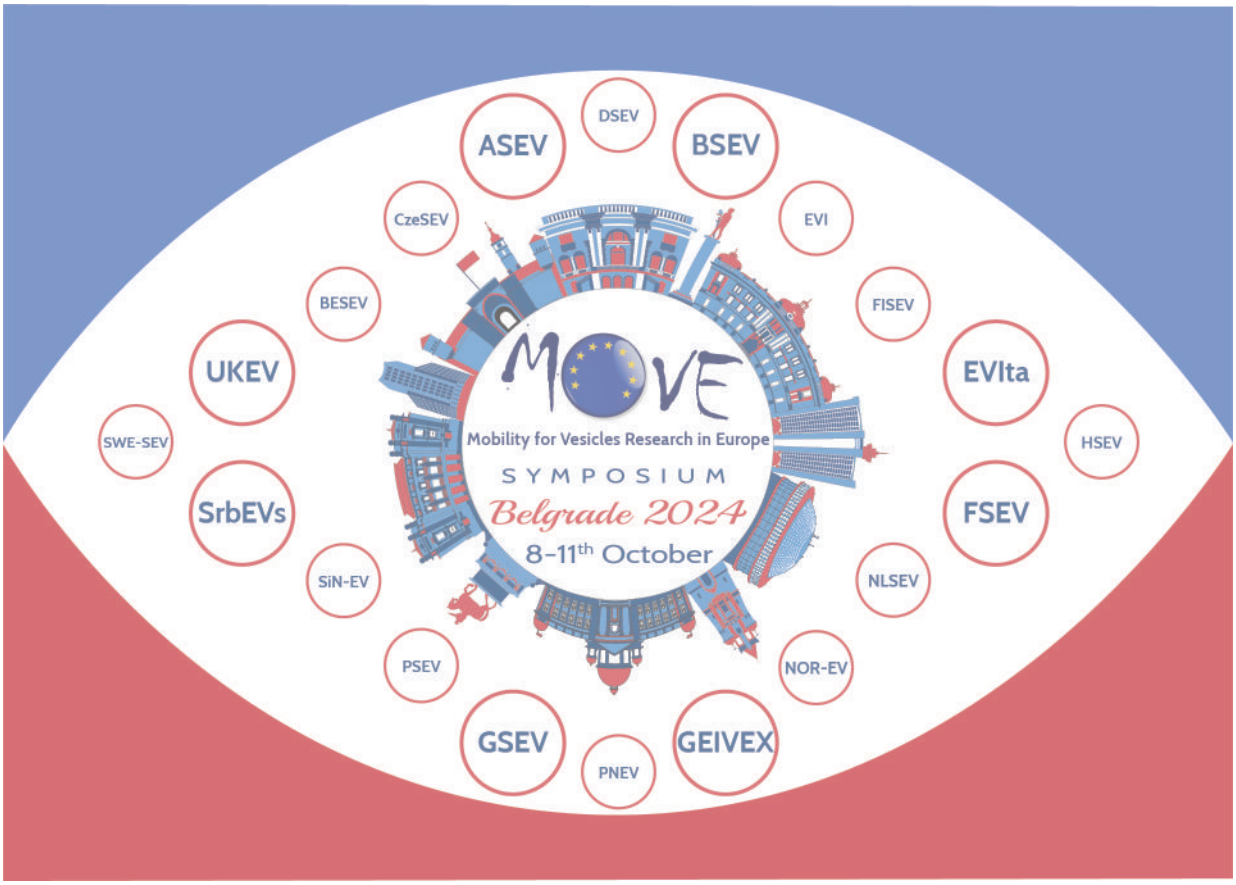
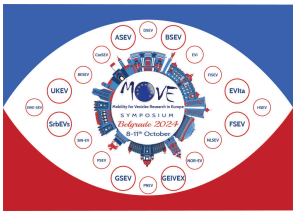
Cancer cells are avid extracellular vesicles (EVs) producers during all disease stages. EVs transport transforming growth factor-β (TGF-β), which has been steadily featured in cancer biology and commonly activated under late stages of cancer progression. Nevertheless, whether TGF-β signaling coordinate EV biogenesis is a relevant topic that remains largely unexplored, so we sought after specific mediators in this pathway that could regulate EV biogenesis. We report that TGF-β, acting via its signaling type I receptor, increased human breast and lung cancer EV release by activating MEK-ERK1/2 signaling. ERK1/2 acted by phosphorylating sterol regulatory element-binding protein-2 (SREBP2) that transcriptionally induces 7-dehydrocholesterol reductase (DHCR7) expression, thus raising intracellular cholesterol abundance. Upon examining several alternative components of EV biogenesis, beyond cholesterol, TGF-β seemed capable of regulating expression of some, yet with inconsistent kinetic or signaling specificity. Proteomic profiling revealed that EVs contained TGF-β pathway-related molecules, including matrix metalloproteinase (MMP)-9. Furthermore, we could monitor specific enrichment of several cargo constituents, and confirmed distinct EV proteomic identity, upon TGF-β stimulation of EV-secreting cells. Additionally, EVs activated TGF-β signaling, even when EV uptake was blocked by heparin, while MMP inhibitor (MMPi) or proteinase treatment blocked EV-mediated TGF-β signaling, suggesting that EVs induced signaling from cell surface TGF-β receptors. Pro-migratory potential transferred by EVs also utilized cargo MMPs, since MMPi abrogated EVs induced invasion and motility. Finally, EVs transferred chemoresistance to recipient cells and inhibition of MEK or cholesterol synthesis, which reduced EV secretion, sensitized cancer cells to chemotherapeutic drugs. Hence, we delineated a novel signaling cascade that leads to high rates of EV generation by cancer cells in response to TGF-β, with cholesterol being a key intermediate step in this mechanism.

Funding: Swedish Cancer Society, grants CAN2018/469 and CAN2021/1506Pj01H (AM); the Swedish Research Council, grant 2018-02757 and 2023-02865 (AM); Lars Hierta Memorial Foundation, grant FO2023-0501 (DMRJr); O.E. och Edla Johanssons Vetenskapliga Stiftelsen (DMRJr).

Basic EV research: Biogenesis/release of EVs and their function in signal transmission

- poster presentations -





P-B-1

Extracellular Vesicles and MBsomes Intercellular Communication in Skin Wound Healing

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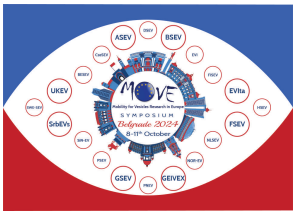
Introduction: Skin wound healing involves interaction between skin cells and consists of four phases: hemostasis, inflammation, re-epithelialization, and tissue remodeling. Orchestrated cross-talk between epidermal and dermal skin cells, keratinocytes and fibroblasts respectively, plays a dominant role in the re-epithelialization phase. Extracellular vesicles (EVs) are membrane bound vesicles released by cells into the extracellular environment carrying proteins, lipids and genetic material. In addition, MBsomes (MBs), could be classified as a new class of large ectosomes that are released by cells, at the final stage of cytokinesis, to act as postmitotic signaling organelles. Whether MBs serve as a mean of intercellular communication in skin wound healing, to our knowledge, is not investigated yet. The aim of this project is to decipher the biogenesis, release and signaling function of MBs, the new EV to be, in skin wound healing. Our hypothesis is that MBs released from hyperproliferating epidermal skin keratinocytes, along with other EVs, are a mean of epidermal/dermal cross-talk in skin wound healing.

Methods: To address our hypothesis, several approaches were used, including purification of EVs by differential ultracentrifugation followed by SEC, and purification of MBs by differential ultracentrifugation, from epidermal keratinocytes. Characterization of EVs and MBs size and concentration by NTA, protein markers by WB, morphological validation by TEM, surface markers expression by IEM, identification of the cargoes of MBs and EVs by transcriptomics and proteomics. In addition, their function in skin wound healing: migration and proliferation were also assessed by scratch-wound assay and Brdu assay, respectively.

Results: Our results show that epidermal keratinocytes release a new subtype of EVs, MBs, from the midbody bridge during cytokinesis. Interestingly, epidermal keratinocytes EVs and MBs promote migration and proliferation of dermal fibroblasts via ERK activation.

Conclusion: Primary keratinocytes release EVs and MBs which play a role in the epidermal/dermal crosstalk in skin wound healing.



P-B-2

Proteomic and lipidomic profiling of extracellular vesicles from tetraspanin-deficient cell lines

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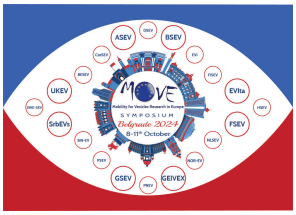
Introduction: Tetraspanins are crucial proteins involved in the biogenesis, release, and uptake of extracellular vesicles (EVs). While recent studies have explored single tetraspanin knockouts, a comprehensive study on the knockout (KO) of three major tetraspanins (CD9, CD63, and CD81) is lacking.

Methods: We isolated EVs derived from four cell lines: the wild type DU145 cell line and three tetraspanin-deficient cell lines (CD9, CD63, CD81 KO) created via CRISPR-Cas9 gene editing. The LC-MS/MS was employed to analyse the proteomic composition of EVs and targeted HPLC-MS/MS determined the levels of phospholipids (PL), sphingolipids (SLs) and glycosphingolipids (GSLs) in the EVs isolated by ultracentrifugation coupled to sucrose cushion.

Results: Our findings indicate that the absence of tetraspanins leads to significant changes in proteomic profiles as well as in lipid metabolism. Biological analyses of upregulated and downregulated proteins from each cell line identified multiple overrepresented processes and pathways. Importantly, this is the first study reporting enrichment of EV GSLs after CD9, CD63 or CD81 suppression.

Conclusion: Each tetraspanin fulfils a distinct role in EV formation and release. Our study provides a detailed description of the proteomic and lipidomic characteristics of EV lacking tetraspanins, contributing valuable insights to the field of EV research.

Funding: This work was supported by the project National Institute for Cancer Research (Programme EXCELES, ID Project No. LX22NPO5102) – funded by the European Union – Next Generation EU. CIISB, Instruct-CZ Centre of Instruct-ERIC EU consortium, funded by MEYS CR infrastructure project LM2018127, is gratefully acknowledged for the financial support of the measurements at the CEITEC Proteomics Core Facility.



P-B-3

Extracellular vesicles act faster than you think; Rapid increase in MFGE8 secretion from endometrial cells is an indicator of embryo maternal cross-talk

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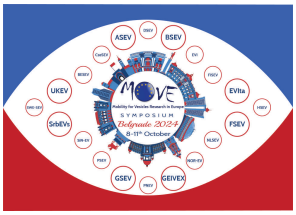
Introduction: Synchronized crosstalk between the embryo and endometrium during embryo implantation is critical for pregnancy establishment. The role of extracellular vesicles (EVs) in facilitating this process has been established. We previously showed that trophoblast-derived EVs can increases the secretion of the Milk fat globule epidermal growth factor 8 (MFGE8) protein from endometrial epithelial cells (EECs) using an *in vitro* embryo implantation model. In the present study, we further evaluated the dynamics of MFGE8 protein secretion during embryo implantation.

Methods: Receptive endometrial cell analogue RL95-2 cells were co-incubated with estrogen and progesterone hormone combinations that mimic luteal and proliferative phases of the menstrual cycle, and MFGE8 expression was analyzed. Then trophoblast cell analog JAr cell-derived EVs were co-incubated with both receptive (RL95-2) and non-receptive EECs (HEC-1A) for 24 h. Furthermore, RL95-2 cells were also co-incubated with JAr EVs in a concentration (1×10^3, 1×10^5 , 1×10^7 and 1×10^9 EVs/ml for 24 h) and time gradient (30 min, 6 h and 24 h), and MFGE8 secretion was analyzed.

Results: The estrogen and progesterone hormone combination suppressed the MFGE8 secretion from RL95-2 cells compared to the untreated control. JAr EVs increased MFGE8 secretion from both receptive and non-receptive EECs. JAr EVs showed dose and time dependent increase in MFGE8 secretion from RL95-2 cells. Notably, the secretion of MFGE8 increased within a short timeframe of 30 min post-EV treatment, suggesting potential rapid binding, receptor-ligand interaction or fusion of embryonic EVs with the EEC membrane.

Conclusion: Increased MFGE8 secretion in EECs was influenced by hormones, endometrial cell receptivity status, EV dose, and the duration of EV-cell interaction. This suggests that MFGE8 secretion may serve as a potential indicator of degree of EV-mediated embryo-maternal crosstalk and could be used to further elucidate the exact mechanisms of EV function.

Funding information: This research was funded by European Union's Horizon 2020 research and innovation programme under grant agreement No 857418 COMBIVET and The European Union through Horizon coordination and support actions under grant agreement No. 101079349 OH-Boost



P-B-4

Physiological vs. Traditional EV Administration: Improved Distribution and Reduced Toxicity with Slow-Release Pumps

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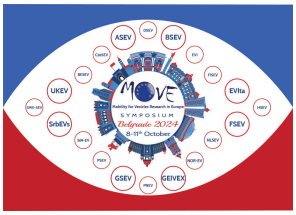
Introduction: Extracellular vesicles (EVs) are essential mediators of intercellular communication, carrying a variety of bioactive molecules such as proteins, RNA, and lipids. The method of EV administration significantly impacts their biodistribution and effectiveness. This study aims to compare the physiological EV secretion rates with common EV injection methodologies and assess the effectiveness of an osmotic slow-release pump in delivering EVs.

Methods: We employed an osmotic slow-release pump (Alzet pump) to administer EVs at a controlled, sustained rate and compared this with the traditional injection methods (intraperitoneal (IP) and intravenous (IV)). Biodistribution and concentration of EVs were monitored in various organs, including the pancreas and liver, over multiple time points (Day 3, Day 6, Day 9, and Day 14). The concentration of EVs in these organs was measured using particle tracking analysis. Additionally, the study evaluated the negative side effects associated with high-concentration EV injections.

Results: The osmotic slow-release pump demonstrated a more consistent and physiological distribution of EVs across the targeted organs compared to traditional injection methods. The concentration of EVs in the pancreas on Day 3 and Day 6 was significantly higher with the pump method, showing 7.88e+08 particles/ml and 1.21e+09 particles/ml respectively. Traditional injection methods led to a localized, short-term, and intense cellular response with potential adverse effects, including cellular saturation and toxicity. The slow-release method mitigated these issues, providing a more stable and prolonged delivery of EVs.

Conclusions: The study highlights the limitations of robust single EV injections, such as localized impact and potential toxicity, emphasizing the benefits of using an osmotic slow-release pump for EV delivery. This method ensures a more physiological and sustained release of EVs, enhancing their therapeutic potential and reducing adverse effects. Future research should focus on optimizing the slow-release delivery system for clinical applications and further exploring its impact on EV-mediated therapies.



P-B-5

Role of the different intracellular pathways in the study of the biogenesis of exosomes

Deborah Polignano[1], Valeria Barreca[1], Valentina Tirelli[2], Lucia Bertuccini[2], Antonella Tinari[2], Massimo Sargiacomo[1], Maria Luisa Fiani[1]

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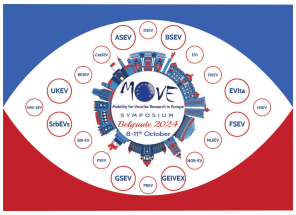
Introduction: Exosomes are small extracellular vesicles (sEV) formed within late endocytic compartments/multivesicular bodies (MVB). Various machineries have been described to regulate their biogenesis, however several aspects of these processes have not been fully elucidated. We developed a methodology to obtain fluorescent exosomes (Bodipy exo) by using Bodipy FL C16. To gain insight into exosome biogenesis we combined this approach with the use of a panel of inhibitors of vesicular trafficking.

Methods: Bodipy exo secreted by melanoma cells pulsed with BODIPY FL C16 and treated with inhibitors of cellular pathways were isolated by differential ultracentrifugation and quantified by Flow Cytometry (FC) and Nanoparticle Tracking Analysis (NTA). Colocalization between Bodipy exo and tetraspanins was determined by FC. Characterization of Bodipy exo protein content was performed by Western Blot analysis. Finally, Transmission Electron Microscopy (TEM) analysis of cells treated with inhibitors was also performed.

Results: Significant differences were observed in the secretion of Bodipy exo as evaluated by FC and NTA upon treatment of melanoma cells with selected inhibitors of cellular pathways (U18666A, Trimeprazine, GW4869, Desipramine, Bafilomycin-A1, Cytochalasin D). Interestingly, with the exception of Bafilomycin-A1 and Brefeldin A treated cells, the percentage of tetraspanins (CD63, CD81, and CD9) positive Bodipy exo, was similar. However, striking differences were observed by Western blot analysis on the total sEV population even when inhibitors treatment did not affect exosomes secretion. TEM analysis showed variations in the intracellular number of MVBs and of intraluminal vesicles within MVBs.

Conclusion: Our results indicate that the use of selected inhibitors of intracellular pathways affects not only the secretion of sEV, but also their protein composition. This suggests that alterations in cellular pathways can induce modifications in protein trafficking, consequently impacting the exosome biogenetic pathway. In conclusion, this approach has the potential to provide further insights into the process of exosome biogenesis.

Funding information: This work was supported by the Italian Ministry of Health (grant RF-2019-12369719)



P-B-6

Anti-Inflammatory and Immunomodulatory Strategies as Therapeutic Approaches for Rab27a-Deficient Pancreatic Cancer

Daniel Lopes[1,2], Nuno Bastos[3], Carolina Dias[1,4], Bárbara Adem[5], Inês Batista[1,6], Sofia Almeida[1], Bárbara Morão[7], Pedro Moutinho-Ribeiro[11,12], Sara Alves[1,4], Carlos A. Melo[8], Guilherme Macedo[11,12], Marília Cravo[9,10], Fátima Carneiro[1,4,11], José C. Machado[1,12,13], Sónia A. Melo[1,12,13]

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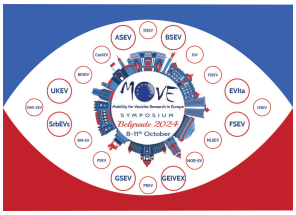
Introduction: Pancreatic ductal adenocarcinoma (PDAC) is among the deadliest cancers, with limited treatment options and poor prognosis. Despite advances in immunotherapy for other cancers, its efficacy in PDAC remains low, necessitating new immunomodulatory strategies to improve patient outcomes. Exosomes, mediators of intercellular communication, transport molecular information between tumor cells and distant organs, reprogramming recipient cells, particularly within the immune system. This study aims to understand how PDAC cells reprogram the immune response to identify novel immunomodulatory treatments for PDAC. Rab27a is crucial for exosomes secretion, and we identified a subset of PDAC patients (~25%) lacking Rab27a expression in their cancer cells.

Methods: Using in vivo PDAC models, we assessed the functional consequences of Rab27a loss in cancer cells. We developed a genetically engineered Rab27a knockout (KO) mouse model that spontaneously develops PDAC, mirroring human disease histopathology.

Results: Rab27a KO in PDAC cells led to earlier disease onset and reduced survival. These tumors exhibited a pro-inflammatory response, characterized by the recruitment of myeloid $CD11b^{+}MRP8^{+}$ cells. This response induced IL-6 production by cancer-associated fibroblasts, leading to the differentiation of $CD4^{+}$ T cells into a Th17 pro-tumor phenotype. Notably, $CD4^{+}$ T cell depletion or dexamethasone treatment impaired tumor growth exclusively in Rab27a KO tumors. Our findings were validated by demonstrating that PDAC patients with low Rab27a expression and high intratumoral $MRP8^{+}$ cell counts have significantly worse prognoses.

Conclusion: Our data suggest that targeting the Th17 response with anti-inflammatory and immunomodulatory strategies in PDAC patients lacking Rab27a expression could serve as a novel therapeutic approach.

Funding information: FCT - Foundation for Science and Technology



P-B-7

EGFR-mutant lung cancer derived EVS induce resistance by activating PI3K/AKT signalling pathway

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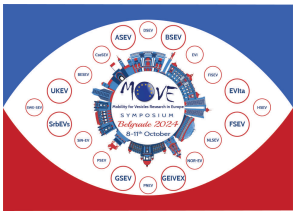
Emerging research suggests that extracellular vesicles (EVs) from drug-resistant tumour cellscan convey a drug-resistant phenotype to sensitive cells. However, it remains unclear whether EVs released by EGFR T790M-mutant-resistant non-small cell lung cancer (NSCLC) cells to gefitinib can transfer this resistance to sensitive cells.

In this study, we isolated EVs from the serum of lung cancer paHents harbouring the T790M mutation and exhibiting resistance to gefitinib and treated the sensitive PC9 cell line withthese EVs. We explored the role and mechanism of EVs in regulating gefitinib resistance in vitro. EV-derived miRNA-128 expression profiles from PC9 cells and patients with lung cancer were analysed using qRT-PCR.

Our results demonstrated that EVs isolated from the serum of patients with lung cancer harbouring the EGFR T790M mutation and resistant to gefitinib could transfer gefitinib resistance to PC9-sensitive cells in vitro by activating the PI3K/AKT signalling pathway. RT-PCR confirmed that miR-128 was differentially expressed in these EVs. Our findings reveal a critical mechanism of acquired resistance to EGFR-TKIs in NSCLC.

Funding information: FCT - Foundation for Science and Technology



P-B-8

Imaging flow cytometry enables studies of the interactions between extracellular vesicles and immune cells

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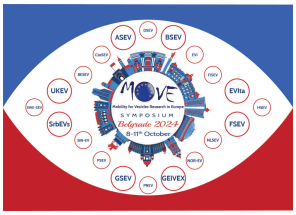
Introduction: Extracellular vesicles (EVs) play a vital role in intercellular communication, and blood serves as a rich source of EVs. We are investigating EVs derived from blood cell concentrates and aim to understand and characterize their biological functions. Few previously published studies show that platelet and red blood cell (RBC) derived EVs can target specifically certain mononuclear cell population, namely monocytes. We have studied the interaction and uptake of platelet and RBC-EVs into prostate cancer cells and mononuclear cells and have seen significant differences between these two types of EVs. Next, we aim to further understand the uptake and influence of these EVs on macrophages.

Methods: Nanoparticle tracking analysis was used to determine particle number and size distribution. Imaging flow cytometer was used to characterize CD235a and CD41 positive particles. In addition, the presence of EV marker proteins and co-isolates were detected with western blot analysis. Cell phenotyping and EV uptake studies were done with imaging flow cytometer and results were confirmed with fluorescence microscopy.

Results: Imaging flow cytometer allowed analysis of EV uptake on a statistically representing number of cells. There was significantly more interaction between platelet EVs and mononuclear cells compared to RBC-EVs. The work with macrophages is on-going but preliminary results show differences also between M1 and M2 macrophages.

Conclusion: We conclude that imaging flow cytometry can be used to study the distinctive differences in the interaction and uptake of EVs with different immune cells.

Funding information: Business Finland Grant EVE Consortium 2346/31/2019



P-B-9

Developing microglia cell lines expressing Nef to study the role of extracellular vesicles in HIV neuropathology

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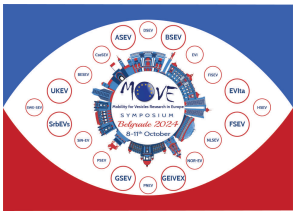
Introduction: Microglia represent one of the main reservoirs of latent HIV-1 in the brain. Greater than 95% of proviruses are defective, however they are translationally competent and produce HIV-1 proteins, including Nef. We similarly showed that HIV-infected microglia release extracellular vesicles (EVs) containing Nef *in vitro*, and that Nef is detected even in the blood of aviremic, infected individuals. Nef, an early HIV-1 protein, is the key factor for viral pathogenesis and is also implicated in HIV-associated neurocognitive disorders (HAND). To better understand the role of Nef-containing EVs in HAND, we here developed human microglia cell lines expressing HIV-1 Nef and characterized EVs released from these cells.

Methods: Cells of human microglia were transduced by lentiviral vectors encoding NefSF2-EGFP and EGFP with inducible promotor for doxycycline. Prior to EV isolation, cells were analyzed for number and viability, transduction efficiency using flow cytometry and morphology via (live cell) fluorescence microscopy. EVs were isolated from conditioned media by sequential centrifugation followed by iodixanol density gradient separation. EVs morphology was characterized by TEM, concentration and size by NTA, and protein content by immunoblotting. Nef-EGFP/EGFP content was quantified using nano-Flow Cytometry (FCM) and an in-house Nef ELISA.

Results: Forty-eight hours after induction, 80–99.9% of transduced microglia were expressing NefSF2-EGFP or EGFP. In the case of Nef-EGFP expressing microglia, $2.89*10^9$ EVs per 10^8 cells and $4.03*10^8$ EVs per 10^8 were detected by NTA and nano-FCM, respectively. Of the latter, 24.3% EVs were positive for Nef-EGFP. In comparison, microglia expressing only EGFP released $1.56*10^9$ per 10^8 cells (NTA) and $6.62*10^8$ per 10^8 cells (nano-FCM), respectively. Microglia Nef-EGFP and EGFP EVs had average mode diameters of 140.5 (±8.7) nm and 132.3 (±5.9) nm, respectively. Both EV populations carried typical EV-associated proteins like Flotillin, Alix, GAPDH, CD81, and CD9, and additional proteins Nef.EGFP or EGFP, and SERINC. As measured by ELISA, 21.8 ng of Nef was detected per 10^9 Nef-EGFP EVs.

Conclusion: Stable Nef-expressing microglia release Nef-carrying EVs. Next, we will use this cellular model to perform Nef EVs functional studies.

Funding information: The Slovenian Research Agency grant P1-0170 and young researcher scholarship.



P-B-10

Investigating the Influence of Cardiomyocyte-Derived Extracellular Vesicles on Macrophage Activation: A Comparative Analysis between Doxorubicin and DL-11

Tamás Bolyky[1], Luigi Menna[1], Annamária Huszár[1], Eszter Lajkó[1], Angéla Takács[1], Zsófia Szász[1], László Kőhidai[1], Gábor Mező[2], Levente Dókus[2], Edit I. Buzás[1] and Hargita Hegyesi[1]

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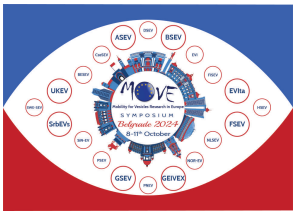
Introduction: Doxorubicin (Dox), an anti-cancer drug, is well-known for its detrimental effects on the heart, leading to severe cardiac damage and local inflammation. Extracellular vesicles (EVs) carry specific cargo molecules that can modulate target cells. This study aimed to explore the impact of cardiomyocyte-derived EVs (CM-EVs) on macrophage activation. We investigated the direct effects of Dox and its conjugate, DL-11, on HL-1 mouse cardiomyocytes and peritoneal macrophages isolated from C57/Bl mice. Additionally, we compared the effects of EVs derived from both Dox-treated and DL-11-treated cardiomyocytes on macrophage viability and gene expression.

Methods: HL-1 cells were exposed to Dox or DL-11, and cell viability was evaluated using the xCELLigence platform. EVs were isolated from the conditioned media. The uptake of CM-EVs was monitored using confocal microscopy and flow cytometry. Macrophages were treated with isolated cardiomyocyte-derived EVs (CM-EVs), and their viability and inflammatory response were assessed. RNA was extracted from macrophages and differences in gene expression were analyzed via qPCR.

Results: Treatment with DL-11 exhibited lower cytotoxicity towards both cardiomyocytes and macrophages compared to Dox. Dox-CM-EVs decreased the viability of targeted macrophages. EVs from Dox-treated cardiomyocytes displayed gene expression patterns associated with early aging and inflammation, while DL-11-treated EVs induced fewer changes.

Conclusion: Our investigation into the impact mediated by cardiomyocyte-derived EVs revealed a propensity towards accelerated cellular aging and the presence of an inflammatory phenotype in macrophages. Nevertheless, this effect was less pronounced when HL-1 cells were treated with DL-11, resulting in fewer significantly altered genes. Furthermore, we observed that macrophages expressed senescent-related genes when exposed to both treatments, albeit with a slower rate of change and milder response to stress signals in the case of DL-11. These findings suggest that DL-11 may offer a more favorable side effect profile and could be integrated into anti-tumor therapeutic approaches in the future.

Fundings: Therapeutic Thematic Programme TKP2021-EGA-23; RRF-2.3.1-21-2022-00003 (National Cardiovascular Laboratory Program); 2019-2.1.7-ERA-NET-2021-00015;



P-B-11

Exploring the Biodistribution of p53-Containing EVs for Targeted Cancer Therapy

Ishai Luz[1], Gil Shalev[2], Doaa Massad[1], Tomer Cooks[1], Alexander Tendler[2]

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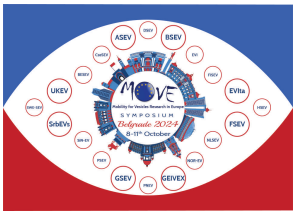
Introduction: Extracellular vesicles (EVs) have emerged as promising tools for targeted delivery of therapeutic agents. EVs derived from corneal epithelial cells (CECs) are of particular interest due to their unique properties. These EVs contain the tumor suppressor protein p53 and play a crucial role in the local anti-cancer defense mechanisms of the cornea. This study aims to investigate the biodistribution of CECs EVs containing wild-type p53 in mice, and to validate their potential for targeting specific organs and tumors, laying the groundwork for future therapeutic applications.

Methods: EVs were harvested from CECs and purified using an IZON qEV10 35nm column. Characterization was performed using nanoparticle-tracking analysis (NTA). EVs were then fluorescently labeled with DiR and injected intravenously to athymic nude or C57Bl/6 mice (˜1x1012 EVs/ml per animal). The biodistribution was monitored using in vivo imaging (NEWTON 7.0 real-time imager) at several time points post-injection (5, 24, and 48 hours).

Results: We found that EVs predominantly accumulate in the liver, lungs, and spleen shortly after injection, with lower presence in the brain and the heart. This effect continued beyond the initial 5 hours, with substantial accumulation in those organs at 24 and 48 hours. Injections of diluted amounts of EVs showed consistent biodistribution patterns, validating that EV staining is dose-dependent and restricted to the blood circulatory system. Both the heart and brain exhibited lower amount of staining across all experimental conditions, yet it was still higher than that of the dye-only control.

Conclusion: This study provides evidence that EVs derived from CECs can be efficiently delivered to specific organs in mice. The consistent biodistribution patterns and effective targeting highlight the potential of these EVs as a delivery vehicle of p53. Future research should focus on optimizing EV formulations and further exploring their therapeutic efficacy in various cancer models and tumor-bearing mice. These findings pave the way for developing p53 replacement therapies targeting challenging cancer types harboring inactivated or mutated p53, leveraging the natural targeting abilities of EVs for precision medicine.

Funding information: *ExoProTher Medical Ltd.*



P-B-12

Unraveling the Role of Galectins in Mesenchymal Stromal/Stem Cell-Mediated Immunomodulation through Extracellular Vesicles

Tanja Jasmin Kutzner[1], Tobias Tertel[1], Yanis Mouloud[1], Bernd Giebel[1]

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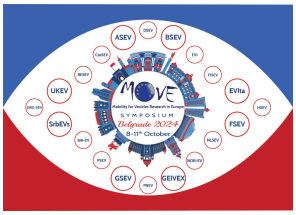
Introduction: Mesenchymal stromal/stem cells (MSCs) possess significant therapeutic potentials due to their ability to modulate immune responses and promote regeneration, primarily through paracrine signaling, especially via extracellular vesicles (EVs). However, the specific mechanism by which MSC-EVs exert their therapeutic function remains unclear. Galectins are crucial players in various physiological and pathological processes by recognizing and bridging glycans on plasma membranes, thereby controlling diverse immune responses. Given that certain galectins, especially galectins 1, 3, and 9, are expressed in MSCs and their function has been linked to EV biology, they might essentially contribute to the therapeutic function of MSC-EVs.

Methods: In our effort to effectively translate MSC-EVs as a new pro-regenerative agent into the clinics, we have established clonally immortalized MSCs (ciMSCs). These cells enable scaled manufacturing of MSC-EV products for the clinical setting and can be effectively genetically manipulated, providing an ideal platform for dissecting the mechanism of action of MSC-EVs. Here, we used CRISPR/Cas-9 technology to delete the coding regions of galectins 1, 3, and 9 in ciMSCs, exploring the impacts of the deletion on the biological properties of the cells and the immunomodulatory function of resulting EV products.

Results: In our ongoing experiments, deletion of galectin-1 did not notably alter the biological properties of the genetically engineered ciMSCs or their EV products. Currently, we explore the impacts of the Galectin 3 and 9 knock-outs, as well as various galectin knock-out combinations in future studies.

Conclusion: In summary, the function of Galectin-1 appears dispensable for the immunomodulatory properties of ciMSC-EV products, as evidenced by the reduction of the activated T cell pool within the mdMLR assay. Further experiments are required to fully understand the role of Galectin-1 for the immunomodulatory potential of MSC-EVs and its contribution to the mechanism of action of ciMSC-EVs.

Funding information: Stammzellnetzwerk NRW, Stammzell-IntraNetz.NRW 005-2305-0037



P-B-13

Characterization of mast cell-derived extracellular vesicles during degranulation

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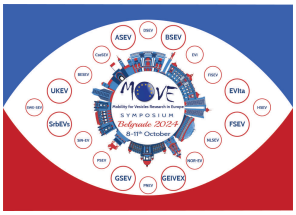
Mast cells (MCs) play a crucial role in allergic reactions and are implicated in numerous diseases, where degranulating MCs are involved in inflammatory reactions including atherosclerosis. Beyond the well documented release of cytokines, degranulating MCs also secrete extracellular vesicles (EVs). However, despite extensive research in the area, the differentiation of EVs and granules from one another remains poorly explored. Therefore, this project aims to characterise and differentiate MC-derived EVs from extracellular granules released during degranulation providing a deeper understanding of their role in diseases. This knowledge could spark the development of novel diagnostic assays, therapeutic targets and improved methodologies.

EVs were isolated from bone marrow derived murine MCs from both wild type and GFP-expressing transgenic mice with differential and density gradient centrifugation and were investigated using Flow cytometry, confocal microscopy, TEM and biochemical assays. MCs were stimulated with calcium ionophore or DNP/IgE. Cell activation and recovery was monitored by beta-hexosamainadase, proliferation and viability assays.

We established a model system to monitor real time EV production during degranulation by flow cytometry and confocal microscopy. Our findings indicate that during degranulation MCs secrete a heterogeneous particle population. These particles exhibit variations in size, concentration and detergent sensitivity suggesting a simultaneous production of EVs, extracellular granules and membrane fragments. Furthermore, the composition of the secreted corpuscles depends on the stimuli used to induce degranulation. These results highlight the challenges and critical importance of distinguishing MC-derived EV secretion and degranulation from one another while emphasizing the necessity for precise characterization in future research.

Funding: NVKP_16-1-2016-0004, VEKOP-2.3.2-16-2016-00002, VEKOP-2.3.3-15-2017-00016, TKP2021-EGA-23, RRF-2.3.1-21-2022-0000, 2019-2.1.7-ERA-NET-2021-00015, EUH2020-739593, ÚNKP-23-3-I-SE-2



P-B-14

EVs biogenesis and secretion machinery in distinct Alzheimer's disease mimicking models

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Introduction: Alzheimer's disease (AD) is a neurodegenerative disorder and the main cause of dementia worldwide. AD is neuropathologically characterized by the aggregation of two proteins, the Aβ peptide into senile plaques and the hyperphosphorylated tau into neurofibrillary tangles, two of the main hallmarks of AD. Extracellular vesicles (EVs), especially exosomes, are secreted by all cell types and recognized as important mediators in cell-to-cell communication. In the context of AD, it has been reported that EVs can carry Aβ and tau, therefore being potentially involved in disease pathogenesis. However, the impact of Aβ itself on EVs biogenesis and secretion in AD in not completely understood.

Methods: Two AD mimicking models were used, namely N2a neuroblastoma cells treated with Aβ peptide and SH-SY5Y cells carrying the Swedish mutation of APP695, which also render in increased Aβ production. The levels of proteins involved in EVs biogenesis and secretion were monitored in these two AD mimicking models by WB.

Results: Significant differences were observed for proteins involved in EVs biogenesis, in both ESCRT dependent and independent pathways; and in EVs secretion, as Rab proteins. Interestingly, different results were obtained for the two AD mimicking models, supporting that distinct cell models uses different EVs-related machinery.

Conclusion: These results support that EVs biogenesis and secretion are altered in AD mimicking conditions. Since EVs can be involved in cell communication and signaling events, data suggest that alterations in the EVs machinery may potentially contribute to abnormal mechanisms underlying AD pathogenesis.

Funding information: This work was supported by iBiMED under Grant UIDB/04501/2020 and UIDP/04501/2020; MV is supported by the individual PhD grant UI/BD/151354/2021 and TMS by the individual PhD grant SFRH/BD/145979/2019.

EVs in health and disease

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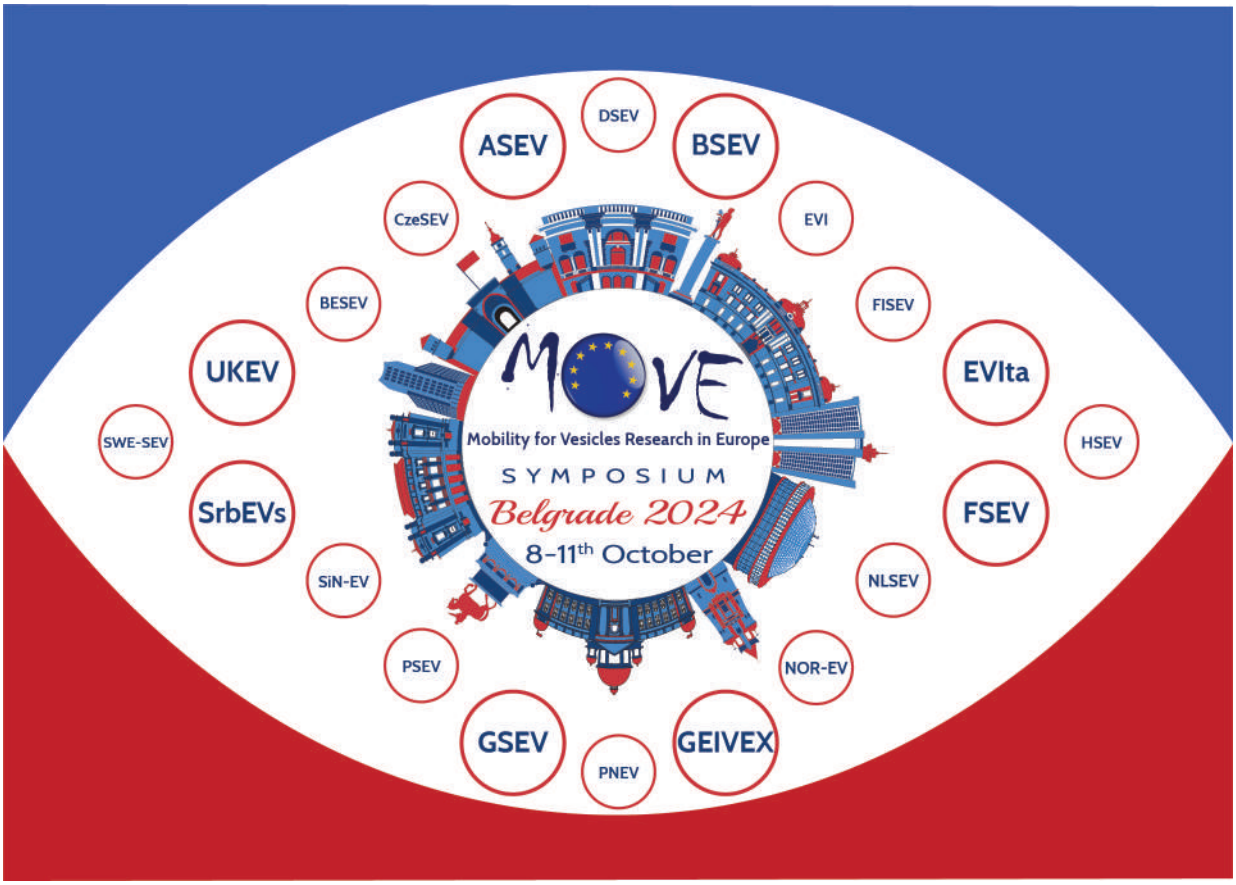

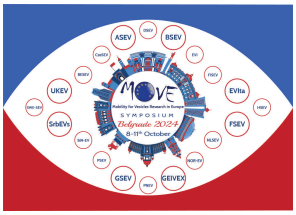

Role of cardiomyocyte-derived extracellular vesicles in post-ischemic cardiac remodeling

Lélia Borowski[1], Paul Alayrac[2], Vincent Duval[3], Jean Sébastien Silvestre[1], Chantal M. Boulanger[1], Xavier Loyer[1], Stéphane Camus[1]

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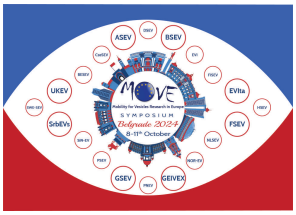
Cardiovascular diseases remain the leading cause of death worldwide. Following myocardial infarction (MI), inflammatory cells are mobilized to the injured myocardium from distant compartments to coordinate tissue remodeling. Soluble mediators are known to drive local inflammation, but their short half-life restricts their range of action. Extracellular vesicles (EV) encapsulate biologically active substances for delivery to target cells, making them ideally fitted for long-range inter-organ communication. In this work, we aimed at determining how cardiomyocyte-EVs (CM-EVs) released during MI shape the inflammatory response and cardiac remodeling.

Using two genetic mouse models expressing CM-specific Cre recombinase, alone (Myh6-Cre) or in combination with mT/mG fluorescent reporters (Myh6-Cre/mTmG), we determined the protein composition of purified CM-EVs by proteomic analysis. We assessed CM-EV biodistribution *in-vivo* by monitoring GFP fluorescence in various remote organs after MI by flow cytometry and 2-photon microscopy and defined the identity of CM-EVs target cells by flow cytometry. To investigate the interaction between CM-EV and their target cells, we incubated CM-EVs with primary resident macrophages and visualized interaction by imaging flow cytometry. We then monitored GFP expression by flow cytometry as a proxy for functional transfer of biological material from CM-EVs to macrophages.

First, we showed that our EV comply with proteins MISEV recommendations. Proteomic analysis shows that CM-EV MI were enriched with mitochondrial proteins. Then, we demonstrate CM-EVs are preferentially targeting splenic and lung macrophages after MI. We validated an interaction between EV and resident macrophages and confirmed CRE transfer from CM-EVs to macrophages as shown by GFP expression in recipient macrophages.

Our data show that CM-EVs generated upon MI establish long-range, inter-organ communication routes and target innate macrophages from the spleen and lungs.

Funding: FRM EQU202003010767



O-HD-2

Extracellular Vesicle-Derived miR-3753p Promotes Fetal Overgrowth in Pregnancies Complicated by Maternal Diabetes

Manon Owen[1], Rachel Quilang[1], **Abigail Byford**[1], Leander Stewart[1], Melanie Reay[1], Naima Endesh[1], Simon Futers[1], Neil Turner[1], Nadira Yuldasheva[1], Jayne Charnock[2], Rebecca Spencer[1], Niamh Forde[1], Beth Holder[3], Susan Ozanne[4], Eleanor M Scott[1], Karen Forbes*[1]

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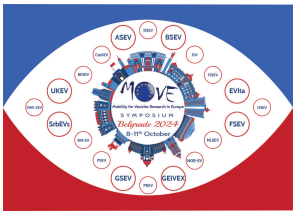
Introduction: Gestational Diabetes (GDM) commonly results in largeforgestationalage (LGA) infants who have increased cardiometabolic risks compared to appropriateforgestationalage infants. We have previously shown that the maternal circulating extracellular vesicle (EV)miRNA signature is altered in GDM pregnancies prior to LGA onset. Some of these EVmiRNAs are also altered in term placental tissue of GDMLGA pregnancies. One of the altered EVmiRNAs, miR3753p, is pancreas specific. We hypothesised that hyperglycaemia increases maternal pancreas release of EVs containing miR3753p which traffic to the placenta, influencing fetal growth in GDM pregnancies.

Methods: Human pancreatic islets were cultured under normal or mild hyperglycaemic conditions (57mM; 3 days; n=3). EVs isolated from conditioned media were characterised following MISEV guidelines, maleimide488labelled and applied to term human placental explants (24hrs). EV uptake and miR-375-3p levels were assessed by fluorescent microscopy and qPCR, respectively. miR3753p (n=7), negative control (NC) (n=6/7) mimics (1mg/kg) or vehicle (n=5) were delivered to healthy pregnant C57BL6/J mice via tail vein injection at E11.5, E13.5, E15.5. At E18.5, pups/placentae were weighed and placental miRNA/mRNA expression assessed by qPCR. Human placental explants from uncomplicated pregnancies were transfected with miR3753p mimics before performing TMT proteomics and functional enrichment analysis (FEA).

Results: Pancreatic isletderived EVs expression of miR-375-3p increased under hyperglycaemic conditions and were internalised into human placental tissue, resulting in increased levels of miR3753p. Pregnant mice treated with miR-375-3p demonstrated increased placental miR3753p levels, placental weight (from 0.0941g to 0.1150g; p=0.00007) and fetal weight (from 1.212g to 1.306g; p=0.022), compared to NC, adjusted for fetal sex/litter size. miR3753p altered the human placental proteome, where differentially expressed proteins were mapped to pathways associated with placental growth and metabolism. These changes were also observed in mouse placenta.

Conclusion: Maternal pancreatic EVs traffic miR3753p to the placenta in GDM pregnancies, where miR3753p contributes towards LGA by altering placental development and function.

Funding Information: This work was supported by funding from the UKRI Medical Research Council (MR/R023166/1) and a British Heart Foundation four-year PhD studentship (FS/4yPhD/F/20/34130).



Extracellular vesicles shed by PDAC cells harboring mutant p53 remodel the metastatic niche and promote hematopoietic differentiation

Tomer Cooks

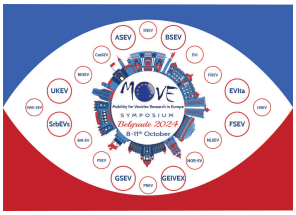
Ben-Gurion University of the Negev

Introduction: Extracellular vesicles (EVs) play a crucial role in intercellular communication within the tumor microenvironment. This study investigates the specific effects of EVs derived from pancreatic ductal adenocarcinoma (PDAC) cells with gain-of-function (GOF) mutant p53 on the metastatic niche and hematopoietic differentiation. Utilizing NIR labelling, biodistribution analysis in immunocompromised mice revealed preferential accumulation of mutant p53-containing EVs in the liver and lungs, consistent with PDAC metastatic tropism.

Methods: Crispr-CAS9 KO of mutp53 allowed us to use several preclinical models (KC and KPC as a murine model, PANC-1 as a human model) to study the metastatic pattern driven by mutant p53 EVs. Full proteomics of the EVs unraveled an ECM-remodeling signature associated with mutant p53 and full blood and bone-marrow analyses was instrumental for looking at immune cell populations.

Results: Mass spectrometry identified upregulated tumor-supporting proteins, notably integrin ß4 (ITBG4), selectively packaged in mutant p53-containing EVs. ITBG4 has been associated with organotropic metastasis, suggesting a novel GOF mutant p53-dependent pathway in EV cargo selection. Functional assays demonstrated that priming with mutant p53 EVs significantly promoted metastasis to the liver and lungs compared to EVs lacking mutant p53, indicating their potency in creating a pre-metastatic niche. Mutant p53 EVs exerted profound effects on immune cell populations. In immune-competent mice, EVs derived from PDAC cells with GOF mutant p53 induced significant increases in granulocytes, monocytes, and MDSC precursor populations in peripheral blood, alongside alterations in B and T-cell subsets.

Conclusions: Overall, this study highlights the role of mutant p53 EVs in remodelling the metastatic niche and modulating hematopoietic differentiation, providing insights into the complex interplay between mutant p53-driven oncogenesis and the tumor microenvironment. Understanding these mechanisms may inform the development of targeted therapies for PDAC metastasis.



O-HD-4

Developing liver spheroids as a model to investigate the role of colorectal cancer derived small extracellular vesicles in hepatic pre-metastatic niche establishment

Elisa Costanzo[1], Marzia Pucci[1], Ornella Urzì[1], Marco Loria[1], Martina Vinci[1], Gabriele Antonuccio[1], Alice Conigliaro[1], Maria Cristina Guerrera[2], Riccardo Alessandro[1], Simona Fontana[1]

[1]Department of Biomedicine, Neuroscience, and Advanced Diagnostics, University of Palermo, Italy; [2]Department of Veterinary Sciences, University of Messina, Italy.

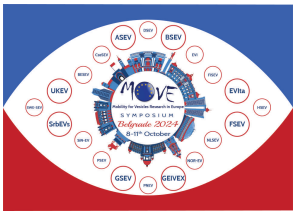
Introduction: Growing evidence shows the key role of colorectal cancer derived small extracellular vesicles (CRC-SEVs) in shaping the hepatic pre-metastatic niche (h-PMN), a supportive and receptive microenvironment for cancer cells colonization. However, the effects of the CRC-SEVs have been widely described on non-parenchymal cells, and only marginally on hepatocytes. Nevertheless, our previous study showed CRC-SEVs' ability to induce in healthy hepatocytes epithelial-mesenchymal transition, early event leading to liver fibrosis (LF), pivotal feature of h-PMN. Being LF overly complex to be represented in bidimensional systems, we developed a tridimensional model of human healthy hepatocytes spheroids (h-HeSphs) for studying the evolution of LF induced by CRC-SEVs.

Methods: h-HeSphs obtained by seeding human healthy hepatocytes (THLE-2) in ultra-low attachment 96 wells, were treated with CRC-SEVs isolated from CRC SW480 cells.

Results: h-HeSphs morphology, physical parameters, and functional properties were examined, revealing a spheric and compact shape, homogenous diameter, weight, and mass density, and the expression of several hepatocyte functional markers. After evaluating the CRC-SEVs uptake by h-HeSphs, the CRC-SEVs' ability to alter h-HeSphs' properties was investigated. The treatment with the CRC-SEVs determined an alteration in h-HeSphs' morphology and physical parameter, and a significant decrease of the expression level of hepatocyte functional markers. Moreover, CRC-SEVs induced an increased expression of mesenchymal markers and a higher deposition of fibronectin, marker of fibrosis. According to the key supportive role that the fibrotic environment can have during the tumor cells invasion, by co-culturing the h-HeSphs with the metastatic CRC cells SW620, we observed that CRC-SEVs treatment enhanced tumor cell invasiveness.

Conclusion: These results show h-HeSphs suitability to study LF and reveal that CRC-SEVs induce hepatocytes to acquire an active role in h-PMN shaping, thus supporting liver metastatic colonization.

Funding information: PRIN 2022, Grant number: 2022ETPX42_002



O-HD-5

Effect of Hypercholesterolemia on circulating and cardiomyocyte-derived extracellular vesicles

Csenger Kovácsházi[1,*], Szabolcs Hambalkó[1,*], Nabil V. Sayour[1], Tamás G. Gergely[1], Gábor B. Brennerv, Csilla Pelyhe[1], Dóra Kapui[1], Bennet Y. Weber[1], Alexander L. Hültenschmidt[1], Éva Pállinger[2], Edit I. Buzás[2,3,4], Ádám Zolcsák[5], Bálint Kiss[5,10], Tamás Bozó[5], Csilla Csányi[5], Nikolett Kósa[5], Miklós Kellermayer[5,10], Róbert Farkas[6], Gellért B. Karvaly[6], Kieran Wynne[7,9], David Matallanas[7], Péter Ferdinandy[1,8], Zoltán Giricz[1,8]

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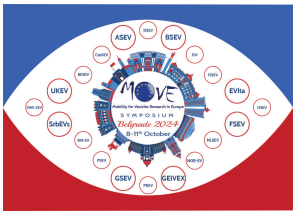
Introduction: Hypercholesterolemia (HC) induces, propagates and exacerbates cardiovascular diseases (CVDs) via various mechanisms that are yet not properly uncovered. Analyzing how HC dysregulates extracellular vesicles (EVs) can lead to further understanding in the connection between HC and CVDs. Our aim is to assess whether and how HC affects circulating and cardiomyocyte (CM)-secreted EVs and how can these changes affect the myocardium.

Methods: Circulating EVs were isolated with density gradient ultracentrifugation followed by size exclusion chromatography using Vezics system from male Wistar rats fed with high-cholesterol or control chow. Plasma and EV metabolome was analyzed using a Biocrates MxP Quant 500 kit. AC16 human CMs were treated with Remembrane HC supplement and EVs were isolated from cell culture supernatant with ultracentrifugation. Samples were analyzed with nanoparticle tracking analysis, and atomic force microscopy. Monocyte activation was measured in THP1-ASC-GFP cells after treatment with AC16-EVs. AC16-EV proteomics was measured with liquid chromatography-tandem mass spectrometry.

Results: HC diet induced hyperlipidemia in rats and reduced the amount of certain phosphatidylcholines in circulating EVs. Furthermore, plasma EV metabolome showed only minor correlation with that of the plasma. HC treatment significantly increased EV secretion of AC16 CMs but did not affect their elasticity and total phosphatidylcholine concentration. AC16 EVs, regardless of the treatment, did not induce the activation of THP1 monocytes. HC treatment modified AC16 EV metabolome greatly, with 77 enriched proteins and 33 proteins with decreased abundance. No specific enrichment or reduction of any well-defined molecular pathways was identified among the dysregulated proteins, however some these proteins contribute to tissue remodeling.

Conclusions: HC greatly affects metabolome of circulating EVs. Furthermore, it induces secretion and modifies proteome of CM EVs. These changes do not affect monocyte activity and may contribute to HC-induced cardiac remodeling.



O-HD-6

Exercise-induced extracellular vesicles in breast cancer

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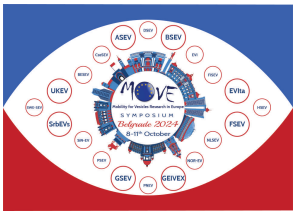
Introduction: Studies demonstrate that physical activity reduces tumor incidence, however, the molecular mechanisms behind these effects are poorly understood. The beneficial effects of physical activity are also mediated by factors secreted into the circulation during exercise, not only as soluble molecules, but also associated to extracellular vesicles (EVs). The aim of this study is to explore the interplay between exercise, EVs, and breast cancer (BC). This may offer insights into EV-based novel therapeutic strategies and preventive measures for cancer.

Methods: Plasma EVs were isolated by size exclusion chromatography (SEC) from both human and mice running-trained healthy females. EVs were characterized using electron microscopy, nanoparticle tracking analysis (NTA), and western blot. Mass spectrometry was used to analyze EV protein composition. Tumor-bearing mice were administered with exercise-induced EVs and the immune response was assed using flow cytometry.

Results: Mass spectrometry analysis on female running-trained human plasma-EVs showed that thioredoxin, a key antioxidant enzyme, was the protein with the highest fold change after exercise. NTA results showed that plasma EV concentration is higher in running-trained Balb/c mice. Moreover, administration of exercise-induced plasma EVs into a triple-negative BC syngenic mice model delayed tumor growth by 19-57%. Finally, we observed differences in tumor-infiltrating immune cells, with a substantial influx of CD8+ T lymphocytes in the exercise-induced EV treated groups.

Conclusion: These results reveal a role for exercise in modulating the presence of antioxidant molecules in EVs, which could protect tissues from oxidative stress and provide a novel mechanism to explain the beneficial effects of physical activity. Moreover, our study demonstrates that treatment with exercise-induced EVs have immunomodulatory effects that may promote an antitumor immune response. These findings support the incorporation of physical activities in the treatment plans of BC patients and provide a rationale for further investigations of EVs as potential exercise mimetics.

Funding information: This work was supported by EEA and Norway Grants under project No EEA-RESEARCH-164 (CancerBeat)



O-HD-7

Horizontal transfer of long non-coding RNA H19 transports splicing factors in recipient cells

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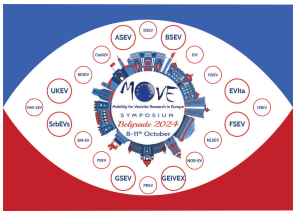
Introduction: cancer progression strongly depends upon intercellular communication established in the tumour microenvironment, and tumour-derived small extracellular vesicles (TD_SEVs) play a pivotal role in this process. It has been demonstrated that TD_SEVs allow pre-metastatic niche formation and promote epithelial-to-mesenchymal transition (EMT). In cancer, the EMT process is prodromal to the metastatic event. Recent evidence highlighted the role of alternative splicing (AS) in the gene expression rearrangement required for EMT, however, there are no data relating TD_SEVs to AS. Our studies are focusing on the molecular cargo transferred by colorectal cancer-derived extracellular vesicles (CRC_SEVs) to the hepatocytes, the most abundant cellular type residing in the liver, to shed light on the molecular mechanisms driving liver metastases of CRC.

Methods: CRC_SEVs were isolated from the conditioned media of SW480, SW620 and HCT-116 cell lines through ultracentrifugation. The SEVs were resuspended in PBS, RNA lysis buffer or RIPA buffer respectively. RNA and protein content were assessed through qRT-PCR and Western Blot. An healthy hepatocytes cell line, the THLE-2 were treated with 20 µg/mL of CRC_SEVs and the biological effects were investigated through qRT-PCR, Immunofluorescence and RNA *in situ.*

Results: our data demonstrated that the long non-coding H19, in CRC, is associated with RNA binding proteins (RNABPs) involved in AS including RBFOX2, whose activity in promoting EMT-related AS has been largely demonstrated. Surprisingly, we found that CRC_SEVs transport both lncH19 and RBFOX2 and the treatment of human hepatocytes (THLE2) with the CRC_SEVs promotes AS of some RBFOX2 target mRNAs involved in cell shape remodelling. Interestingly, EVs deprived of lncH19 lack also RBFOX2, thus reducing their effects in AS dysregulation.

Conclusion: our data indicated, for the first time to our knowledge, that lncH19 carries splicing factors inside EVs, affecting AS related to EMT processes in recipient cells and thus enforcing a pre-metastatic microenvironment.



O-HD-8

Extracellular vesicles from a triple negative breast cancer (TNBC) / paclitaxel-resistant cell model carry chemoresistance and inflammation signals

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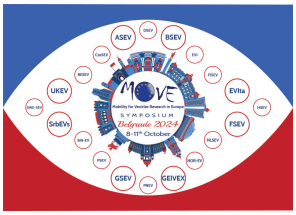
Introduction: Triple negative breast cancer (TNBC) is a heterogenous aggressive type of breast cancer with poor prognosis and high possibility of relapse. Chemoresistance can be endogenous to specific TNBC types or developed during chemotherapy. Interestingly, extracellular vesicles (EVs), can vectorize chemoresistance-associated and pro-inflammatory proteins in the presence or absence of LPS, as a sepsis model.

Methods: Crude EV fraction was collected from the 120,000g sediment of the 30,000g supernatants from SUM159 sensitive (parental) or resistant to paclitaxel (SUM159-ptx) cell models. Morphological evaluation was performed through Dynamic Light Scattering (DLS). The presence of small EVs was evaluated through exosomal markers. Relative changes of proteins from cell lysates and EVs under normal conditions or after LPS treatment were determined by high resolution mass-spectrometry. Appropriate controls were included in this study.

Results: DLS revealed a polydisperse sample, including vesicles ranging in size from 35 to 400nm. EVs from LPS-treated cells shifted to smaller diameter. Over 4000 proteins were identified in EVs, of which, 506, from ptx cells were not present in EVs from parental cells. LPS-treatment increased the number of EVs-associated unique proteins (593 and 212 in parental and ptx cells, respectively). ABCG2 and TUBB3 and PRDX6 proteins were increased in EVs from SUM159-ptx vs SUM159-parental cells. Both PLA2G2A and PLA2G15 were detected in EVs and not in cell lysates, exhibiting lower levels in SUM159-ptx compared to SUM159. However, LPS induced an increase in EV-PLA2G2A from SUM159-ptx, in contrast with EV-PLA2G15, which was elevated in SUM159_LPS compared with SUM159-ptx_LPS.

Conclusion: Proteomic analysis is a useful tool to investigate the possible involvement of EVs on the establishment of resistance to paclitaxel in TNBC cell models and indicate a transport system of inflammatory signals. In vitro LPS treatment induces changes highlighting the differential activation of signaling pathways and the regulation of EV-proteins associated with drug-resistance.

Funding information: “Establishment of "capacity building" infrastructures in Biomedical Research (BIOMED-20)” (MIS 5047236) which is implemented under the Action “Reinforcement of the Research and Innovation Infrastructure”, funded by the Operational Programme "Competitiveness, Entrepreneurship and Innovation" (NSRF 2014-2020) and co-financed by Greece and the European Union (European Regional Development Fund), “An Open-Access Research Infrastructure of Chemical Biology and Target-Based Screening Technologies for Human and Animal Health, Agriculture and the Environment (OPENSCREEN-GR)” (MIS 5002691) which is implemented under the Action “Reinforcement of the Research and Innovation Infrastructure”, funded by the Operational Programme "Competitiveness, Entrepreneurship and Innovation" (NSRF 2014-2020) and co-financed by Greece and the European Union (European Regional Development Fund), Operational Programme «Human Resources Development, Education and Lifelong Learning 2014-2020» in the context of the project, “Chemistry and Technologies for Pollution Control and Environmental Protection-CTePCEP”, MIS: 5162213, Code: 83218), which is co-financed by Greece and the European Union (European Social Fund- ESF).



P-HD-9

Influence of platelet extracellular vesicles on T cell function and endothelial integrity in allergic inflammation

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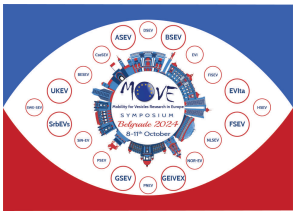
Introduction: Platelet extracellular vesicles (PL-EVs) have recently been shown to have different metabolic cargo depending on severity in respiratory allergy patients. As EVs are a means of cell-cell communication by delivering their content to recipient cells and lipids are critical immune system signals, PL-EV released in severe allergic patients' blood could cross the endothelial barrier and modify T-cell functions in different tissues. However, the influence of these PL-EVs on the allergic inflammatory response remains unknown. Here, we aim to decipher the effects of PL-EVs on T cell functions and endothelial barrier stability.

Methods: PL-EVs were extracted by serial ultracentrifugation from platelet-rich plasma obtained from non-allergic subjects (n=3), mild allergic patients (n=4) and severe allergic patients (n=3). PL-EVs were characterized according to MISEV 2023 and added to activated (CD3/CD28 beads) $CD3^+$ cells to assess their proliferation (RealTime-Glo™ MT cell viability assay), viability (Annexin V and 7AAD staining) and differentiation into regulatory T cells ($CD4^+$ $CD25^+$ $FOXP3^+$) at different time points. Furthermore, the effect of PL-EVs on the endothelial barrier integrity was evaluated by measuring the trans-endothelial electrical resistance (TEER) on human lung microvascular endothelial cells through an EndOhm chamber in individual cups.

Results: Our data showed that PL-EVs exerted a proliferative, rather than apoptotic effect on activated T-cells, which positively correlate with the degree of allergic inflammation, but did not alter the number of regulatory T cells. Remarkably, PL-EVs induced a strong endothelial barrier disruption, independently of their phenotype.

Conclusion: These results suggest that PL-EVs, affecting both T cell proliferation and endothelial barrier integrity, may play a role in modulating the immune response in patients with respiratory allergy. However, further analyses are needed to improve our understanding of the implications of PL-EVs in inflammatory allergy.

Funding information: SEAIC (23A_Izquierdo), ISCIII (PI19/00044), cofounded by FEDER "Investing in your future" for the thematic network and cooperative research centres ARADyAL (RD16/0006/0015 and RD16/0006/0013) and RICORS "Red de Enfermedades Inflamatorias (REI)" (RD21/0002/0008), Fundación Mutua Madrileña (AP177712021), Universidad CEU San Pablo.



P-HD-10

Mechanisms of extracellular vesicle uptake in G. intestinalis and host cell interactions: role of clathrin and caveolin-mediated endocytosis

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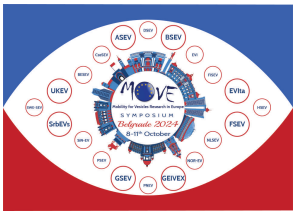
Introduction: Giardia intestinalis is a flagellated protozoan responsible for giardiasis, one of the most common parasitic diseases in humans. This infection is prevalent worldwide and recognized as a neglected disease by the World Health Organization. Giardiasis imposes a significant global burden due to morbidity and insufficient research and therapeutic development. The pathogenesis is multifactorial and begins with the adhesion and colonization of the parasite to the intestinal epithelium, leading to gastrointestinal symptoms. This process is facilitated by the release of extracellular vesicles (EVs), which include microvesicles and exosomes. These EVs, surrounded by a lipid bilayer, play a crucial role in intercellular communication. The objective of this study is to understand the mechanism of EV uptake and communication during interaction with host cells.

Methods: We defined three types of EVs during parasite-host cell interaction: Giardia-originated EVs (gEV), host cell-released EVs (hEV), and parasite-host cell interaction EVs (intEV). Uptake assays were performed with these purified EVs labeled with PKH26 in Caco-2 cells. Dose-dependent and kinetic titrations of EVs were conducted in contact with host cells, and results were analyzed by flow cytometry and confocal microscopy. The pathways involved in EV uptake were analyzed using endocytosis inhibitors, comparing the internalization of vesicles in treated and untreated host cells.

Results: Our results demonstrated that different EVs are internalized by Caco-2 intestinal cells in an energy and dose-dependent manner, exhibiting distinct patterns. Notably, intEVs are internalized more quickly than other EVs, which could be important in the disease's pathophysiology. Among several inhibitors, Dynasore and genistein most effectively blocked EV internalization by host cells.

Conclusions: This study confirms that EVs are taken up by host cells and suggests that endocytosis is the primary mechanism of EV capture, opening the possibility of blocking this pathway as an alternative strategy to control the infection.

Funding: Capes, CNPq, UFPR and Carlos Chagas Institute. Acknowledgments: UFPR and Carlos Chagas Institute.



P-HD-11

Endothelial cell derived extracellular vesicles in chemotherapy

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Introduction: Despite advancements in cancer treatments, colorectal cancer (CRC) remains the third most diagnosed tumor and a leading cause of death globally. Chemotherapy, particularly with 5-fluorouracil (5-FU) combined with folinic acid, oxaliplatin, or irinotecan, is a key treatment, enhancing survival rates. However, 5-FU is associated with cardiotoxicity, affecting up to 20% of patients. The exact mechanisms of this cardiotoxicity are not fully understood. Anti-tumor drugs like 5-FU impact endothelial cells (EC) and cardiomyocytes, leading to the production of molecules such as pro-inflammatory cytokines, chemokines, reactive oxygen species (ROS), and endothelial-derived extracellular vesicles (EVs). Cardiotoxicity is often detected through imaging after irreversible damage has occurred, highlighting the need for early diagnosis through new biomarkers. EVs, which transport and protect molecules in the bloodstream, could serve as critical biomarkers for assessing cardiovascular risk in cancer patients undergoing chemotherapy.

Methods: 5-FU cytotoxicity on DLD, HCT116 and HUVEC at 24h and 48h was assessed by MTS assay (Promega). Cells was treated with increase concentration of 5-FU including the clinical dose (426 uM). Optical microscope pictures (magnification: 10X; ZEISS) and Countess 3 Automated Cell Counter (Thermo) was used to evaluate morphological changes and quantify cell numbers. DLD-1 cells and HUVEC (vehicle and 5-FU treated) supernatants were used to measure CD9, CD81, CD63 EV markers by Exoview array analysis.

Results: Using a clinically relevant dose, cell viability assays showed a significant decrease in the viability of HCT116 and DLD-1 cells after 24 and 48 hours (27% and 44% respectively) of 5-FU treatment, while HUVECs viability remained largely unaffected (77%) ($P<0,005$). Morphological examinations revealed that HUVECs maintained their density comparable to vehicle-treated cells, unlike the cancer cell lines. Exoview analysis indicated decreased expression of CD9, CD63, and CD81 markers in EVs from 5-FU-treated DLD-1 and with an even greater reduction for HUVECs derived EV ($P<0,005$).

Conclusion: Our results suggests that 5-FU significantly disrupts normal EC-EV biology by affecting their generation and release profiles, without impacting cell viability. Understanding how 5-FU influences EC-EV could offer insights into 5-FU-induced cardiotoxicity and help develop EV blood-based biomarkers for cardiovascular monitoring during treatment.

Funding information: NA acknowledges support by research grants from the British Heart Foundation (BHF) Centre of Research Excellence, Oxford (RE/13/1/30181 and RE/18/3/34214); British Heart Foundation Project Grant (PG/18/53/33895) and a British Heart Foundation Intermediate Fellowship (FS/IBSRF/22/25110); the Tripartite Immunometabolism Consortium, Novo Nordisk Foundation (NNF15CC0018486 and NNF20SA0064144); Oxford Biomedical Research Centre (BRC); Nuffield Benefaction for Medicine and the Wellcome Institutional Strategic Support Fund (ISSF) and a Health Research Bridging Salary Scheme (HRBSS).
Giulia Artemi was awarded the MOVE Fellowship, granting her the opportunity to spend three months working on this project in the Akbar lab.

EVs in health and disease

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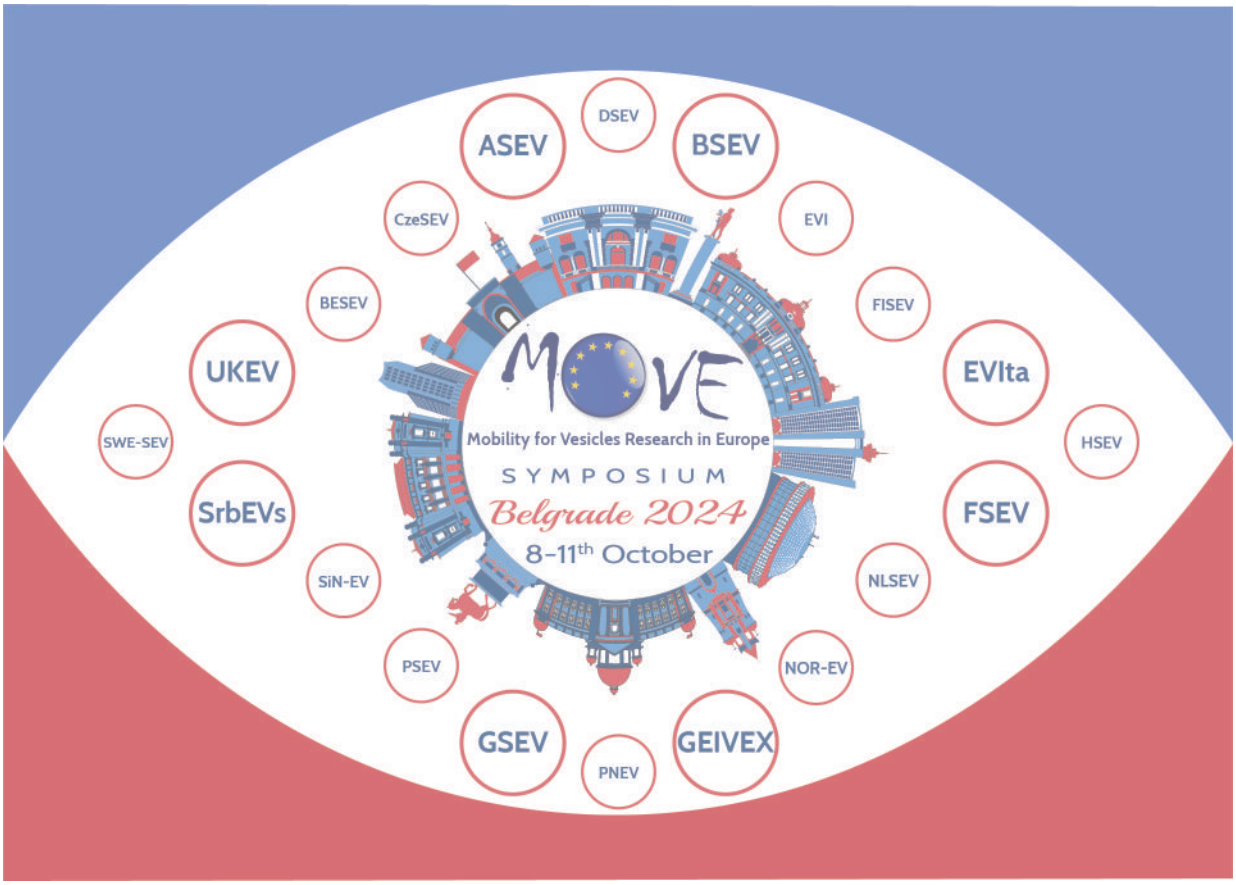

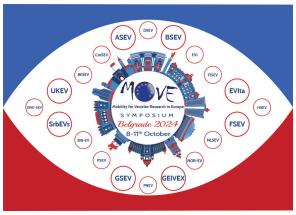

P-HD-1

The interaction of Trypanosoma cruzi with myoblasts and intestinal cells induces extracellular vesicles of different composition and functionality

Izadora Volpato Rossi[1,2], Rafael Fogaça de Almeida[3], Bruna Sabatke[1,2], Lyris Martins Franco de Godoy[3], **Marcel Ivan Ramirez**[2]

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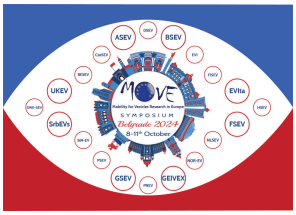
Introduction: Trypanosoma cruzi is the protozoan that causes Chagas disease (CD), an endemic parasitosis in Latin America spreading worldwide. The chronic phase of CD may progress to cardiac, digestive or neurological manifestations and the gastrointestinal tract represents an important reservoir for T. cruzi in Chronicity . The contact between T. cruzi and host cells induces the release of extracellular vesicles (EVs) that modulates the immune system and enhances the infection, but the dynamics of secretion of host and parasite molecules through different tissues is unknown. We hypothesized that the parasite differentially interacts with myoblast and intestinal epithelial cells to release EVs and we analysed the protein content of these EVs.

Methods: We used two cell lines to simulate the environments found by the parasite in the host: C2C12 cell, myoblast and Caco-2 cell, intestinal epithelium. We isolated large EVs (LEVs) from the interaction of culture-derived trypomastigotes (TCTs) of T. cruzi (CL Brener and Dm28c) in contact with C2C12 and Caco-2 cells for 2 and 24 hours of infection. Peptides of LEV proteins were analyzed by mass spectrometry and in MaxQuant platform.

Results: Our data showed that at two hours there is a strong cellular response mediated by EVs, both in the number, variety and enrichment of proteins found in LEVs for diverse functions. Qualitative and quantitative analysis showed that proteins exported in LEVs of C2C12 and Caco-2 have different patterns. We found a predominance of host proteins at early infection. The parasite-host cell interaction induces a switch in the functionality of proteins carried by LEVs and a heterogeneous response depending on the tissues analyzed.

Conclusions: These data provide new evidence that the interaction with T. cruzi leads to a rapid tissue response through the release of LEVs, reflecting the enrichment of proteins that could modulate the infection environment.

Funding: CNPq, Capes, Fundação Araucária, Fiocruz; **Acknowledgments:** We would like to thank the Mass Spectrometry Platform at the Carlos Chagas Institute and all the project funders (CNPq, Capes, Fundação Araucária, Fiocruz).



P-HD-2

Endothelial progenitor cells-derived extracellular vesicles mitigate the inflammation in septic model

Luigi Menna[1], Nóra Fekete[1], Boglárka Nagy[1], Kristóf Csont[1], Eszter Lajkó[1], Zsófia Szász[1], László Kőhidai[1], Edit I Buzás[1], Hargita Hegyesi[1]

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Introduction: Endothelial Colony-Forming Cells (ECFCs), characterized by progenitor properties, possess an augmented capacity for repair damaged vasculature. Recent research has demonstrated that ECFCs secrete EVs, and these endothelial progenitor cell-derived EVs could promote M2 macrophage polarization.

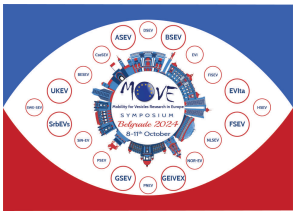
This study aimed to characterize ECFCs and their derived EVs. Additionally, we examined the anti-inflammatory effects of ECFC-derived EVs on neonatal cardiomyocytes (NCMCs) pre-treated with lipopolysaccharide (LPS) and assessed the therapeutic benefits of ECFC-EV administration in a sepsis model.

Methods: ECFCs were isolated from CDH5 Cre/mTmG mice, the GFP+ ECFCs obtained from bone marrow and cultured in supplemented EBM-2 medium for 6 weeks. EVs were harvested from the conditioned medium of ECFC cultures using differential ultracentrifugation (dUC). Flow cytometry was employed to identify both ECFCs and their EVs. In vitro ECFC-EV mediated effects were measured on NCMC cells. Expression of Il-1β, IL-6, TNFα, CCL-2, CCL-11 was assessed by qPCR, IL-6 and troponinI was detected by ELISA. In the in vivo sepsis model, the effect of administration of ECFC-EVs were measured by immunophenotyping the PBMCs.

Results: ECFCs exhibited endothelial progenitor markers CD31, CD34, and VEGFR2, while GFP+ ECFC-EVs expressed CD31 and classical vesicular markers such as CD63 and Annexin V. GFP+ ECFC-EVs were internalized by LPS-treated NCMCs. Treatment with GFP+ ECFC-EVs for 24 hours significantly reduced LPS-induced CCL-2 and TGFβ expression in NCMCs. In vivo, the number of endothelium-derived vesicles increased following LPS injection, and the reduction in CD4 and CD8 cell counts was mitigated by peritoneal administration of ECFC-EVs four hours post-LPS administration.

Conclusion: ECFCs can be efficiently maintained in primary culture and identified by specific endothelial progenitor markers and GFP expression. The anti-inflammatory properties of ECFC-derived EVs highlighting their potential therapeutic role in sepsis-induced cardiac dysfunction.

Fundings: Therapeutic Thematic Program TKP2021-EGA-23; RRF-2.3.1-21-2022-00003 (National Cardiovascular Laboratory Program); 2019-2.1.7-ERA-NET-2021-00015; HUN-REN-SU Translational Extracellular Vesicle Research Group the program



P-HD-3

Caveolin-1-overexpression affects extracellular vesicle loading and modulates tumour microenvirnment in a rhabdomyosarcoma model

Rachele Agostini[1], Emanuela Polidori[1], Paola Ceccaroli[1], Laura Graciotti[2], Stephanie Fondi[1], Michela Battistelli[1], Francesca Luchetti[1], Silvia Codenotti[3], Gabriella Pocsfalvi[4], Massimiliano Bonafè[5], Alessandro Fanzani[3], Vilberto Stocchi[1], Michele Guescini[1]

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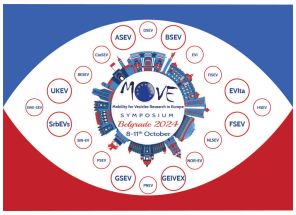
Introduction: Extracellular vesicles (EVs) are lipid-bound vesicles secreted by cells into the extracellular space and have a pivotal role in cancer disease. Caveolin-1 (CAV1) is a 22 kDa protein located in strategic areas of the plasma membrane, such as caveolae and cholesterol-enriched lipid rafts. In the context of rhabdomyosarcoma (RD) CAV1-overexpression promotes tumour growth and metastatic diffusion, acting as a tumour enhancer. The present work aims to investigate if EV machinery is affected by CAV1-overexpression and if the EVs released by RD cells overexpressing CAV1 (RD-CAV1) can contribute to their increased aggressiveness.

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 (WB) and Flow Cytometry Analysis (FC). Proteomic Analysis has been performed on EV subpopulations. Migration and proliferation assays were conducted on HUVEC cells.

Results: The obtained data showed that RD-CAV1 cells release more EVs with an entirely different protein profile compared to RD-Ctrl ones. WB and FC analysis revealed that RD-CAV1 small extracellular vesicles (*s*EVs) do not exhibit the typical exosomal markers CD63, CD81, and CD9. Proteomic analysis extended this alteration to many other proteins showing an overall reduction in protein loading and expression in these *s*EVs compared to the control. These findings are combined with an impairment in the RD-CAV1 intracellular vesicular trafficking, suggesting that CAV1-overexpression induces an alteration of EV biogenesis and secretion. Moreover, the treatment of HUVECs with RD-CAV1 EVs showed a significant increase in cell proliferation and migration compared to the control.

Conclusion: Taken together, these data demonstrate that CAV1-overexpression critically affects RD-intracellular trafficking and EV cargo-release, leading to an increase in their aggressiveness. Future studies will focus on the characterization of RD-EV lipid- and miRNA-loading and on the evaluation of RD-EV effects in other cell types, typical of tumour niche.

Funding information: European Union – NextGenerationEU under the Italian Ministry of University and Research (MUR) National Innovation Ecosystem grant ECS00000041 - VITALITY - CUP H33C22000430006



P-HD-4

Assessing T Cell-Mediated Immunity to SARS-CoV2 infection in extracellular vesicles through affinity capture

Teresa Valero[1,2], Fabian Vergara[1,2], Bernardino Alcazar Navarrete[2,3], José Antonio Sánchez Martínez[2,3], Beatriz Carolina Gómez[2,3], Concepción Morales García[2,3], Juan José Díaz Mochón[1,2], Rosario María Sánchez Martín[1,2], Francisco Gabriel Ortega Sánchez[2,3]

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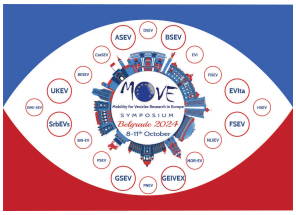
Introduction: Cellular immunity mediated by T cells plays a crucial role in infectious, autoimmune, oncological, and degenerative processes(1). However, its analysis is not applied due the complexity of conventional methods (ELISPOT, FLUOROSPOT, or Flow-Cytometry kits)(2), which require fresh blood, cell culture, specialized equipment, and highly trained personnel(2). This study aims to assess the feasibility of using the specific binding of a peptide to its T cell receptor for the selective isolation of extracellular vesicles (EVs) to evaluate specific cellular responses to SARS-CoV2 infection or vaccination.

Methods: Plasma samples were collected from three distinct cohorts: 1: Plasma from 5 donors obtained in 2019; 2: Plasma from 5 donors less than a month after recovering from the disease (COVID-19); 3: Plasma from 5 donors vaccinated against COVID-19. Magnetic beads were functionalized with a pool of peptides from the spike protein subunit 2 of the SARS-CoV2(3). EV-capture was performed by direct incubation of EVs isolated from plasma and functionalized beads. EV-quantification was carried out using a colorimetric ELISA method, with CD81 as the quantified protein(4). Specific binding of EVs to the antigenic peptide was confirmed through TEM and competitive tests. Finally, results were compared across the three cohorts.

Results: The outcomes demonstrated a successful functionalization of magnetic beads with a pool of peptides of the subunit 2 of the SARS-CoV2 spike protein, though protein quantification. The magnetic beads decorated with the pool of peptides efficiently captured T-cell-derived EVs of plasma of previously infected or vaccinated individuals, which was confirmed through Scattering Electronic Microscopy (SEM). The extent of this affinity capture can be assessed with a colorimetric ELISA-like assay, finding significant differences between vaccinated or previously infected individuals vs pre-pandemic controls. Moreover, this quantification can be performed in frozen samples and the signal is reverted in competitive conditions, where the co-incubated free peptide significantly reduces the colorimetric signal.

Conclusion: This innovative approach to assess T cell-mediated immunity could enhance speed, efficiency, and versatility compared to conventional methods. Its simplicity and versatility make it a promising tool for investigating specific immune responses in diverse clinical contexts.

Funding information: European Commission (H2020-MSCA-IF-895664), Next Generation Europe funds (FEDER-C-EXP-160-UGR23), Carlos III Research Institute (DTS23/00030).



P-HD-5

Vesicle-like particles extracted from ginger antagonize staurosporine-induced apoptosis

Delaram Khamari[1,2], Nora Fekete[1], Meir Kestecher[1,3], Panna Kiralyhidi[1,2] Bernadett Réka Bodnár[1,3], Luigi Menna[1,2], Ririka Tamura[1,4], Bence Erdos[1,4], Tunde Barkai[1,2], Maximilien Bencze[5] , Edit I Buzás[1,2,3]

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Apoptosis is a form of programmed cell death. Inappropriate apoptosis can have detrimental effects on tissue homeostasis leading to various pathological conditions such as cancer, ischemia, autoimmune, and neurodegenerative diseases. Extracellular vesicles (EVs) are known regulators in an array of pathologies and play a significant role in apoptosis. Plant-derived EVs, such as those from Zingiber officinale (Ginger root) are biocompatible, easily obtained, and stable. It was shown earlier that these vesicle-like particles also play a role in blockage of inflammasome and inhibit the release of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β). In this project, ginger vesicle-like particles were isolated by different methods to find the best isolation approach. These vesicle-like particles were then characterised by nanoparticle tracking analysis to determine the particle size distribution and concentration. Micro-BCA protein- and SPV lipid assays were performed to determine the protein and lipid contents, respectively. Transmission electron microscopy was used to show the ultrastructure and to identify fractions containing EV-like particles. By comparative analysis using flow cytometry and UV-VIS, we distinguished EV-like particles and autofluorescent particles (containing e.g. flavonoids, and phenolic compounds). Our results showed that ginger root-derived EV-like particles were similar to mammalian EVs in their basic parameters. Finally, we demonstrated in our studies that cardiomyocytes or monocytes co-cultured with ginger EV-like particles protected the cells from staurosporine-induced apoptosis. Our data suggest that ginger EVs can confer cytoprotective properties against apoptosis. Taken together, our data provide evidence that it is feasible to isolate biologically active ginger-derived extracellular vesicle-like particles similarly to mammalian cell-derived extracellular vesicles.

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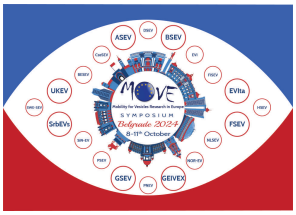

P-HD-6

The Effect of Extracellular Vesicles Originated from Mesenchymal Cells of Peritoneal Dialysate on the Mechanism of Fibrosis

Péter Bokrossy[1], Beáta Szebeni[1,2], Apor Veres-Székely[1,2], Domonkos Pap[1,2], Zoltán Varga[3], Judith Mihály[3], Éva Pállinger[4], Mária Bernáth[1], György S. Reusz[1], Attila J. Szabó[1,2] and Ádám Vannay[1,2]

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Introduction: The literature is abundant in the topic of benefits of cell therapy in different experimental fibrosis models. Using extracellular vesicles (EVs) as an alternative to cell therapy promises benefits like lower imunnogenicity, a possible crossing of the blood-brain barrier, and not inducing acute immune rejection. Aims: We investigated the effect of EVs originated from mesenchymal cells (MCs) of peritoneal dialysate (PDE) on the activation of primary MCs and fibroblasts.

Methods: MCs were isolated, characterised and cultured from PDE. From the serum free cultures supernatant EVs were isolated by tangential flow filtration and size exclusion chromatography. After the isolation EVs were characterized based on their particle number, size distribution, morphological features, surface markers, and the composition of cargo proteins. Their effect on fibroblast activation was tested by in vitro experiments on primary peritoneal fibroblasts (pFBs) isolated from peritoneal biopsy collected during removal of the Tenchoff catheter. The effect of EVs on the pFBs were examined by using functional assays such as MTT proliferation assay, Sirius Red assay and Transient Agarose Spot assay.

Results: The mesenchymal cells isolated in the study displayed positive expressions of CK-18, α-SMA, CD73, CD105, and CD90, while lacking the CD34, HLA-DR, CD45, and CD19 markers as indicated by immunofluorescent staining and RT-PCR analysis. The isolated EVs exhibited stem cell and CK18 positivity, implying that their original source cells were MCs that had undergone mesothelial mesenchymal transition. The EVs successfully internalised by pFBs and reduced their PDGF induced proliferation, TGF-β induced collagen accumulation and EGF induced migration as shown by various functionals assays.

Conclusion: Due to the potential antifibrotic properties exhibited by these extracellular vesicles (EVs), they could hold therapeutic promise. Nevertheless, further in vivo testing is required to substantiate this hypothesis.

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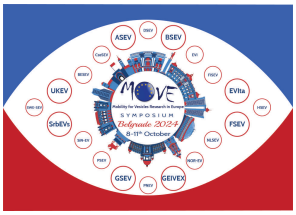

P-HD-7

Investigating the effects of neutrophil derived extracellular vesicles on lung injury using human precision cut lung slices

Jessica Ghobrial[1], HuiXin Loh[1], Antonella Pellicoro[1], Pamela Holland[1] , Richard O'Connor[1], Asta Valanciute[1], Stuart Dickson[1], Manu Shankar-Hari[1], Christopher Lucas[1], Christopher Gregory[1], Kev Dhaliwal[1]

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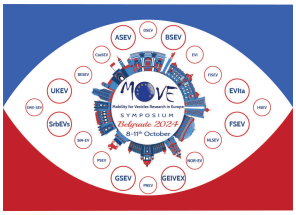
Introduction: Acute lung injury (ALI) is a challenging respiratory condition due to its high mortality rate and the absence of effective targeted therapies. One of the hallmarks of ALI is the accumulation of recruited neutrophils in alveolar space, which augment lung injury through the release of their cytotoxic molecules. However, there are currently no effective therapies targeting neutrophils in ALI. Recent studies have shown that neutrophils isolated from chronic obstructive pulmonary disease patients, secrete extracellular vesicles (EVs) with a high concentration of cytotoxic molecules that could initiate lung injury and inflammation in murine models. Yet, there is still a lack of understanding of the effects of neutrophil derived EVs (neu-EVs) during human lung injury.

Methods: In this investigation, the potential role of neu-EVs in inducing lung injury was explored using the human ex vivo model, human precision cut lung slices (hPCLS). Initially, Neutrophils were isolated from healthy human blood and activated with fMLF. Neu-EVs were then extracted using size exclusion chromatography and characterized by nanoparticle tracking analysis and western blot, indicating that neutrophils mostly secrete EVs smaller than 200 nm with CD63 and Alix expression. hPCLS were cultured in the presence of Neu-EVs for 14 days to investigate their effects on tissue inflammation.

Results: Preliminary results show that fMLF-treated neu-EVs increase tissue damage and cellular infiltration into the alveolar space and trigger the release of high levels of pro-inflammatory cytokines, including, MCP-1, MMP-9, IL-6 and IL-8. These results demonstrate that activated neu-EVs could be involved in initiating lung injury in humans.

Conclusion: However, further investigation is still required to assess the effects neu-EVs have on the functions of lung resident and recruited immune cells. These findings will enable a greater understanding of the role of neu-EVs in ALI pathogenesis, which makes neu-EVs a potential therapeutic target for ALI in future.

Funding information: Translational Healthcare technologies, University of Edinburgh



P-HD-8

CD63+ extracellular vesicles are reduced in hypercholesterolaemic mice and humans

Brachyahu M. Kestecher[1,2,3], Krisztina Németh[1,2], Nabil V. Sayour[4,5], Tamás G. Gergely[4,5], Sayam Ghosal[1,3], Bernadett R. Bodnár[1,3], András I. Försönits[1], Barbara W. Sódar[1,3], Zoltán V. Varga[4,5], Zoltán Giricz[4,5], Péter Ferdinandy[4,5], Edit I. Buzás[1,2,3], Xabier Osteikoetxea[1,3]

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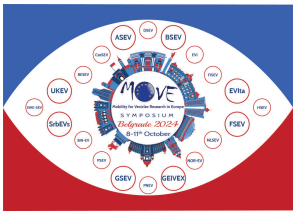
Introduction: The association and co-isolation of low-density lipoproteins (LDL) and extracellular vesicles (EVs) have been shown in blood plasma. Here we study the consequence of this association and of EVs in atherogenesis.

Methos and Results: Wild type (WT), PCSK9$^{-/-}$, and LDLR$^{-/-}$ C57BL/6J mice were used in this study. Eleven week-old male mice were fed high-fat diet (HFD) for 12 weeks or kept on normal diet until old age (22-months). Cardiac function was assessed by ultrasound, cholesterol was quantified with a commercial kit, and circulating EVs were measured by flow cytometry. Plaques were analysed post-mortem using Oil-Red-O staining of the aortic arch. EVs were measured from platelet free blood plasma of patients with normal cholesterol or hypercholesterolaemia. Based on annexin V and CD63 staining, we found a significant increase in EV levels in LDLR$^{-/-}$ and PCSK9$^{-/-}$ mice after HFD, but CD81 showed no significant change in either group. There was no significant change in plaque formation after HFD. PCSK9$^{-/-}$ mice show a favourable cardiac function after HFD. Blood cholesterol levels progressively increased during HFD, with LDLR$^{-/-}$ mice always elevated whereas PCSK9$^{-/-}$ were maintained lowered cholesterol compared to WT animals. In old age mice, similar cholesterol levels were observed to that of 11-week old animals. At old age, ejection fraction was decreased in all groups of mice, as were CD63^{+} and annexin V^{+} EVs. Here, LDLR$^{-/-}$ mice showed significantly increased plaques. Patients with hypercholesterolaemia showed significantly lower CD63^{+} EVs.

Conclusions: This research demonstrates an inverse relationship between circulating EVs and cholesterol, making EVs a potential marker for cardiovascular disease (CVD). HFD causes reduced cardiac function, but atherosclerotic development is slow progressing, even in a hypercholesterolaemic model, thus only observed with old-aged animals. These results also bring further evidence for the benefit of using of PCSK9 inhibitors as therapeutic agents in CVD.



P-HD-9

Synovial Fluid-Derived Extracellular Vesicles: Orchestrators of Endothelial Cell Function and Inflammation

Riham Osman[1], Michella Battistelli[1], Eleonora Olivotto[2], Sabrina Burattini[1], Maria Gemma Nasoni[1], Serena Benedetti[1], Michele Guescini[1], Francesca Luchetti[1]

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Introduction: The relationship between tight junctions (TJs), adherens junctions (AJs), and the endothelial monolayer is crucial for maintaining tissue integrity and barrier function. Disruption of this barrier leads to plasma leakage and neutrophil infiltration, a pathological process leading to inflammatory response. Extracellular vesicles (EVs) are lipid-bound nanoscale contributors to physiological and pathological processes. They have been detected in a variety of biological fluids, such as urine, plasma, and synovial fluid (SF).

Methods: SFs were collected from the joints of patients with femoroacetabular impingement (FAI) classified as having “low or high” levels of inflammation based on cartilage damage score, and from patients with osteoarthritis (OA). SFs levels of interleukin-6 (IL-6) were measured by ELISA immunoassay. Isolation of the vesicles was performed using ultracentrifugation (UC). The viability of Human umbilical vein endothelial cells (HUVECs) after being treated with different SF-EVs concentrations was tested with the trypan blue exclusion test. Vesicle morphology and internalization were investigated by transmission electron microscopy (TEM), whereas the expression and localization of β-catenin and VE-Cadherin were investigated by immunofluorescence. Total mRNA expression of the chemokine monocyte chemoattractant protein- 1 (MCP-1) and IL-6 was assessed by qRT-PCR analysis.

Results: A higher expression of IL-6 levels in the SFs can be observed moving from a low inflammatory condition to OA. As for cell viability, results showed a significant decrease after being treated with the highest concentration of HI-EVs and OA-EVs for 24h. Moreover, TEM observations interestingly showed the presence of Weibel–Palade Bodies (WPBs) when HUVECs were treated with OA-EVs. β-catenin and VE-Cadherin fluorescence levels were significantly decreased when cells were treated with LI-EVs and OA-EVs. MCP-1 and IL-6 mRNA levels show a marked increase in cells treated with LI-EVs.

Conclusion: Our preliminary data suggest that SF-EVs affect endothelial cell functions by modulating endothelial permeability and the inflammatory response.

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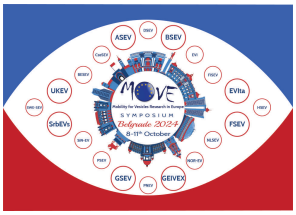

P-HD-10

The death signals mediated by extracellular vesicles between glioblastoma multiforme (GBM) cells during temozolomide treatment depend on the bioactive cargo specifically loaded by the different GBM lines

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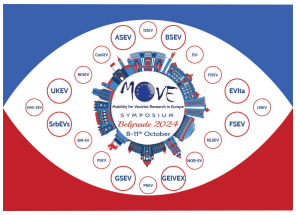
Introduction: Extracellular vesicles (EVs) of glioblastoma multiforme (GBM), a highly malignant primary brain tumour, may contribute to cancer cell proliferation and survival by shaping the tumour microenvironment. Resistance of GBM to the chemotherapeutic drug temozolomide (TMZ) leads to poor survival of patients, highlighting the importance of further research. We have previously demonstrated cell-specific properties of EVs isolated from various TMZ-treated or TMZ-untreated GBM cell lines. Here, we tested the ability of EVs to promote or inhibit the induction of cell death in recipient GBM cells and determined which class of molecules, i.e. proteins, lipids, or nucleic acids, of the EV cargo might be primarily responsible.

Methods: UF/SEC isolated EVs from untreated and TMZ-treated U87MG and U251MG cells were characterized by proteomics, NTA, TEM, and cryo-EM analysis. Non-TMZ treated GBM cells were challenged with EVs in which proteins and RNAs of EVs were simultaneously or separately inactivated by heat and RNase A. Cell response was assayed by WB of cell death markers for apoptosis, ferroptosis, and autophagy.

Results: EVs released from both sensitive (U87MG) and chemotherapy-resistant (U251MG) GBM cells differ in their properties, which are further influenced by TMZ treatment and when incubated with recipient GBM cells modify their physiological response. For example, EVs from TMZ-untreated U87MG cells significantly increase caspases 3/8/9 and CD71 and GPX4 apoptotic and ferroptotic markers in recipient U251MG cells. Heat and/or RNA-inactivated EVs modulate the effect described for non-inactivated EVs, and the intensity of modulation is related to the specific GBM cell.

Conclusion: Our data further confirms the physiological heterogeneity between GBM cells. Indeed, the EVs produced by the two GBM cell lines not only differ in size, size distribution, shape, and cargo, but also in the spread of death signals with different contributions of proteins, lipids, and RNA depending on the specific GBM cell.

Funding information: 000301_RICER CA_SAPIENZA_ 2022_Grande Progetto di Ateneo to LD



P-HD-11

Extracellular Vesicle-Encompassed MyomiRs Contribute to Placental Vascular Immaturity in Pregnancies Complicated by Maternal Diabetes

Margeurite G Kennedy[1], **Abigail R Byford[1]**, Jayne C Charnock[2], Beth Holder[3], Eleanor M Scott[1], Karen Forbes[1]

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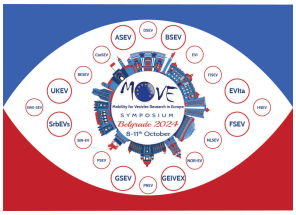
Introduction: Gestational diabetes (GDM), the most common medical complication of pregnancy, is associated with adverse outcomes including increased rates of stillbirth, fetal growth complications, and a predisposition for offspring developing cardiometabolic complications later in life. The underlying cause of these complications is unclear, however, GDM placentas display abnormal morphology indicative of vascular immaturity. We have shown that extracellular vesicles (EVs) in maternal circulation contain vascular regulatory miRNAs and that maternal EVs can enter, and influence events in the placenta. We hypothesise that EVs containing vascular regulatory miRNAs traffic to the placenta from maternal circulation, to influence placental vascularisation and that this is altered in GDM pregnancies.

Methods: EVs were isolated from maternal plasma (24–32 weeks) from uncomplicated or GDM pregnancies and characterised by TEM, Western blotting and NTA. Levels of vascular regulatory miRNAs in EVs and placenta were quantified by QPCR. Primary placental mesenchymal stromal cells (PMSCs) were isolated from uncomplicated term placentas and characterised by flow cytometry (FC), immunocytochemistry (ICC) and trilineage differentiation. PMSCs were induced towards a vascular smooth muscle cell (VSMC) lineage by plating on collagen and culturing in differentiation media for 14 days. AntimiRs were used to inhibit miR-1-3p and miR-133a-3p in PMSCs. QPCR and ICC were used to assess PMSC and VSMC markers.

Results: miR-1-3p and miR-133a-3p, were decreased in plasma EVs and in placental tissue from GDM pregnancies with fetal overgrowth. Induction of PMSCs towards VSMC lineage was confirmed by FC, ICC and QPCR. VSMC differentiation was accompanied by increased levels of miR-1-3p and miR-133a-3p. Inhibition of miR-1-3p and miR-133a-3p reduced the ability of PMSCs to differentiate towards a VSMC lineage.

Conclusions: EV encompassed myomiRs, miR-1-3p and miR-133a-3p are altered in maternal circulation in GDM. Trafficking of these EVs/miRNAs to the placenta may contribute to placental vascularisation immaturity and adverse outcomes in GDM.

Funding Information: MGK was funded by a Mary & Alice Smith Memorial Scholarship from University of Leeds. This work was also supported by funding from UK Research and Innovation (UKRI) Medical Research Council (MR/R023166/1).



P-HD-12

Interaction of cancer-associated exosomes with glyconanotherapeutics and study to cross blood tumor and blood brain barrier

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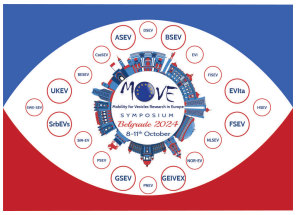
Introduction: Cancer-derived exosomes (EXs) play an important role in intercellular communication regulating tumor malignancy. They can reprogram recipient cells to make them actively participate in metastasis or angiogenesis. Due to their crucial role in these processes, EXs are emerging as a prospective target for various types of therapeutics. Here we focused on the glyconanotherapeutics and their impact on EXs fate. Glycopolymers and glycodendrimers can efficiently bind to galectins, which are known modulators of tumor cell migration, proliferation and antitumour immunity. Moreover we studied the essential questions of transmission EXs via the blood tumor (BTB) or blood brain barier (BBB).

Methods: We evaluated impact of lactose decorated polyoxazolines and carbosilane dendrimers on exosomes isolated from three different tumour cell lines (prostate adenocarcinoma, mammary gland or glioblastoma cell line) using DLS and cryoTEM. Exosomes were isolated by ultracentrifugation and characterized by NTA, DLS and WB. Transmission of fluorescently labeled Exs was studied using in vitro model of BBB/BTB.

Results: We observed distinct interaction between 1-3rd generation carbosilane dendrimers and polyoxazoline-based copolymers decorated with lactose and tumor EXs isolated from all tumor cell lines. Carbosilane dendrimers in contrast to polyoxazoline copolymers, without a difference in the decoration with lactose, disrupted the EXs in a concentration dependent manner. Recombinant galectin 3 decreased the disrupting potential of lactose-decorated carbosilane dendrimers, whereas it did not affect the binding of polyoxazoline copolymers to EXs. Furthermore, we evaluated the efficiency of glycopolymers and glioblastoma derived Exs transfer across the BBB/BTB for their further therapeutic use.

Conclusion: The glycodendrimers and glycopolymers exhibited different behaviour in interaction with EXs-galectin-3 depending on their structure and lactose decoration. This findings could open a new strategies for EXs modification or cancer treatment and together with study of EXs transmission via BBB/BTB could open new view for EXs involvement for therapeutic or diagnostic purposes.

***Funding information**: Czech Health Research Agency – NU23-08-00307 and UJEP-SGS-2022-53-005-3*



P-HD-13

Endothelial dysfunction alters extracellular vesicles' character and secretion profile

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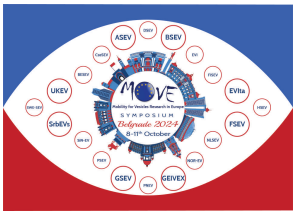
Introduction: Endothelial cells (ECs) are diverse, ubiquitous groups of cells that line the lumen of blood vessels throughout the body, participate in many regulatory processes and play a critical role in vascular and tissue homeostasis. It is recognized that endothelial extracellular vesicles (EEVs) are produced by ECs and transported to recipient cells, where they unload bioactive cargo. EEVs have been shown to induce cytoprotection against ischemia-reperfusion injury (IRI) and supplement cardiomyocytes with proteins involved in cellular metabolism and stress response. Patients suffering from metabolic diseases such as diabetes and obesity suffer from endothelial dysfunction (ED), leading to cardiovascular complications. We hypothesize that ED associated with metabolic disease is linked to changes in the properties of the associated EEVs, thereby altering the cytoprotective effects of EEVs and their homeostatic function. In this study, we induce ED by exposing ECs to hyperglycemic or hyperlipidemic conditions, and investigate alterations in function, secretion patterns, and content of EVs derived from these cells.

Methods: Human umbilical vein endothelial cells (HUVECs) were cultured and expanded under normal, high glucose, high lipid, or a combination. EEVs were isolated using differential ultracentrifugation and size exclusion chromatography. HUVEC morphology and function were assessed to confirm dysfunctional phenotype. EEV enumeration and cargo analysis were performed. And EEVs surface modifications were assessed.

Results: HUVECs grown in these conditions show altered morphology, reduced expression of cell junction proteins, and decreased barrier function. We also show alterations in EEV secretion profiles, EEV cargo, and EEV surface, which may hint at disease-modulated function in EEVs isolated from our ED model.

Conclusions: These results show that HUVECs cultured under diabetes-mimicking conditions exhibit a dysfunctional phenotype and that their secreted EEVs have altered secretion profiles and cargo. This may suggest a novel mechanism of endothelial dysfunction and its association with the increased susceptibility to cardiovascular injury.

Funding: This project is funded by the Israeli Science Foundation, grant number 1802/23, and supported by the Zuckerman STEM Leadership Program.



P-HD-14

Exploring the connections between tumor cell DNA and EVs

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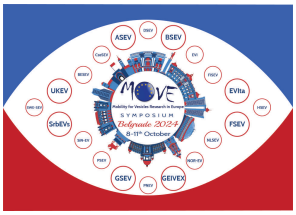
Introduction: Tumor cells profit of EV secretion for communication with their microenvironment. Indeed, EV shedding is augmented in most cancer cell types compared to their non-transformed counterparts. DNA is an intriguing content of tumor-derived EVs and it has been suggested that its loading in EVs could be a mechanism for eliminating toxic DNA. In this work, we aimed to explore the interplay between EV secretion and DNA dynamics.

Methods: EV isolation from melanoma models was performed by differential ultracentrifugation. DNA purification was carried out with a commercial kit. EV proteomic profiles were obtained through LC/MS-MS and subsequent pathway enrichment analysis. The interplay between tumor cell DNA and EV cargo was explored by immunoblotting, immunofluorescence and flow cytometry assays. In addition, we have engineered melanoma models to express CD9, CD63 and ARF6 coupled with fluorescent proteins and studied their colocalization with DNA-related proteins through a high-content screening imaging system.

Results: DNA was present in melanoma EVs but much lower levels were detected in melanocyte-derived EVs. Proteomic analysis revealed that melanoma EVs differentially contain histones and other DNA-related proteins compared to melanocyte-secreted EVs. Interestingly, inhibition of EV secretion promotes cellular and vesicular changes in DNA-associated proteins. Colocalization of EV markers and micronuclei is promoted in DNA stress-induced contexts. Combination of EV inhibitors and DNA repair inhibitors further enhances DNA damage and cell death. Remarkably, these processes occur preferentially in melanoma cells, while primary melanocytes were significantly less sensitive to the inhibition of these pathways.

Conclusion: Tumor cells need to cope with considerable DNA-related stress due to their fast replication. Our results suggest that EVs could be an additional mechanism for alleviating cell-threatening situations involving hazardous DNA.

Funding information: HP: Ministry of Science and Innovation (PID2020-118558RB-I00), Fundación Científica AECC (PRYCO223002PEIN) and Mission Cancer within HORIZON EU Programme (PANCAID-101096309). EB: predoctoral fellowship from the Ministerio de Ciencia, Innovación y Universidades and Agencia Estatal de Investigación (AEI, PRE2021-098062).



P-HD-15

Targeting pro-tumoral stromal exosomes by disrupting the syntenin-CD138/syndecan pathway as a novel therapy in Multiple Myeloma

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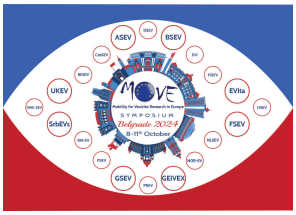
Introduction: Exosomal communication between bone marrow stromal cells (BMSCs) and Multiple Myeloma (MM) cells has been extensively studied as a critical step in the progression and drug resistance of myeloma. However, the specific targeting of pro-tumoral exosomes remains elusive. The PDZ protein syntenin which couples to syndecan 1, has been identified as an exosome marker which can regulate the biogenesis of exosomes. In this study, we aimed to explore whether specific inhibition of syntenin alters exosomal output by BMSCs and whether this could counter BMSC-induced drug resistance in MM.

Methods: Syntenin knockout bone marrow stromal cells (BMSCs) were generated by CRISPR/Cas9 technology. Their exosomes were isolated by ultracentrifugation and protein markers were identified by western blot. The effects of syntenin knockout in BMSCs on myeloma cells were determined by cell viability, apoptosis assay and western blot analysis. A small chemical compound targeting the PDZ2 domain of syntenin, termed SyntOFF was used to block syntenin loading into exosomes and its therapeutic effect in combination with bortezomib was evaluated *in vitro* **and** in the preclinical 5T33MM mouse model.

Results: Syntenin (SDCBP) expression correlates to poor survival in MM patients and is enriched in bone marrow stromal cells. Knockout of syntenin in BMSC alters the exosomal output and abolishes BMSCs-induced bortezomib resistance of MM cells via regulation of STAT3, MAPK, and AKT-mTOR pathways. SyntOFF decreases syntenin sorting into exosomes and enhances the therapeutic effect in vitro and in vivo.

Conclusion: Here we show that syntenin controls the secretion of pro-tumoral exosomes in BMSCs. Blocking syntenin therefore disrupts the communication between BMSC and MM cells and serves as a possible novel target in multiple myeloma.

Funding information: Kom op Tegen Kanker 3M220408



P-HD-16

Towards an understanding of melanoma EV heterogeneity, target cell interactions, and molecular functions in the tumor-draining lymph node

Pragati Lodha[1,2], Sarah Hofmann[1], Alperen Acari[1,2], Jochen Rieck[1], Shruthi Hemanna[1], Emma Reynaud[1,2], Lothar C. Dieterich[1]

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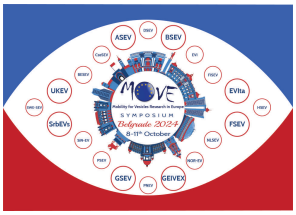
Introduction: Tumor cell-derived extracellular vesicles (TEVs) play an important role in tumor progression and metastasis. Previous studies have shown that TEVs circulate in the blood, interact with distant organs and induce vascular permeability, enhancing metastasis. TEVs are also abundant in tumor-draining lymph and travel via lymphatic vessels to draining lymph nodes (LNs), where they interact withlymphatic endothelial cells (LECs) and macrophages, promoting proliferation and suppressing tumor immunity. However, TEVs are very heterogenous, and the progression-promoting functions ofindividual EV subsets in the LN microenvironment are currently unknown.

Methods: In this study, we aim to unravel the complexity of TEVs within the lymphatic system in order to identifytarget cells and functions of molecularly defined EV subsets in pre-metastatic LNs in melanoma. We have established a multi-parameter flow cytometry-based approach to probe surface proteins on individual EVs derived from cultured melanoma cells, allowing the definition of individual EV subsets based on molecular markers. In addition, we are developing genetic tools to perturb the release of selected EV subsets by melanoma cells in order to decipher their functions in vivo.

Results: Unsupervised clustering of the flow cytometry data suggests that Podoplanin and IFNgR1 mark individual EV subsets. These and other markers will be used to track individual EV subsets and to identify their target cells in vivo in the future. Overall, decoding the interplay between TEV subtypes, their interactions with recipient LN LECs and other cell types, and their influence on tumor progression and tumor immunity holds great promise for identifying therapeutic targets and diagnostic biomarkers that may pave the way for innovative interventions to improve cancer patient prognosis.



P-HD-17

Is Placental Extracellular Vesicle Uptake Modulated by Factors Associated with an Obese Diabetic Pregnancy?

Andreea D Cristian[1,2], Denise Fernandez-Twinn[1], Adriana Paz Córdova Casanova[1], Laís Vales Mennitti[1], Lila M Oyama[3], Catherine E Aiken[1,2 4], Susan E Ozanne[1,2]

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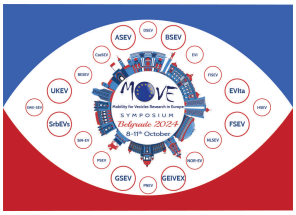
Introduction: Placental-derived small extracellular vesicles (sEVs) and their content have been increasingly recognised as a means of maternal-fetal communication. Moreover, sEVs can travel between tissues and alter metabolism of the target organ, by delivery of sEV-packaged miRNAs. This exosomal cargo is also impacted by conditions such as Gestational Diabetes Mellitus and obesity. However, it is not known if the dynamics of EV fusion with target tissues is also regulated by factors associated with an obese diabetic environment. Using cultured human trophoblasts, we aimed to investigate the target cell specificity of placental sEVs fusion and explored if this was modulated by factors such as glucose and insulin that are altered in obese diabetic pregnancies.

Methods: Human placentas were collected from consenting participants undergoing full-term elective Caesarean section at Addenbrookes Hospital in Cambridge UK who met the study inclusion and exclusion criteria. Study-specific ethical approval was granted under REC21/SC/0025 and the placental collections were obtained via the biobank of the Centre for Trophoblast Research (REC17/EE/0151). Isolated primary cytotrophoblasts were allowed to differentiate into syncytiotrophoblasts over 5 days. Placental sEVs were isolated from the culture media by centrifugation and size-exclusion chromatography. Different human-derived cell lines were then cultured to study the cellular uptake of human trophoblast-derived sEVs under different conditions (glucose, metformin, insulin) using high throughput microscopy.

Results: The placental sEVs were preferentially taken up by the brain cell line with no significant effect of treatment.

Conclusion: These findings suggest that sEVs are taken up with different kinetics by different target cell types. This suggests specificity of communication between the placenta and other tissues via sEVs. With the potential for this to be regulated by factors associated with an obese diabetic intrauterine environment this could provide an additional mechanism by which the placenta could impact on maternal metabolism during healthy and complicated pregnancies.

Funding information: Centre for Trophoblast Research



P-HD-18

The characterization and variability of blood-derived extracellular vesicles in healthy humans and the influence of recent mild COVID-19

Marija Holcar[1], Ivica Marić[2,3], Tobias Tertel[4] Katja Goričar[1], Urška Čegovnik Primožič[5], Darko Černe[5], Bernd Giebel[4], Metka Lenassi[1]

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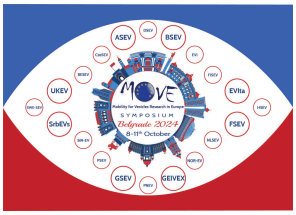
Introduction: Circulating blood extracellular vesicles (EVs) are central to EV biomarker research, yet their characteristics in healthy individuals remain poorly understood. We comprehensively quantified circulating EVs by cellular origin in a large cohort of healthy blood donors, assessed the influence of subject characteristics on interindividual variability, and examined the impact of recent mild COVID-19.

Methods: Blood, clinical (height, weight, blood pressure, menopause), demographic (sex, age), and lifestyle (exercise, smoking) data were collected from 208 healthy pre-COVID-19 (HDC19-) and 60 healthy post-COVID-19 (HDC19+) donors (ethically approved, informed consent). HDC19- samples underwent CBC, lipoprotein, CRP, and insulin analysis. HDC19+ samples were analysed for CBC and anti-SARS-CoV-2 antibodies. Imaging flow cytometry was used to phenotype HDC19- plasma EVs by 25 surface proteins. EVs were enriched from all plasma samples (ultracentrifugation on a 20% sucrose cushion), then analysed for concentration (NTA) and 37 surface proteins by bead-based flow cytometry.

Results: HDC19- subjects, evenly distributed by sex and age, had normal CBC, insulin, lipoprotein, and CRP levels. The average concentration of EV subsets in HDC19- plasma was 2.3*10^7/mL with high interindividual variability and variability between different EV subsets. Specific EV subsets correlated with sex, smoking, menopausal status, and blood type, but not with their cell of origin concentration. Enriched HDC19- EVs (conc: 5.7*10^9/mL; mode size: 151.7 nm) displayed predominantly platelet-derived markers, correlating with smoking (increased EVs from platelet, endothelial, and leukocyte origins) and age (increased EVs of endothelial origin). Compared to HDC19-, HDC19+ samples had significantly fewer (conc: 3.6*10^8/mL) and smaller (121.8 nm) EVs, and different pattern of surface marker expression on enriched tetraspanin+ EVs, however the results did not correlate with anti-SARS-CoV-2 levels or time since recovery.

Conclusion: There is a high interindividual variability in plasma EV concentrations in healthy adults, correlating with some patient's characteristics. Mild COVID-19 altered EV size and concentration.

Funding information: Slovenian Research and Innovation Agency (ARIS): P1-170, Blood Transfusion Centre of Slovenia: grant number 5966.



P-HD-19

Cerebral ischemia alters the microRNA and protein cargo of brain-derived extracellular vesicles

Nea Korvenlaita[1], **Olga Neustroeva**[1], Shaila Eamen[1], Luca Giudice[1], Paula Korhonen[1], Cristiana Pistono[1], Martin E van Royen[2], Guido W Jenster[3], Laura J Vella[4,5], Lesley Cheng[6], Andrew F Hill[6,7,8], Katja M Kanninen[1], Rashid Giniatullin[1], Tarja Malm[1]

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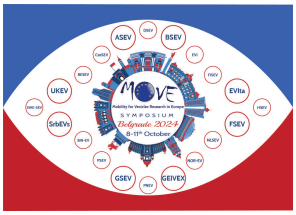
Introduction: Hypoxia alters the secretion of neuronal extracellular vesicles (EVs) but the effects of cerebral ischemia on the *in vivo* secretion of brain-derived EVs (BDEs) is unclear. Studying of changes in microRNA (miRNA) and protein composition of BDEs upon ischemia could help to better understand the pathological mechanisms of stroke and aid in the discovery of novel biomarkers.

Methods: Cerebral ischemia was modelled in mice by permanent middle cerebral artery occlusion (pMCAO). BDEs were isolated from brain hemispheres using sucrose density gradient ultracentrifugation. BDEs were quality controlled by Western blot, transmission electron microscopy (TEM) and Nanoparticle Tracking Analysis (NTA). The number of particles was quantified using both NTA and confocal imaging based EVQuant assay. The levels of miRNAs were measured with qPCR and quantitative proteomics were performed by nano-LC Ultimate 3000RS coupled with the Orbitrap Fusion Lumos.

Results: TEM revealed increased debris in BDE isolates at 12h, but not at 2h or 6h, post-pMCAO. Cerebral ischemia induced a modest increase in BDE particle number at 6h measured by EVQuant. The level of BDE miR-223-3p, a myeloid specific miRNA, was increased at 6h post-pMCAO. Proteins dysregulated at both the BDE and whole tissue level were detected but, in some cases, protein was found to be increased in the tissue and reciprocally decreased in the BDEs. In addition, proteins altered only at the BDE level were detected. The analysis of cell type specific proteome showed positive enrichment of the microglial proteins in the BDEs upon cerebral ischemia.

Conclusion: Microglia might acutely respond to cerebral ischemia by increasing the release of EVs. The consequences of the microglial EVs on the ischemic brain injury and the biomarker potential of microglial EVs for the detection of ischemic stroke and estimation of the severity of cerebral ischemia require further investigation.

Funding information: Saastamoinen Foundation, Paavo Nurmi Foundation, Instrumentarium Science Foundation, Era-Net Neuron iBioStroke



Analysis of the subcellular localization of the transcripts CD81-205 and CD81-215

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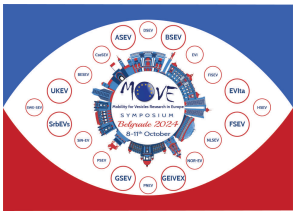
Introduction: Previous research suggested that transcripts CD81-205 and CD81-215 derived from the same gene promoter may be a long non-coding RNAs of stromal origin with potential tumor-promoting role in colon cancer. *In silico* analyses indicated non-coding potential of these transcripts and their elevated expression in tumor vs. non-tumor tissue of colon and rectum. The analysis of the transcripts' expression in tumor cells and tissue samples suggested low expression levels, especially in tumor cells, pointing to the stromal cells as a major source of these molecules. The aim of the study was to analyze the subcellular localization of transcripts CD81-205 and CD81-215.

Material and Methods: The expression of transcripts CD81-205 and CD81-215 was analyzed in the subcellular compartments of the colon cancer cell line SW480 and the normal colon cell line with fibroblast morphology CCD-18Co. The RNA was extracted from nucleus and cytoplasm of SW480 and CCD-18Co cells, and also from exosomes collected from the cell culture media. The qPCR was performed using oligonucleotides targeting the sequence present in both transcripts of interest and not present in other transcripts from this gene.

Results: The expression of transcripts CD81-205 and CD81-215 was undetectable in cytoplasms of cells SW480 and CCD-18Co, while their expression in nuclei of both cell lines and exosomes of SW480 was detectable, but low. The prominent expression was detected in exosomes of the CCD-18Co cells, and this expression was around 5 times elevated in comparison to the exosomes of the SW480 cells.

Conclusion: The prominent expression of transcripts CD81-205 and CD81-215 in exosomes of the CCD-18Co cells confirms the suspected stromal origin of these molecules. Considering that non-coding RNAs may act as signaling molecules even at low concentrations, the study also points to the potential role of CD81-205 and CD81-215 in intercellular signaling between stromal and malignant cells in the tumor tissue.

Funding informarion: Science Fund of the Republic of Serbia, PROMIS, #6052315, SENSOGENE; Ministry of Science, Technological Development and Innovation of the Republic of Serbia, 451-03-66/2024-03/200042



P-HD-21

Role of Extracellular Vesicles in Parkinsonian disorders

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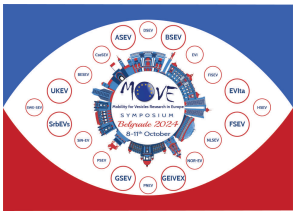
Introduction: Parkinson's disease (PD) is the fastest-growing neurodegenerative disease, of which both α-synuclein (αSyn) aggregation and propagation are known to be a major event in its pathophysiology. PD diagnosis is based on clinical motor symptoms, which only arise after considerable and irreversible neuronal damage has already occurred. Of note, the neuropathological changes in the brain can precede symptoms by decades, as such highlighting a still unmet need for new non-invasive diagnostic and prognostic PD biomarkers. In this context, mounting evidence points to extracellular vesicles (EVs) as a source of biomarkers being important mediators of intercellular communication and carriers for disease-associated proteins including αSyn. More specifically, neuronal-derived EVs (nEVs) separated from peripheral biofluids can provide a snapshot of ongoing pathological changes in the brain, thereby constituting a promising diagnostic tool of PD.

Methods: EVs were isolated from human plasma samples via a combination of size exclusion chromatography and density gradient centrifugation, whereafter EV concentration, size, morphology and tetraspanin distribution were characterized with nanoparticle tracking analysis, negative staining electron microscopy and ExoView. To enrich for nEVs, immunoprecipitation with the neuronal marker L1CAM is explored. To validate whether αSyn levels in (n)EVs are representative for αSyn brain pathology, we established a preclinical mouse model based on the intrastriatal injection of αSyn PFFs in wildtype mice.

Results: Preliminary results indicate successful EV isolation from plasma. Furthermore, we confirmed the presence of several pathological hallmarks including the deposition of αSyn aggregates, neuroinflammation and loss of dopaminergic neurons in our PD mouse model, supporting its feasibility to validate whether plasma (n)EVs can represent a liquid brain biopsy.

Conclusion: This study will demonstrate if αSyn (n)EV levels hold promise as diagnostic biomarker for PD and if αSyn (n)EV levels correlate with severity of PD pathology in both preclinical mouse models and human samples.

Funding information: VLAIO: HBC.2023.1016, FWO: 1295223N, King Baudouin Foundation: 2023-J1991080



P-HD-22

Comparative analysis of surface glycosylation of prostasomes from human seminal plasma of normozoospermic and asthenozoospermic men

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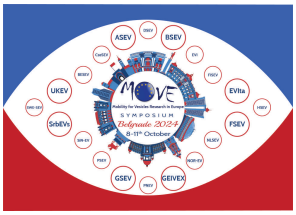
Introduction: Seminal prostasomes are extracellular vesicles secreted by prostate epithelial cells. Using lectin affinity-chromatography, previous studies showed that mannosylated and sialylated glycans contribute to the prostasomal surface. Taking advantage of nanoparticle tracking analysis (NTA), we further investigated the difference of surface glycosylation and size distribution of prostasomes isolated from seminal plasma of normozoospermic (sProN) and asthenozoospermic (sProA) men.

Methods: A panel of fluorescently labeled plant lectins with different sugar specificity, along with antibodies for the tetraspanins CD9 and CD63 were used to analyze sProN and sProA by NTA.

Results: NTA analysis showed that majority of CD9/CD63 positive populations of both sProN and sProA were within the range of 100-200 nm. Wheat germ agglutinin (WGA), recognizing sialic acid and N-acetylglucosamine, and Ricinus communis agglutinin I (RCA I), that binds to terminal galactose or N-acetylgalactosamine, both exhibited high reactivity to sProN and sProA. Concentration (particles/mL) of WGA-positive prostasomes was equal in sProN (3.55E+11) and sProA (3.50E+11). Their size distribution matched that of tetraspanin-positive populations. Concentration of RCA-positive prostasomes was slightly higher in sProN (4.05E+11) compared to sProA (3.4E+11). Their size distribution shifted in relation to tetraspanin-positive populations towards sizes larger than 200 nm, most notably in sProA. Concanavalin A (ConA), specific for mannose and glucose, as well as peanut agglutinin (PNA), specific for galactosyl (β-1,3) N-acetylgalactosamine, showed low reactivity to both sProN and sProA, hindering number and size distribution analysis. Ulex europaeus agglutinin specific for α-linked fucose, Dolichos biflorus agglutinin specific for α-linked N-acetylgalactosamine and soybean agglutinin specific for terminal N-acetylgalactosamine showed no reactivity to both sProN and sProA.

Conclusion: Unlike RCA-, the size distribution of WGA-positive populations of sProN and sProA corresponded to that of tetraspanin-positive populations. RCA-positive populations of sProN and sProA showed slight differences in their size distribution.

Funding information: The study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia - Grant No. 451-03-66/2024-03/200019.



P-HD-23

Extracellular release of a disintegrin and metalloproteinase correlates with periodontal disease severity

Ahmad Aljohmani[1], Hakon Heinze[1], Federico Guillermo Gharzia[1], Bashar Reda[2], Ahmed Mohamed, Mostafa Abdrabou[3,4], Sören Becker[3], Markus Bischoff[3], Matthias Hannig[2], Daniela Yildiz[1]

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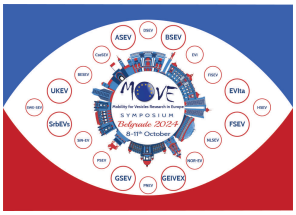
Introduction: Extracellular vesicles (EVs) like exosomes play an essential role in orchestrating the immune response and influencing the performance of immune cells. Periodontal disease is driven by oral pathogens, including Porphyromonas gingivalis, and the release of inflammatory cytokines. These cytokines (e.g. TNF) or their receptors (e.g. IL-1R) are substrates of ADAMs. In this study, we aimed to determine the effects of ADAMs and their release in EVs on periodontal disease phenotypes.

Methods: Exosomes were isolated from sulcus fluid of patients, primary oral keratinocytes and primary human neutrophils by ultracentrifugation followed by sucrose fractionation. The activity of ADAM proteases in EVs and their impact on the recipient cells were evaluated by Western blot, FRET-based activity measurements, ELISA and live cell imaging.

Results: In the sulcus fluid of patients, ADAM8 protein expression and activity were correlated with disease stage, whereas ADAM10 protein expression was inversely correlated with disease stage. Infection and the resulting cytokine release orchestrated the release of soluble ADAM8 by oral keratinocytes and primary neutrophils as soluble ectodomain and on exosomes, respectively. This exosomal release regulated oral tissue destruction and wound healing. Furthermore, ADAM8 regulated the release of ADAM10 and MMP9.

Conclusion: Dysregulation of cell-associated and extracellular ADAM proteolytic activity may be an essential regulatory element in the progression of periodontal disease driven by ADAM8, , influencing wound healing and tissue destruction. The influence of ADAM8 on disease onset and the evaluation of targeting ADAM as a potential and novel local treatment option should be addressed in future translational in vivo studies.



P-HD-24

Phenotypic Alterations in Tissue Extracellular Vesicles from Renal Cell Carcinoma Under Mechanical Stress

Gaia Bianchi[1,2], Elisa De Lorenzis[3], Giovanni Montini[1,2], Emanuele Montanari[1,3], Giancarlo Albo[1,3], Benedetta Bussolati[4] and Federica Collino[1,2]

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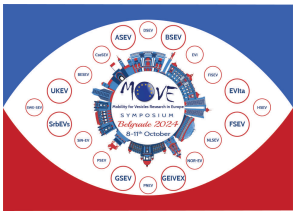
Introduction: Renal cell carcinoma (RCC) accounts for 90% of all kidney malignancies, and nephrectomy represents the mainstay treatment. However, 20-30% of patients with RCC experience recurrence after surgery, with up to 10% reporting a higher risk for distant metastases. Tumor mechanical stresses—namely fluid shear and solid stresses—have been described to induce immune evasion and metastasis formation. Extracellular vesicles (EVs) are relevant players in the tumor microenvironment, with roles in angiogenesis, immune modulation, metastases, and drug resistance. Their composition can change upon external stimuli and stresses, including those occurring during surgical intervention for tumor exportation. Our study aims to define the phenotypic changes occurring in EVs released by the tumor after a mechanical stress and their potential effects on the surrounding microenvironment during tumor progression.

Methods: Twenty-five RCC patients with tumor grade 2, 3 and 4 (pT1a-pT3a stage) were recruited. EVs were extracted from the tumor using an optimized enzymatic protocol followed by either simple dropping (tissue-EVs) or application of a mechanical stress to the tissue (stress tissue-EVs). Particle number was determined by nanoparticle tracking analysis, and the expression of 37 surface proteins was analyzed by flow cytometry using the MACSPlex kit.

Results: Application of a mechanical stress (compression and shears) during the extraction procedure produced an increase in the number of released particles. The three tetraspanins were equally expressed on the surface of EVs obtained through either isolation methods. Stress tissue-EVs presented a general increase of surface markers, particularly of immune-related, inflammatory, and cell adhesion molecules. The only marker that decreased in comparison with tissue-EVs, was the epithelial molecule CD326.

Conclusions: We optimized a protocol for EVs isolation from kidney biopsies. The application of mechanical stress affected the number of vesicles released and the expression of immune and inflammatory related markers. RNA sequencing analysis of stress tissue- and tissue-EVs molecular content will complete their characterization.

Funding: This research was supported by PSR 2022, PI Starting Grant from University of Milano (UNIMI); and Grant P-0038 from IMPACTsim S.p.A.



P-HD-25

Extracellular vesicles from saliva of Rheumatoid Arthritis patients have a distinct profile compared to healthy controls

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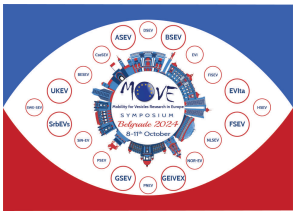
Introduction: Rheumatoid arthritis (RA) is a systemic inflammatory disease that not only affects the joints but can also cause significant disability and impacts overall health, including oral health. Clinical studies have demonstrated that patients with RA often have worse oral health compared to healthy individuals. Saliva has been recognized as a valuable diagnostic tool for detecting both local and systemic diseases, including RA. However, using whole saliva presents several challenges, such as variability in composition and potential contamination. Therefore, salivary extracellular vesicles (EVs) have been investigated to overcome these barriers. The aim of this study was to isolate and characterize salivary EVs from patients with RA in comparison to healthy controls.

Methods: Salivary EVs were isolated from saliva pools of healthy volunteers and patients with RA by differential centrifugation, followed by characterization using SDS-PAGE, dot-blot, Western blot and NTA.

Results: Analysis of the number, size, and zeta potential showed that the number and size of RA salivary EVs were greater than those isolated from healthy donors. Additionally, the zeta potential of RA salivary EVs was negative, unlike the zeta potential of salivary vesicles isolated from healthy volunteers. Within the overall similarity in protein composition and CD9- and CD63-immunoreactivity in the examined groups, a difference was observed in the presence of CD81, which was found only in RA salivary EVs.

Conclusion: The obtained results indicate distinct differences between EVs isolated from patients with RA and healthy donors. It can be assumed that the observed differences may be a consequence of the disease mechanism, its duration, or therapy. Further studies will determine the diagnostic potential of these differences and possibly lead to novel diagnostic or monitoring assays for RA.

Funding information: This study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Agreement no. 451-03-66/2024-03/200019).



P-HD-26

Cargo of circulating extracellular vesicles regulates CDKN1A gene expression according to the MS disease severity

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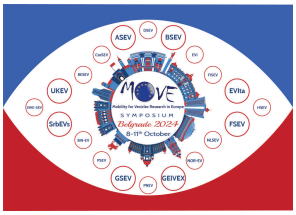
Introduction: Using targeted RNA-Seq of ferroptosis-related genes we have previously identified *CDKN1A* as the top upregulated gene in PBMCs of patients with secondary progressive (SPMS) compared to mild relapse-remitting (RRMS) MS. Herein, we aimed to investigate if treatment with circulating extracellular vesicles (EVs) from MS patients could induce the observed gene expression changes, *in vitro*.

Methods: Plasma samples (V=1ml) of 6 MS patients (SPMS n=3, RRMS n=3) and 3 controls have been used for purification of EVs according to Plasma/Serum Exosome Purification Mini Kit protocol (Norgen Biotek). Three pools of EVs (SPMS, RRMS and Control) were used for assessment of nanoparticle size distribution and concentration using Nanoparticle Tracking Analysis (NTA) on a ZetaView® instrument. Healthy peripheral blood lymphocytes (PBLs) were cultured in 6-well plates at 1×10^6 cells/well using RPMI 1640 with L-Glutamine, with 25mM HEPES medium. Treatment with EVs was performed in triplicate per group for 48h at 37°C using two concentrations, 1×10^9 (lower) and 1×10^{10} (higher) particles/ml. Total RNA was extracted using TRI Reagent. *CDKN1A* mRNA levels were quantified using TaqMan® method on qRT-PCR.

Results: *CDKN1A* was significantly upregulated in PBLs treated with EVs from SPMS patients compared to treatment with EVs from controls, for both EV concentrations (FC_{lower}=1.6, $pValue_{lower}$=0.036; FC_{higher}=1.7, $pValue_{higher}$=0.036). We observed trend in *CDKN1A* upregulation between PBLs treated with EVs from SPMS and RRMS patients using lower EV concentration (FC_{lower}=1.4, $pValue_{lower}$=0.1) but, the treatment with higher EV concentration was followed by significant upregulation of *CDKN1A* in SPMS EVs cell culture ($FC=_{higher}$1.4, $pValue_{higher}$=0.05).

Conclusion: Results suggest difference in circulating EVs cargo from SPMS and RRMs patients with diverse capacity to influence gene expression (e.g. by miRNAs in EVs) and replicate gene expression changes observed *ex vivo*. Brain-originating EVs are present in circulating EVs thus, differences in their cargo could reflect the brain processes related to different MS course.

Funding information: This research was funded by the Science Fund of the Republic of Serbia, grant number #Grant no. 7753406, identification and functional characterization of extracellular and intracellular genetic regulators of ferroptosis related processes in multiple sclerosis, FerroReg.



P-HD-27

The Role of Extracellular Vesicles in Renal Cell Carcinoma Progression

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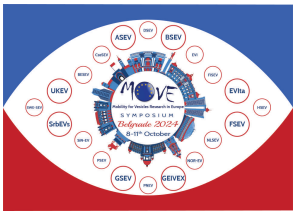
Introduction: With approximately 430,000 cases, kidney cancer accounted for 4.6% of total cancer diagnoses in 2020, according to WHO. Ninety percent of these are Renal Cell Carcinoma (RCC). The 5-year relative survival rate significantly drops when the tumor metastasizes. Extracellular vesicles (EVs) have emerged as key players in cell-cell communication, supporting cancer progression. Given the limited knowledge about EVs in RCC, our aim is to profile EVs and investigate their role in RCC progression.

Methods: Given RCC heterogeneity, the study evaluated two RCC cell lines, 786-O and 769-P, with human embryonic kidney (HEK293) and prostate cancer (DU145) cells as controls. The 3D culture was used to mimic tumor architecture. Molecular and functional analysis (Western Blot, live-dead staining, staining for caspase 3/7 activity, wound-healing, and transwell) characterized the cell lines. EVs were isolated by ultracentrifugation and characterized by NTA, TEM and EVQuant. Spheroids were co-incubated with fluorescently labeled EVs and the uptake was assessed using the Opera Phenix HCS confocal microscope. Functional assays and molecular evaluation of EV-treated spheroids are currently underway.

Results: 786-O exhibit active HIF-1α and higher levels of pro-tumoral markers (vimentin and MMP-2, -3, -8, -9). They migrate faster than 769-P in wound-healing and transwell assays, indicating a more aggressive phenotype. Co-incubation of spheroids with EVs demonstrates uptake in 3D, validating the concept. In ongoing functional and molecular assays, we intend to explore the impact of 786-O-derived EVs on promoting aggressiveness.

Conclusion: In summary, *in vitro* data shows a spectrum of RCC aggressiveness that is related to cellular phenotype. Given this, we hypothesize that these RCC phenotypes are reflected in its derived EVs, potentially influencing cancer progression. Functional and molecular assays are being conducted to explore this hypothesis. Furthermore, our goal is to analyze EVs' content via their unique RNA and protein profiles to identify contributors to hypoxia and metastasis. This could open door for investigation of therapeutic targets.

Funding information: FAPESP Grants 2021/12951-6 and 2023/09378-8



P-HD-28

Extracellular Vesicles in Amyotrophic Lateral Sclerosis: Identification of Hub Regulatory Genes and Their Role in Disease Development Mechanisms

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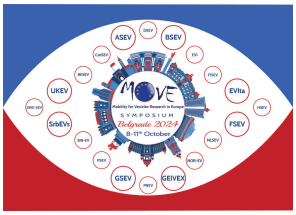
Introduction: Amyotrophic lateral sclerosis (ALS) is a fatal progressive neurodegenerative disease characterized by a permanent degeneration of both upper and lower motor neurons and consequent progressive atrophy and weakness of the skeletal muscles. The ethiopathogenesis of ALS involve multiple altered pathways including perturbations in extracellular vesicle (EV) biogenesis, secretion and functions. The aim of this screening study was to identify the hub regulatory genes connected to the GO term "extracellular vesicle" and its relation to the ALS development, as well to prioritize interactions between genes, involved pathways, and relevant gene-regulators.

Methods: In silico approach included the usage of Comparative Toxicogenomics Database, Cytoscape, ToppGene, MIENTURNET, ChIP-X Enrichment Analysis version 3.

Results: From 98 annotated genes related to EV and ALS development, 10 hub genes were prioritized: NR1H3, PPARGC1B, RXRA, ESRRA, PPARGC1A, FUS, NCOA1, ESRRG, NR4A1, TP53. The main types of interactions among target and 20 related genes - physical interactions (52.09%), predicted (19.63%), genetic interactions (13.24%), shared protein domains (7.88%), co- expression (4.11%), and pathway (3.05%). The prioritized 1) molecular functions were: nuclear receptor binding, RNA polymerase II-specific DNA-binding transcription factor binding, DNA-binding transcription factor binding, transcription regulator activity, transcription factor binding; 2) biological processes were: positive regulation of DNA-templated transcription, positive regulation of RNA biosynthetic process, cellular response to hormone stimulus, hormone-mediated signaling pathway, positive regulation of transcription by RNA polymerase II; 3) pathways were: Regulation of lipid metabolism by PPARalpha, Energy metabolism, Nuclear Receptor transcription pathway, Transcriptional activation of mitochondrial biogenesis. Key regulatory miRNAs were: hsa-miR-148a-3p/hsa-miR-148b-3p/hsa-miR-152-3p, hsa-miR-216b-5p, hsa-miR-376c-3p, while transcription factors were: CEBPA, ZNF385B, MYOD1, E2F4, ZNF391, CRX, KLF4, ESRRB, NR2E3, BBX.

Conclusion: This study identified hub regulatory genes and interactions related to EV and ALS development, providing insights into molecular mechanisms (transcription, lipid and energy metabolism and mitochondrial biogenesis) and regulatory elements that could contribute to disease progression.

Funding information: The author would like to acknowledge the support from the project NIMOCHIP-PRISMA No.4242, Science Fund of the Republic of Serbia and the support from the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (451-03-66/2024-03/ 200178).



P-HD-29

Exosome processing in the context of Alzheimer´s disease

Petra Riegerová[1], Petr Fojtík[2], Dáša Bohačiaková[2] and Martin Hof[1]

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Introduction: Exosomes are extracellular vesicles that play a pivotal role in the development of neurodegenerative disorders, including Alzheimer's disease (AD). By transmitting signal molecules, they regulate neuronal communication, and serve as a rich source of biomarkers for diagnostic purposes. However, their biogenesis in response to neuropathological conditions remains poorly understood. To gain deeper insights into the molecular mechanisms and risk factors contributing to AD development, we investigated exosome processing under disease conditions characterized by non-specific oxidative stress and the dysfunctional SORLA, the newly discovered causative gene in AD.

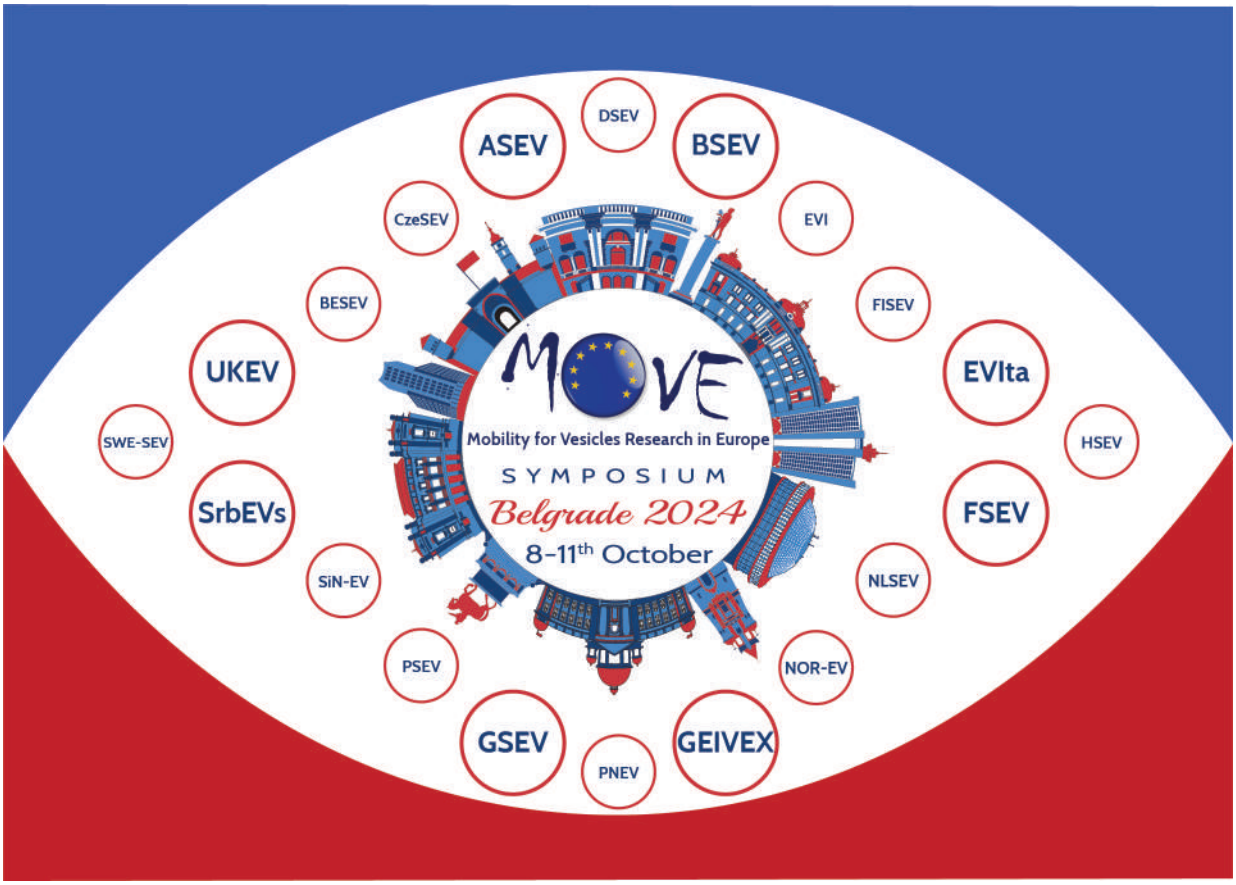
Methods: The effect of AD condition to exosome biogenesis was analyzed in human neurons which were either treated with diethyl maleate to induce oxidative stress or genetically edited to investigate the effect of dysfunctional AD hallmark protein. The subcellular distribution and release of exosomes were monitored by super-resolution fluorescence microscopy, and the obtained signals were subjected to quantification analysis.

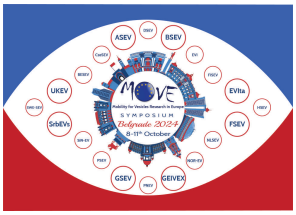
Results: Both oxidative stress and the introduction of specific genetic mutations in the SORLA protein result in altered exosome production and release. Moreover, our results indicate that dysfunctional SORLA affects exosome biogenesis at early stages of the endo-lysosomal pathway.

Conclusion: Conditions associated with Alzheimer's disease significantly alter the intracellular processing and release of exosomes in human neurons.

EV-based biomarkers

- oral presentations -





O-BM-1

Predicting Kidney Injury Post-Transplant: The Role of urine EVs and EV-DNA as Biomarkers

Maja Vodušek[1,3], Ivana Sedej[1], Maja Štalekar[2], Katja Goričar[3], Marija Holcar[3], Nika Kojc[4], Polona Kogovšek[2], Vita Dolžan[3], Metka Lenassi[3], Miha Arnol[1]

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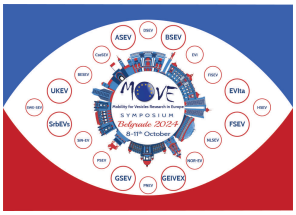
Introduction: Allograft biopsy is the gold standard for assessing transplanted kidneys. However, current non-invasive methods lack sensitivity and specificity. Our study aimed to determine whether urinary extracellular vesicles (uEVs) and uEV-DNA can predict kidney injury at the time of surveillance biopsy approximately 1 year after transplantation (Tx), serving as early, non-invasive biomarkers of kidney allograft injury.

Methods: In a prospective longitudinal study, we followed 49 well-defined adult kidney transplant patients at 1, 3, 6, and 12 months after Tx (ethically approved, informed consent). Blood and second-morning urine samples were collected at each visit. At the time of surveillance biopsy (approximately 1 year after Tx), patients were categorised into two groups based on histologic and molecular analysis (MMDx): normal histology (NH, n = 33) or kidney injury (KI, n = 16). uEVs were isolated using size exclusion chromatography and analysed by nanoparticle tracking, genotyping, and digital droplet PCR for their characteristics and DNA parameters.

Results: Three months after Tx, the KI group had higher donor-derived uEV-DNA levels (p=0.0296), and after six months, they also showed increased uEV size (p=0.0229) and uEV-DNA (p=0.0055) compared to the NH group. A multivariable model with uEV parameters at 6 months predicted KI diagnosis at the time of biopsy with an AUC of 0.74 (95% CI: 0.56 to 0.92), a sensitivity of 0.46, and a specificity of 0.96 (p=0.0001). The negative predictive value (NPV) was 79% and the positive predictive value (PPV) was 86%. Adding serum creatinine in the model increased the AUC to 0.88 (95% CI: 0.77 to 0.99), sensitivity to 0.69, and specificity to 0.89 (p=0.0001). The NPV was 86% and the PPV was 75%.

Conclusion: uEVs and uEV-DNA are highly promising as early biomarkers for kidney allograft injury and as predictors of kidney injury. They have the potential to enhance non-invasive monitoring beyond current biopsy standards.

Funding information: Slovenian Research and Innovation Agency (ARIS), J3-50117



Characterization of circulating extracellular vesicles in patients with major depressive disorder: are them good biomarkers of severity and response to therapy?

Fabiola Stolfi[1], Claudio Brasso[2], Riccardo Sinella[1], Virginia Landra[2], Davide Raineri[1], Giuseppe Cappellano[1], Paola Rocca[2], Annalisa Chiocchetti[1]

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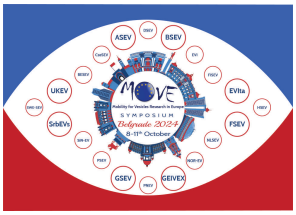
Introduction: Numerous studies have highlighted Extracellular Vesicles (EVs) as diagnostic/prognostic biomarkers; however, they have been scarcely studied in major depressive disorder (MDD). Our objectives were to characterize the most abundant circulating-EVs (leukocyte, endothelial, and platelet-derived) and glia-derived EVs in MDD patients during a major depressive episode (MDE); to evaluate possible associations between MDE severity, antidepressant therapy response, and amount of circulating-EVs.

Methods: 80 MDD patients were recruited during an MDE. During recruitment (baseline), blood sample collection, a clinical evaluation and therapy changes were performed. Patients were clinically reassessed after 3 months (follow-up). EVs isolated at baseline were characterized and quantified by flow cytometry. The severity of depressive symptoms (HAM-D, MADRS), functioning (GAF, WSAS, LEAPS), and patients' subjective quality of life (Q-LES-Q-SF) were assessed at both timepoints. Initially, patients were divided between those not taking antidepressants (NT) and those showing an inadequate response to ongoing psychopharmacological therapy (IR), and at follow-up between responders (≥50% HAM-D score reduction) and non-responders. Differences in circulating-EVs quantity between groups and associations between EVs and clinical variables were evaluated.

Results: At baseline, astrocyte-derived EVs (GLAST+) were associated with worse MADRS score; microglia-derived EVs (TMEM-119+) were associated with better GAF and LEAPS scores and leukocytes-derived EVs (CD45+) were associated with worse Q-LES-Q-SF score. Additionally, compared to the NT group, IR subjects had more platelet-derived EVs (CD41+CD31+). At follow-up, microglia-derived EVs (TMEM-119+) and those generated by B lymphocytes (CD19+) were found to be positive and negative predictors, respectively, of response to the initiation or modification of antidepressant therapy.

Conclusions: In MDD patients during an MDE, platelet-derived, leukocytes-derived, microglia-derived, astrocyte-derived, and B lymphocyte-derived EVs appear to vary with the severity of depressive symptoms, impairment of functioning, patients' subjective quality of life and response to therapy. They could therefore represent useful biomarkers to stratify MDE severity and response to therapy.

Funding information: This project was funded by CARIPLO Foundation (ref. 2019_3277).



O-BM-3

Elevated GLAST+ and Proteoglycan-4+ Extracellular Vesicles, with Proteoglycan-4+ Linked to Cognitive Protection in Amyotrophic Lateral Sclerosis

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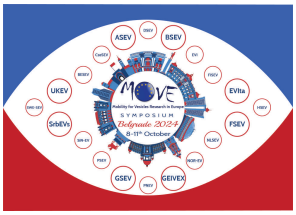
Introduction: Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disorder that leads to the degeneration and death of motor neurons in the brain and spinal cord. Among the pathogenetic mechanisms implicated in ALS, one is glutamate excitotoxicity, which involvesexcessive glutamate accumulation in neurons. In astrocytes, glutamate transporter (GLAST) is the key glutamate transporter responsible for keeping its levels below neurotoxic levels. In ALS, therei s an urgent clinical unmet need related to the lack of reliable biomarkers for early diagnosis, monitoring disease progression, and evaluating new treatments for ALS. Extracellular vesicles (EVs) are small membrane-bound particles that carry proteins, lipids, and nucleic acids. They have emerged as promising biomarkers for various human diseases. The main aim of this study was to search for tissue-specific EVs and to apply proteomic analysis of EVs cargo to identify potential biomarkers for ALS.

Methods: We enrolled 61 ALS patients and 30 healthy age-matched controls (HC) (ethical committee approval n. 184/20). EVs were identified in fresh whole blood and plasma by flow cytometry. Shotgun proteomic was applied to analyze EVs' content.

Results: We found increased levels of GLAST+EVs in the plasma of ALS patients. Proteomic analysis revealed seven upregulated proteins in ALS'EVs compared to HC such as fibrinogen α (FIBA), fibrinogen β (FIBB), fibrinogen γ (FIBG), von Willebrand Factor (VWF), Complement Component 9 (C09), Proteoglycan 4 (PRG4), and Lipopolysaccharide Binding Protein (LBP). Notably, PRG4 levels in EVs resulted significantly increased in ALS patients with normal cognitive function, suggesting its protective role.

Conclusions: These findings highlight the potential of EVs as diagnostic and predictive tools for ALS monitoring. Further functional characterization of GLAST and PRG-4+ EVs could significantly contribute to the development of novel diagnostic and therapeutic strategies.

Funding information: European Union's Horizon 2020 Research and Innovation Program under Grant Agreement No. 953121, project FLAMIN-GO, and the Italian Ministry of Education, University and Research (MIUR) Program "Departments of Excellence 2018-2022," FOHN.



Immunodepletion to enrich and select for rare extracellular vesicle populations: pitfalls and suggestions for best practice

Adam P.S. Bennett[1], Turgay Dalkara[1]

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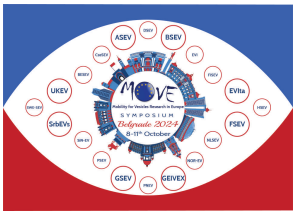
Introduction: Immunodepletion of abundant EV populations is a promising strategy to select for biomarkers associated with rare EVs, epecially when combined with positive selection. However, underappreciated technical factors can affect the interpretation of results for the depletion efficacy of immunodepletion candidates. Here, challenges are described from employing a novel immunodepletion candidate for negative selection of brain-derived neuronal EVs (nEVs).

Methods: EVs were separated from gentleMACS-dissociated murine brains or human plasma using a commercial precipitation reagent or nickel-based isolation. Immunodepletion was performed with concentrated EVs and antibody-coated beads. For proteinase K treatment, EV preparations were incubated for 10 min at 37°C. EV proteins were assessed by Western blot.

Results: Western blotting showed depleting brain-derived EVs bearing the candidate protein led to enrichment of nEV markers in the depleted fraction (compared to non-depleted total EVs). To verify that enriched proteins were constituents of EVs, brain-derived EV preparations were treated with proteinase K and analysed by Western blot. This showed that some assumed nEV markers were likely enriched in the depleted fraction because they were co-isolated free proteins which had been enriched due to the removal of abundant EV proteins from the sample. Next, EVs from plasma were incubated with antibody-coated beads at different concentrations. Western blotting with equal protein loading showed increasing bead concentrations enhanced depletion of the candidate protein in EV preparations compared with non-depleted total EVs. However, Ponceau staning showed additional protein bands in depleted fractions (absent from total EV fractions). These were inferred to be stabiliser proteins from the antibody-bead buffer. Thus, with the addition of these proteins with increasing bead concentrations, Western blot EV protein loading was not equal between groups.

Conclusion: In immunodepletion experiments, EV preparations should firstly be characterised and nEV markers validated to verify constituent EV proteins. Controls for buffer proteins should also be used.

Funding information: Horizon Europe, 101067960; TÜBİTAK, 120C122



O-BM-5

miR-146a-5p and miR-21-5p from extracellular vesicles as potential biomarkers in gestational diabetes

Jovana Stevanović[1], Ognjen Radojičić[2], Ninoslav Mitić[3], Ana Penezić[1], Dragana Robajac[1], Miloš Šunderić[1], Goran Miljuš[1], Danilo Četić[1], Milica Mandić[2], Daniela Ardalić[2], Vesna Mandić Marković[2,4], Željko Miković[2,4], Olgica Nedić[1], Zorana Dobrijević[1]

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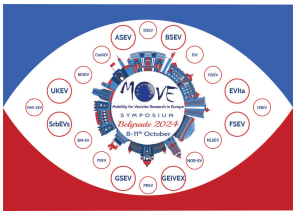
Introduction: The importance of dysregulation of the anti-oxidative system as the driving force of severe damage and pregnancy complications in gestational diabetes (GDM) is well supported by numerous lines of evidence. MicroRNA-based regulatory mechanism demonstrate impairment associated with GDM, as well as disturbances related to the presence of (glyco)oxidative stress ((g)OS) interconnected with inflammation (IFM). Therefore, we hypothesized that microRNAs may act as potential sensors and/or effectors of (g)OS/IFM in GDM and we selected known (g)OS/IFM-associated microRNAs mir-146a-5p and miR-21-5p as candidates for GDM biomarker analysis. The aim was to assess the biomarker potential of these microRNAs from serum-derived extracellular vesicles (EVs).

Methods: EVs were isolated by differential centrifugation from serum of 36 patients with GDM and 36 healthy controls (pregnancy weeks 24-30). EVs sizing and quantification were conducted by nanoparticle tracking analysis, while the shape and size were confirmed by transmission electron microscopy. The presence of CD63 in EVs isolates was determined by dot-blot and Western blot. Quantitative RT-PCR was used for relative quantification of mir-146a-5p and miR-21-5p.

Results: Both miR-146a-5p and miR-21-5p were found to be significantly upregulated in GDM samples ($p=0.014$ and $p=0.017$, respectively). More prominent was the increase in the expression of miR-21-5p, which was two-fold, while the expression levels of these microRNAs in GDM EVs were correlated ($r=0.49$). The expression of miR-146a-5p negatively correlated with the values of anthropometric characteristics of the newborn of GDM patients. MiR-21-5p, on the other hand, showed correlation with CRP values in GDM patients, while in controls it negatively correlated with newborn weight and BMI and positively with the values of maternal HOMA index.

Conclusion: The presented results illustrate the potential of (g)OS/IFM-related microRNAs serve as indicators of GDM. Results suggesting that the expression of mir-146a-5p and miR-21-5p in EVs correlates with newborn characteristics warrants the evaluation of their predictive potential in a larger study.

Funding information: This study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Agreement no. 451-03-66/2024-03/200019).



O-BM-6

Brain-derived blood extracellular vesicles as potential biomarkers in multiple sclerosis (MS): Pilot results from relapsing MS patients receiving anti-CD20 therapy

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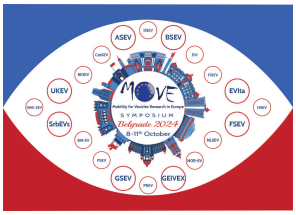
Introduction: Suppression of inflammatory activity with disease-modifying therapies (DMT) is the cornerstone of multiple sclerosis (MS) treatment. Many DMTs, such as anti-CD20 monoclonal antibodies, are administrated as maintenance therapies indefinitely, due to lack of precise biomarkers that reflect the treatment response. Over time, this increases the risk of infections and other immune-mediated side-effects. The specific ability of brain-derived blood extracellular vesicles (EVs) to cross the blood-brain-barrier into the bloodstream, reflecting the current immune status of the central nervous system (CNS), has kindled interest in them as potential biomarkers. Aims: We aimed at defining the potential of MS-specific brain-derived blood EVs biomarker panel to tailor anti-CD20 therapy (rituximab) regimens to individual relapsing MS patients. The overall goal is to maintain a consistent high treatment effect combined with a reduced risk of adverse immune-mediated events during biomarker guided dosing intervals.

Methods: Serum samples (n=5) from baseline (month 0) and after 6 months, were examined from the rituximab (RTX) arm of an ongoing randomized clinical trial OVERLORD-MS (comparing anti-CD20 therapies in relapsing-remitting multiple sclerosis - RRMS patients), in comparison with serum samples from health individuals (n=5). Baseline cerebrospinal fluid (CSF) samples from the same study RRMS cohort were also examined. Morphology, size, distribution of total and L1CAM$^+$ (brain-derived) EVs from both serum and CSF samples were characterized using transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). Deep immunophenotyping of EVs surface receptors were analyzed with conventional flow cytometry using human cytofluorimetric bead-based MACSPlex exosome kit.

Findings: Characterization of blood and CSF EVs revealed a typical round-shaped morphology with a size range of 50-150nm, and expressed all three tetraspanin markers, CD9, CD63 and CD81. L1CAM$^+$ serum EVs from treatment-naïve baseline RRMS patients showed higher tetraspanin expression levels prior to anti-CD20 therapy. Immune receptor profiling revealed a similar expression pattern in L1CAM$^+$ serum EVs and CSF EVs, including distinct pattern change in the surface receptor expression of CD8 and CD19 during therapy towards the pattern of healthy controls. Serum EVs were increasingly expressing EBNA1 following rituximab treatment.

Conclusion: This study indicates the biomarker potential of L1CAM$^+$ blood EVs, as they express a similar immune receptor profile as observed in the CSF EVs, providing a possibility to examine the CNS immune status. The specific expression pattern of CD8 and CD19 in L1CAM$^+$ blood EVs shows promise as anti-CD20 treatment response biomarkers in RRMS patients, prompting further studies.



O-BM-7

Tumour-Derived Extracellular Vesicles Enriched in CAIX Indicate Hypoxia in Drug-Sensitive and Drug-Resistant Breast Cancer

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Introduction: Neratinib benefits many patients with HER2+ breast cancer, but innate or acquired resistance to such drugs undermines their benefit. Our group has previous discovered that expression of hypoxia-inducible carbonic anhydrase 9 (CAIX), which is associated with poor outcome in breast cancer, is increased in neratinib-resistance compared to neratinib-sensitive HER2+ cells[1]. Separately, in prostate cancer, our group also made the seminal discovery that extracellular vesicles (EVs), which are nanosized membrane surrounding packages of proteins and nucleic acids released from cancer cells, have potential as minimally invasive biomarkers reporting on the drug-sensitive versus drug-resistant nature of their cells of origin. Thus, we aimed to evaluate CAIX in EVs to determine if this cargo reflects the hypoxic versus normoxic and/or the drug-sensitive versus drug-resistant nature of these cells.

Methods: Neratinib-sensitive (HCC1954, SKBR3, EFM192A) and neratinib-resistant (HCC1954-NR. SKBR3-NR, EFM192A-NR) cells, established in our laboratory[3], were cultured in normoxic (21% oxygen) or hypoxic (1% oxygen) conditions. Cells were cultured in medium containing EVs-depleted FBS. EVs were collected from their conditioned media using a differential ultra-centrifugation method and the corresponding cells were lysed. EVs were fundamentally characterised using Immunoblotting, NTA and TEM in line with MISEV2023. Cellular and EVs CAIX was quantified by ELISA.

Results and Discussion: Hypoxic compared to normoxic conditions significantly (1.5-4.6-fold; p=0.01-0.0001) increased the expression of CAIX in all cell line variants, as expected. Induction of CAIX was not substantially different whether the cells were neratinib-resistant or neratinib-sensitive. Interestingly, CAIX was detected as cargo of the corresponding EVs, significantly (p=0.03-0.004) more so when the corresponding cells were cultured under hypoxic compared to normoxic conditions.

Conclusion: The CAIX cargo of EVs may have potential as a biomarker reflecting the hypoxic environment of a tumour.

Funding: This research was supported by the Research Council Advanced Laureate Award EVIC to LOD [IRCLA/2019/49].

EV-based biomarkers

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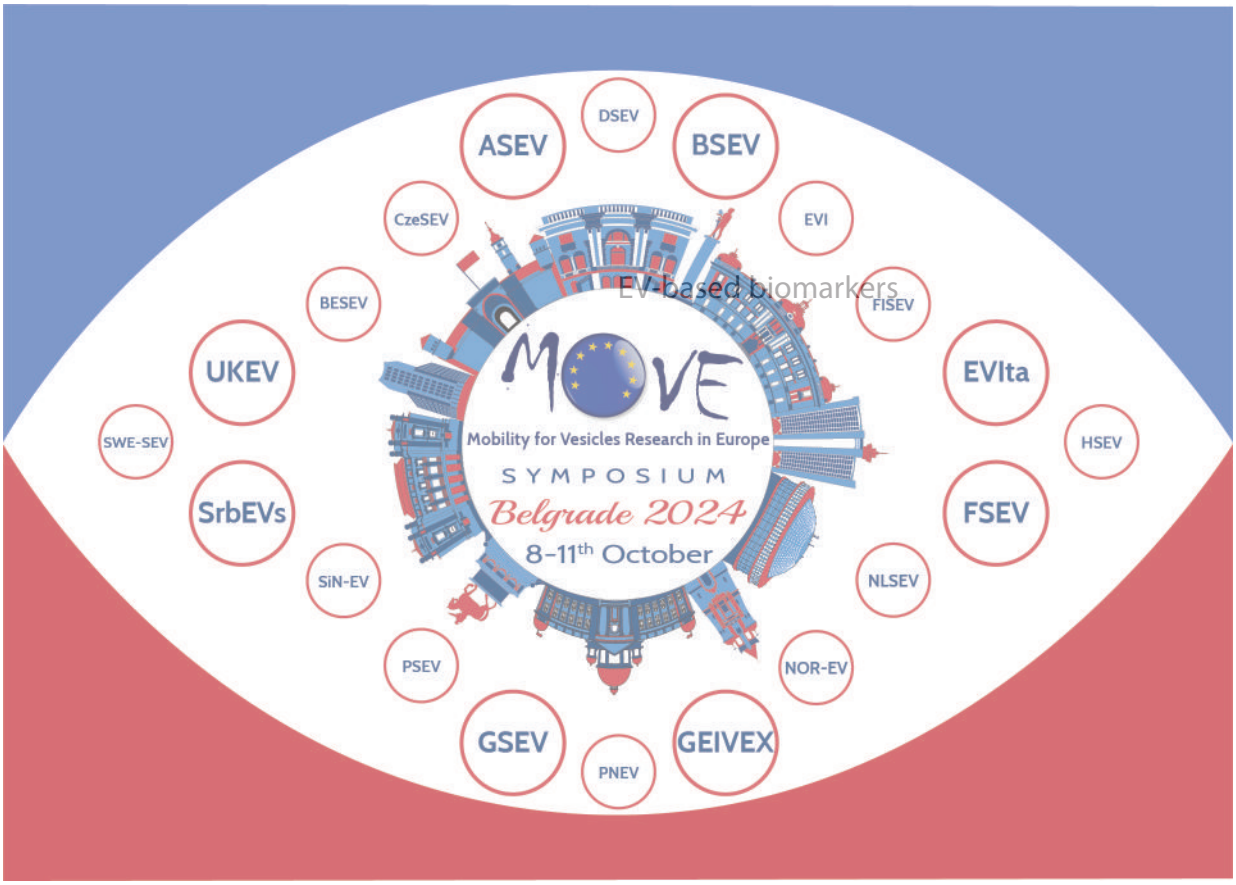

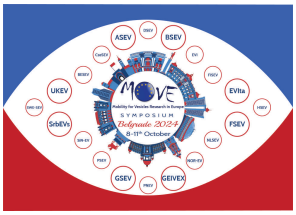

P-BM-1

MARCO as a potential EVs biomarker for Leishmaniasis

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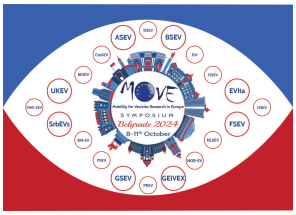
Introduction: Leishmania, an intracellular protozoan parasite, causes leishmaniasis, a vectorborne disease prevalent in 97 countries with an estimated annual incidence of 700,000 to 1,000,000 cases. Leishmania can also be a major pathogen in dogs with canine Leishmaniasis (CanL) being highly prevalent in endemic areas and a major veterinarian concern. Novel biomarkers and management strategies are urgently needed to enhance diagnosis, treatment, and prognosis in humans and dogs. Extracellular Vesicles (EVs), are considered a resource with great potential for a higher understanding of complex biological processes and as a source of biomarkers.

Methods: To evaluate the potential of plasma-derived EVs in the context of Leishmaniasis and to find new possible molecules of interest for disease management EVs were recovered from the plasma of an HIV+VL+ patient over a two-year period, and compared to age and sex-matched HIV and HIV-VL- controls. The approach selected for plasma EVs recovery was size-exclusion chromatography followed by fractions selection using common EVs markers and proteomic analysis of fractions of interest by mass spectrometry. EVs from CanL and non-CanL dogs were also recovered for further validation of MARCO.

Results: The proteomic analysis confirmed the identification of classical EVs markers. The capacity to detect Leishmania proteins was limited, with no identifications involving more than one unique peptide. Moreover, no Leishmania protein identification was common between time points. Regarding human proteins, differences in abundance were observed in the patient compared to both control groups. Using the Gene Ontology approach, these proteins were associated with specific biological processes, such as MHC-I, providing additional insights into the patient's immunological status. Notably, the macrophage receptor with collagenous structure (MARCO) was consistently detected in the patient over multiple time points and in plasma-derived EVs from five other VL patients. Moreover, it was also only detected in plasma EVs from dogs with CanL. The presence of MARCO in plasma EVs was confirmed by Western Blot.

Conclusion: Overall, EVs analysis in the context of VL aids in understanding the ongoing pathological process and may serve as a source of potential biomarkers for disease monitoring. MARCO, uniquely identified in the VL patients, warrants further investigation as a potential diagnostic marker. Future studies are needed to gather comprehensive data on EVs in various infections, aiming to enhance clinical management in vulnerable populations.

Funding information: This work was financed by the Portuguese Foundation for Science and Technology (FCT) through PTDC/CVT-CVT/6798/2020 and the PhD scholarships SFRH/BD/140177/2018, SFRH/BD/140119/2018 and by Individual Support Grant CEECIND/04304/2017 awarded to Inês Costa, Sofia Esteves, and Ana Isabel Pinto, respectively. Nuno Santarém is an assistant researcher funded by national funds through FCT and co-funded through the European Social Fund within the Human Potential Operating Programme 2021.04285.-CEECIND/CP1663/CT0004. This work also had support from the Portuguese Mass Spectrometry Network, integrated in the National Roadmap of Research infrastructures of Strategic Relevance ROTEIRO/0028/2013; LISBOA-01-0145-FEDER-022125). Research on extracellular vesicles in the laboratory of Carmen Fernandez-Becerra is funded by CIBER-Consorcio Centro de Investigación Biomédica en Red (CB 2021), Instituto de Salud Carlos III, Ministerio de Ciencia e innovación, and Unión Europea–NextGenerationEU; Ministerio de Ciencia e Innovación (PID2022-142908OB-I00) and by AGAUR-SGR (2021 SGR 01554).



P-BM-2

Effect of ultramarathon race on Circulating Extracellular Vesicles release and their potential role as exercise biomarkers

Stephanie Fondi[1], Paola Ceccaroli[1], Emanuela Polidori[1], Rachele Agostini[1], Francesca Luchetti[1], Serena Benedetti[1], Vilberto Stocchi[2], Michele Guescini[1]

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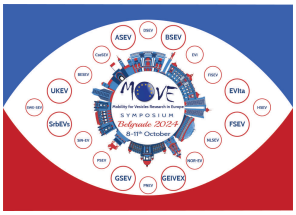
Introduction: Regular physical exercise promotes systemic adaptations which enhance skeletal muscle health, prevent atrophy, and promote regeneration, a highly coordinated process involving inflammation, regeneration and remodeling. Extracellular vesicles (EVs) have recently emerged as key mediators of the inflammatory processes associated with exercise, essential for initiating tissue repair. EVs activate resident and circulating immune cells, mostly macrophages, at the injury site by transferring inflammatory cytokines and signaling molecules, thus modulating the immune response. Moreover, EVs are also involved in the activation of satellite cells, essential for muscle repair and remodeling, by delivering signals that promote their proliferation and differentiation, thereby enhancing muscle regeneration. In this context, the present study aims to investigate the modulation of circulating EVs in response to high intensity and prolonged exercise in order to predict or detect systemic exercise adaptations.

Methods: Serum samples of 19 ultramarathoners were collected (pre- and post-race) and processed by Size Exclusion Chromatography (SEC). SEC fractions were characterized with BCA assay for total protein content, Nanoparticle Tracking analysis (NTA) for particle counting and size distribution, TEM for EV imaging and Dot blot assay for EV markers detection.

Results: EV size and concentration resulted comparable with those reported in literature and EVs showed structural integrity with no morphological differences between pre- and post-race. In addition, there is a significant post-race increase in the expression of tetraspanin CD9, muscle-related marker CAV3 and stress-related mitochondrial marker HSP60.

Conclusion: The obtained results suggest that post-exercise adaptations may promote a shift of the EV phenotype towards an increase in CD9-enriched EVs. Moreover, the increase of CAV3 and HSP60 expression indicates that circulating EVs might mediate muscle remodeling in response to the metabolic stress of high-intensity, prolonged exercise. EVs could therefore represent novel exercise biomarkers that could lead to personalized treatments for muscle injuries and degenerative conditions.

Funding information: University of Urbino Carlo Bo PhD Scholarship funded by PNRR EX DM 351/2022, 38-411-63-DOT19MJNRL-1858



P-BM-3

Flow Cytometric Detection of Extracellular Vesicles Subpopulations in Liquid Biopsies

Seán Patmore[1,2,3], Szilárd-Krisztián Belényesi[1,2,3], Rebecca O'Brien[3,4,5], Eabha O'Sullivan[3,4,5], Faye Lewis[3,6,7], Marika Kanjuga[3,6,7], Sinead Hurley[3,4,5,6,7], Maeve Lowery[3], Sharon O'Toole[3,6,7], Kathy Gately[3,4,5], Lorraine O'Driscoll[1,2,3]

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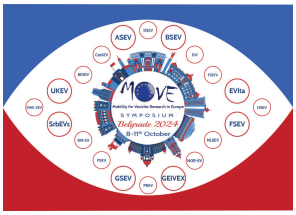
Introduction: Extracellular vesicles (EVs) are heterogenous non-replicating lipid bilayer particles. Released by most cell types, EVs play a role in cell-cell communication and delivery of cargo molecules. The presence of EVs in biofluids makes them ideal liquid biopsy markers for cancer diagnosis. As EVs are direct correlates of the parental cell and can be acquired non/minimally-invasively in biofluids, direct assessment may confer quick and precise clinical diagnostic capabilities. Our approach used flow cytometry to detect the presence of EVs markers, using plasma samples and organoid conditioned media (OCM).

Methods: Plasma from patients with ovarian, gastric and lung cancers were processed and collected for EVs using size exclusion chromatography (SEC). Using a Cytek-Aurora flow cytometer, EVs markers (CD9, CD63 and CD81) were evaluated and the size of particles categorised in matched plasma, OCM from healthy and tumour samples, and collected EV fractions. EVs were also characterised as per the MISEV2023 guidelines, using immunoblotting, TEM and NTA.

Results: Flow cytometry of EVs samples was found to effectively measure size distribution and prevalence of EVs markers. Interestingly, our analysis determined that EVs measured directly in plasma showed significantly higher concentrations than paired EV samples (3.65x108 vs 1.2x107 CD9+EVs/mL, p>0.0179). Furthermore, as tumour biopsies represent the current gold standard for cancer diagnosis, we assessed our EV flow-detection method on tumour OCM. Notably, we found higher levels of EVs markers in OCM from tumour organoids than from patients' matched OCM from normal tissue. Moreover, work is ongoing to characterise the size distribution and EVs marker profile from matched plasma and OCM with immunoblot results.

Conclusion: Flow cytometry offers a powerful method of detecting and quantifying EVs subpopulations in cancer patients' samples. The detected EVs markers provide distinguishable differences between tumour and non-tumour tissues and may offer quicker, more clinically appropriate alternatives to cancer diagnosis and its ongoing monitoring.

Funding information: This research was supported by the North-South Research Programme administered by the Higher Education Authority on behalf of the Department of Further and Higher Education, Research, Innovation and Science and the Shared Island Fund (CLuB: The All-Ireland Cancer Liquid Biopsies Consortium https://www.clubcancer.ie)



P-BM-4

Mirnomic analysis of urine extracellular vesicles from type 2 diabetic patients under SGLT2 inhibitors treatment: identification of biomarkers for therapeutic response

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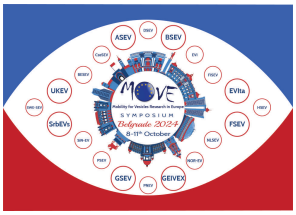
Introduction: Sodium-glucose cotransporter-2 inhibitors (SGLT2i), or gliflozins, lower blood glucose in type 2 diabetes mellitus (T2DM) and improve renal and cardiovascular outcomes in patients with and without T2DM. SGLT2i were also implicated in reducing cell senescence and have shown potential neuroprotective effects. Extracellular vesicles (EVs) have emerged as important mediators of intercellular communication, playing a role in various physiological and pathological processes, including diabetes and its complications. This study aimed to investigate the mechanism(s) of SGLT2i-mediated renal-protection in T2DM patients through miRNA-omics analysis of urine EVs.

Methods: We conducted a clinical trial to identify potential markers of response to treatment of a standard dose of Dapagliflozin (10 mg once a day) in T2DM patients. Patient samples before treatment as well as four to seven months follow up were collected. EVs were isolated from urine using ultracentrifugation. Small RNA sequencing analysis was performed to characterize the EV miRNA cargo.

Results: The differential expression analysis was performed using DESeq22 demonstrating that 16 miRNAs are differentially expressed in urine's EVs, before and after treatment. Two of the significantly upregulated miRNAs target key mediators of the NLRP3 inflammasome pathway and pro-inflammatory cell recruitment, as identified by the Reactome 2022 database. The results of this first bed-to-bench evaluation using EVs revealed significant changes in the miRNA profile of urine EVs obtained from T2DM patients under treatment with SGLT2i.

Conclusion: Our findings suggest that EVs may serve as potential biomarkers for monitoring therapeutic response in T2DM and provide the basis to uncover the biological process associated with SGLT2i-induced renal protection.

Funding information: University of Turin, BRIM_RILO-19_01



P-BM-5

Characterisation of thyroid-originating extracellular vesicles from thyroid cell culture medium and plasma of patients harboring thyroid tumors

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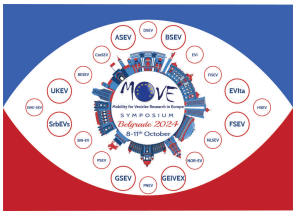
Introduction: Postoperative follow-up of differentiated thyroid carcinoma patients based on measuring thyroglobulin (Tg) levels is unreliable in patients with Tg autoantibodies (TgAt). Detecting thyroid markers on extracellular vesicles (EVs) might be a potent way to overcome these issues. We analysed the presence of CD63 (EVs marker) and thyroid-specific markers: thyroid-stimulating hormone receptor (TSHR) and Tg in EVs of thyroid cell lines to optimize detection and later in plasma EVs of thyroid nodule patients.

Methods: EVs were isolated by differential ultracentrifugation from thyroid normal (Nthy-ori-3-1) and cancer (TPC-1, OCUT2) cell lines and from patient's plasma (four benign, three malignant, and one recurrent). EVs number and size were measured with NTA. The presence of protein markers was determined using dot-blot.

Results: Nthy-ori-3-1 and OCUT2 cells were not viable after 6h of growth in serum-free medium, while TPC-1 cell line was viable until 24h, which influenced the number of isolated EVs. CD63 was present on EVs isolated from all cell lines. TSHR was confirmed in the cell lysate of all cell lines, while only EVs from TPC-1 harbored TSHR on their surface. Tg was present in the cell lysate and EVs originating from Nthy-ory-3-1 and TPC-1 cells, but not OCUT2. Marker analysis showed that plasma EVs from both malignant and benign groups were positive for CD63 and TSHR, and that they contain Tg, while levels of TSHR were higher in the malignant group. Plasma EVs from recurrent patient were positive for all three investigated markers.

Conclusions: TSHR found on the surface of thyroid-originating EVs from medium and plasma could allow immunoaffinity-based isolation of thyroid-specific EVs while the presence of Tg in these EVs holds a promise in detecting reliable Tg levels in TgAt recurrent patients. These promising findings need to be validated on a higher number of samples with analysis of Tg localisation.

Funding information: The study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia Grant No. 451-03-66/2024-03/200019.



P-BM-6

Modelling the impact of extracellular vesicle cargoes in the diagnosis of CAD

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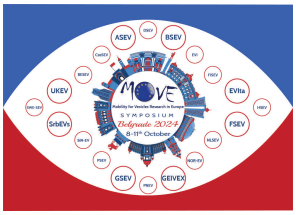
Introduction: Extracellular vesicles (EV) are increasingly focused on as potential biomarkers. We aimed to assess the relationship between circulating EVs, hypoxia-related proteins and conventional CAD risk factors to find novel biomarkers.

Methods: Patients enrolled in the study were categorized based on the results of coronary CT angiography (CCTA). The patient and control groups were matched by age, gender, height, and body weight. The study included 40 individuals, 26 with CAD (mean age 68, ±1.85) and 14 control subjects (mean age 62±2.68. Biochemical and hematological parameters were determined using standardized routine laboratory methods. Characterization of circulating EVs and analysis of plasma concentration of GDF15 were performed by multicolor or bead-based flow cytometry. Plasma protein levels of PYGM, clusterin, CPN1 were determined by ELISA. Logistic regression was used to determine the association of the biomarkers with the CAD outcome after accounting for established risk factors. The analysis was built in three steps: first, we included the basic clinical and laboratory variables (Model 1), and then we integrated the plasma protein values (Model 2), and finally complemented it with the circulating EV pattern (Model 3). To assess the discrimination value of the models, an area under the receiver-operating curve was compared across the three models.

Results: The AUC values were 0.68, 0.77 and 0.84 in Models 1, Model 2 and Model 3, respectively. The variables with the greatest impact on the AUC values were Hemoglobin 0.2 (0.16-0.26) in Model 1, CPN1 0.12 (0.09-0.14) in Model 2, and circulating CD41+/CD61+ EVs 0.31 (0.15-0.5) in Model 3. Correlation analysis showed a significant impact of circulating CD41+/CD61+ platelet-derived EVs (p=0.03, r=-0.4176) in Model 3.

Conclusion: The circulating EV profile can be used as an additional biomarker along with the conventional laboratory markers of CAD, and it enables a more sensitive, non-invasive diagnostic pathway of CAD.

Funding information: Therapeutic Thematic Programme TKP2021-EGA-23; RRF-2.3.1-21-2022-00003 (National Cardiovascular Laboratory Program); 2019-2.1.7-ERA-NET-2021-00015; HUN-REN-SU Translational Extracellular Vesicle Research Group the program



P-BM-7

Neutrophil-derived extracellular vesicles serve as potential biomarkers for carbon monoxide poisoning: a pilot study

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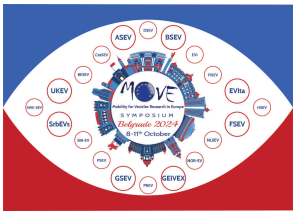
Introduction: Carbon monoxide (CO) poisoning is a serious condition that can occur in various circumstances. CO binds to hemoglobin in the blood, forming carboxyhemoglobin (HbCO), which leads to both neurocognitive and cardiac sequelae. Research has shown that neutrophils interact with platelets during CO poisoning, leading to the deposition of peroxy-nitrate and causing endothelial damage. Upon activation, neutrophils release extracellular vesicles (EVs), that are microparticles involved in cell-to-cell communication by carrying proteins, DNA, RNA etc. derived from the parental cell. EVs are present in all biological fluids and serve as diagnostic/prognostic biomarker of diseases. This work is based on findings from a mouse model, which described an increased count of neutrophil-derived extracellular vesicles (nEVs) following high CO exposure. To our knowledge, there is no data available regarding their involvement in patients intoxicated with CO. Thus, we investigated whether nEVs may serve as biomarker of CO poisoning, and the relation with the development of severe delayed neurological sequelae (DNS).

Methods: 39 patients and 20 healthy controls (HC) were enrolled at the Hospital "Maggiore della Carità", Novara, Italy. We evaluated nEVs counts expressing on the surface CD66 marker, by flow cytometry. Statistical analyses were performed using the Mann-Whitney test.

Results: We found that nEV counts were significantly higher in CO-intoxicated patients compared to HC. Interestingly, among the patients, one showed the highest level of nEVs, which were reduced after normobaric treatment. However, she was hospitalized again one month later due to the development of DNS, and nEV levels were increased concurrent with the onset of neurological complications.

Conclusion: Our preliminary findings suggest that nEVs may serve as diagnostic biomarker for CO poisoning, and possibly as prognostic indicators for DNS. Further studies are needed to validate their utility in clinical practice.

Funding information: Piano Riparti Piemonte", Azione n. 173 "INFRA-P. Realizzazione, rafforzamento e ampliamento Infrastrutture di ricerca pubbliche–bando" INFRA-P2-TECHNOMED-HUB: 378-48



P-BM-8

Bioinformatic analysis predicts serum EV miRNA hsa-miR-378a-3p as potential downregulator of Ceramide signaling pathway in peripheral blood CD8+ T cells of glioblastoma patients

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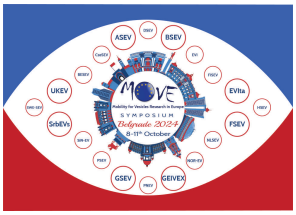
Introduction: Glioblastoma is the most common malignant tumor of the CNS in adults. CD8+ T cell (CTL) tumor infiltration is associated with improved survival in glioblastoma patients. Recent studies have suggested that extracellular vesicle (EV) miRNA cargo assists tumor survival via promotion of immunosuppression of CD8+ T cells. Ceramide signaling negatively affects glioblastoma survival, both as an inducer of apoptosis and as a promoter of CTL activity. We have bioinformatically analyzed which differentially expressed miRNAs (DEmiRNAs) from glioblastoma serum EVs may have the potential effect on dysregulation of ceramide signaling molecular pathway in peripheral blood CTLs of glioblastoma patients.

Methods: Glioblastoma differentially expressed genes (DEGs; glioblastoma patients vs. healthy controls) were obtained from publicly available RNASeq datasets (Huff et al., 2020), serum EV DEmiRNAs (glioblastoma patients vs. healthy controls) were obtained from Ebrakhimkhani et al., 2018. Signaling pathway regulation was analyzed with Qiagen's Ingenuity Pathway Analysis (2023, spring release) tool, while potential miRNA-DEG interactions were analyzed with the gene2mir tool at miRNet software (last accessed in April 2023). The potential effect of serum EV DEmiRNAs was assessed assuming the canonical mRNA-miRNA interaction which leads to mRNA downregulation.

Results: Ceramide signaling was one of the most strongly downregulated adaptive immunity pathways in glioblastoma CTL DEGs (33 DEGs, $p = 1x10\text{-}6$, Z-score = -2.117). Hsa-miR-378a-3p had the greatest potential effect on ceramide pathway downregulation as it potentially regulates the expression of 6 downregulated positive regulators (CTSD, CYCS, JUN, KSR1, RAP2B and PPM1L).

Conclusion: Bioinfomatical predictions point to EV miRNA hsa-miR-378a-3p as having the greatest potential to downregulate Ceramide signaling in peripheral blood CTLs of GBM patients. Knowing that decreased ceramide content interferes with cytotoxic activity of CD8+ T cells, further research should be focused on potential orchestration of CD8+ T cell's by EV cargo, secreted from glioblastoma.

Funding information: The Ministry of Education, Science and Technological Development, Republic of Serbia #451-03-66/2024-03/200017 and Science Fund of the Republic of Serbia, Grant no. 7753406, Identification and functional characterization of extracellular and intracellular genetic regulators of ferroptosis related processes in multiple sclerosis, FerroReg.



P-BM-9

Extracellular vesicles in liquid biopsies: do different size exclusion chromatography columns yield different results?

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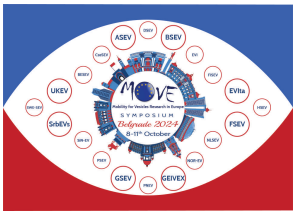
Introduction: Extracellular vesicles (EVs) are heterogenous lipid bilayer-surrounded particles released by a variety of cell types. Notably, EVs contribute to cancer progression through cell-cell communication and delivery of cargo molecules throughout the tumour microenvironment. Growing evidence has shown that EVs can be isolated from minimally invasively obtained samples – such as plasma - where they may be able to serve as disease biomarkers. However, commonly used separation methods such as ultracentrifugation are often inaccessible in a clinical setting. Our approach, using size exclusion chromatography (SEC), a more clinically applicable technique, sets out to characterise EVs from two cancer patient cohorts (lung and ovarian) and explore their diagnostic, prognostic and/or predictive potential.

Methods: Longitudinal plasma samples were taken from patients with either lung or ovarian cancer. EVs were collected from each by SEC, using columns with a 35nm cut-off (qEV35) or 70nm cut-off (qEV70). The resulting EVs isolates were characterised in accordance with the MISEV2023 guidelines using five parameters: nanoparticle tracking analysis, transmission electron microscopy, flow cytometry, immunoblotting, and ELISA.

Results: Our collection method reproducibly isolated plasma EVs from patients of two different cancer types. The resulting EVs demonstrate consistent physical and biochemical properties across these cancer types, as shown by morphology, size and biomarker expression (CD9/CD63 and Syntenin). In the ovarian cancer cohort, the amount of CD63+ EVs was significantly higher in qEV35 isolates as compared to qEV70 isolates (13299±11106 objects/mL for qEV35, 5051±2406 objects/mL for qEV70 respectively, $p<0.005$). A similar trend could be observed in the lung cancer cohort, however not significant. Interestingly, both collection methods yielded similar amounts of apolipoprotein B, as assessed by ELISA.

Conclusion: SEC may be a suitable method for collecting EVs, from liquid biopsies, for utility as biomarkers in cancers. The use of different columns may yield different EVs populations.

Funding information: This research was supported by the North-South Research Programme administered by the Higher Education Authority on behalf of the Department of Further and Higher Education, Research, Innovation and Science and the Shared Island Fund (CLuB: The All-Ireland Cancer Liquid Biopsies Consortium https://www.clubcancer.ie).



P-BM-10

Neuronal extracellular vesicles as nanotools to study and monitor Angelman Syndrome neurodevelopmental disorder

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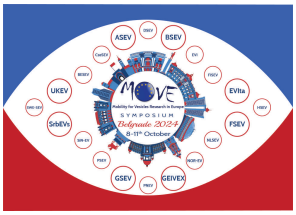
Introduction: Angelman syndrome (AS) is a neurodevelopmental disorder caused by mutations or deletions of the maternally inherited Ube3a gene, which encodes for the ubiquitin ligase E3A (UBE3A) in neurons. AS is characterized by severe intellectual disability, speech impairment, epilepsy, and behavioural abnormalities. UBE3A plays a key role in neurodevelopment but still little is known about its role in the pathogenesis of AS. Today a therapy for AS, based on the unsilencing of the paternal Ube3a allele via antisense oligonucleotides to restore UBE3A expression, is in trial. Monitoring neurodevelopmental disorders' progression and the effects of therapies is often complex and requires invasive procedures or behavioural observations (highly prone to subjective bias). Nowadays there is a high need for reliable and quantitative biomarkers of brain function, for monitoring preclinical and clinical trials, particularly in AS.

Methods: Here, we present a clinical assay based on the selective selection and enrichment of neuronal small Extracellular Vesicles (sEVs) from biologic fluids, with the aim to identify molecular biomarkers deregulated or defective in AS. The assay allows the collection of neuronal sEVs, through size exclusion chromatography and immunocapture, and their subsequential proteomic and transcriptomic analysis. We set up and validate this assay from different wild-type (WT) and AS samples: neuronal cell cultures, murine and human blood samples.

Results: Proteomic results show a significant difference in the levels of several proteins present in the neuronal sEVs between WT and AS neuronal cells. The differentially expressed proteins are meanly involved in cell-cell adhesion, cell-matrix adhesion, and cell movement processes. Interestingly, UBE3A is present in WT neuronal sEVs.

Conclusion: The identified biomarkers will be useful: I) to identify new potential therapeutic targets in AS, clarifying the role of UBE3A in neuronal cells; II) to monitor patients and their therapies, avoiding multiple invasive or semi-invasive procedures.

Funding: this work is supported by Regione Toscana - Bando Giovani*SI* 2021 and Fondazione Pisana per la Scienza ONLUS "Isolation and molecular characterization of neuronal exosomes in models of neurodevelopmental disorders"



P-BM-11

Three methods of isolating EVs from pleural effusion samples of patients with advanced lung adenocarcinoma - potential applications in clinical practice?

Miodrag Vukovic[1], Lidija Filipovic[2], Nina Petrovic[1,3], Andrej Zecevic[4], Maja Kosanovic[5], Miljana Tanic[1], Radmila Jankovic[1], Tatjana Stanojkovic[1], Milica Popovic[2], Aleksandra Korac[6], Milena Cavic[1]

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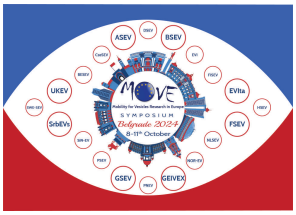
Introduction: Pleural effusion (PE) occurs in 17-23% of lung cancer (LCa) patients and it contains extracellular vesicles (EVs) from cancer cells, representing “liquid biopsy” of LCa. Isolated PE-EVs could be used for LCa diagnosis or monitoring its progresion/therapy. However, there is still no standard method for isolation of EVs from pleural fluid. The aim of this work is to compare PE-EVs isolation methods that could be employed both in research or clinical settings.

Methods: PE samples diluted in PBS (1:1) from patients with advanced non-small cell lung cancer (NSCLC) were utilized. Three methods for isolating EVs were employed: an in-house spherical porous methacrylate-based copolymer coupled with VHH antibodies (chromatography method-CH), ultracentrifugation (UC), and the Norgen Plasma/Serum Exosome Purification and RNA Isolation Mini Kit (Commercial kit-CK). For each EVs isolation method, efficiency was monitored in terms of the amount of starting sample, time required for vesicle isolation, yield, quality of the obtained isolates and overall cost.

Results: In terms of the amount of starting sample, the CH and CK have an advantage, allowing work with as little as 500µL, whereas UC required several milliliters of sample (in our case, due to the rotor type, 12mL minimum). The shortest isolation time was with the CK, followed by the CH, while UC took the longest. Vesicle isolates were cleanest when isolated by CH, followed by the CK, with the lowest purity obtained through UC. When comparing costs per sample, they were approximately: UC–23 euro/sample, CH–20 euro/sample, CK–20 euro/sample.

Conclusion: Depending on downstream analyses, the CH method proved to be the most effective for characterizing the vesicles or analyzing their proteome. For analyzing non-coding RNAs as potential biomarkers in lung cancer, due to the short isolation time of vesicles and the designation of the kit, the CK is the preferable choice.



P-BM-12

Immune checkpoint profiles on circulating extracellular vesicles predict response to immunotherapy in hepatocellular carcinoma

Ramsha Masood[1], Gustav Buescher[1], Joao Gorgulho[1], Franziska Giehren[1], Francesca Pagani[1],Lorenz Kocheise[1], Vincent Jörg[1], Constantin Schmidt[1], Lorenz Adlung[1], Moritz Waldmann[1], Thomas Renne[1], Jenny Krause[1], Guido Sauter[1], Franz Ricklefs[1], Cecile Maire[1], Mohsin Shafiq[1], Ansgar W.Lohse[1], Samuel Huber[1], Henning Wege[1], Carolin Zimpel[2], Jens U. Marquardt[2], Bernhard Scheiner[3], Lorenz Balcar[3], Matthias Pinter[3], Enrico De Toni[4], Ignazio Piseddu[4], Najib Ben Khaled[4], Kornelius Schulze[1], Johann Von Felden[1]

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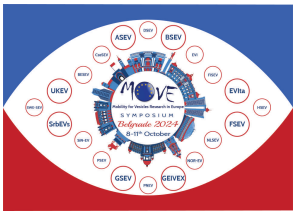
Introduction: Atezolizumab and bevacizumab (AB) is the new standard of care for advanced hepatocellular carcinoma (HCC). However, with only 30% objective response, predictive biomarkers are urgently needed. Tissue expression of PD-L1 is associated with better response, but with limited access to tissue specimens, blood-based biomarkers would be preferable. The aim was to correlate immune checkpoint (IC) expression of tumor-derived circulating extracellular vesicles (EV) with response to AB.

Methods: This multicentre study included 4 independent cohorts with 158 HCC patients with 414 sequential blood samples and 50 tissue specimens (2 more cohorts collected: analysis of another 104 patients and 284 samples pending). Tissue specimens were stained for PD-L1 and PD-1 (CTLA-4 pending). EV were extracted from serum using differential ultracentrifugation and quality control of isolates was performed using nanoparticle tracking analysis and electron microscopy. IC were quantified using bead-based multiplex immunoassays, normalized to total protein input, and presented as pg/µl.

Results: Membrane-bound immune checkpoints (ICs) were significantly enriched in extracellular vesicle (EV) isolates compared to whole serum or EV-depleted serum in HCC patients (Cohort 1, n=40). There was a strong correlation between IC levels in tumor tissue and paired serum EVs (Cohort 2, n=50). Baseline EV-IC levels were higher in non-responders compared to responders in HCC patients receiving AB (Cohort 3, n=49), with distinct EV-IC dynamics over time showing increases in non-responders and decreases in responders. These findings were validated in an independent cohort (Cohort 4, n=43), where baseline IC levels predicted response with high accuracy.

Conclusion: Immune checkpoint levels on circulating extracellular vesicles from blood samples are able to predict response to atezolizumab and bevacizumab in two independent cohorts, both before initiation of therapy and based on early dynamics after initiation. Our results hold promise for the development of liquid biopsy-based biomarkers for treatment prediction in HCC.



P-BM-13

Exosomal Galectins as Biomarkers and Therapeutic Targets of Glioblastoma

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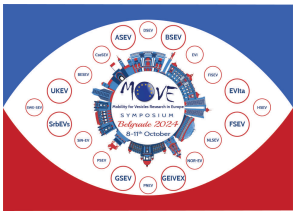
Introduction: Glioblastoma is the most aggressive and lethal primary brain tumor, characterized by increasing resistance to the therapy and neoplasm re-growth. The heterogeneity of glioblastoma and selective permeability of blood-brain barrier present significant challenges for treatment. Recent research has highlighted the major role of exosomes in migration, proliferation, angiogenesis and immune response. Among the various molecules carried by exosomes, galectins have emerged as one of the key modulators of tumor progression and immune response. Our study investigates the role of exosomal galectins in glioblastoma as biomarkers for therapy and potential therapeutic targets.

Methods: We collected exosomes from the blood of healthy volunteers, glioblastoma patients before, immediately after and three months after therapy, as well as exosomes from post-resection tumor tissues. Isolation of exosomes was performed by ultracentrifugation. We characterized exosomes by Nanoparticle tracking analysis, Dynamic light scattering, Bicinchoninic acid assay and Western blotting. The proliferation and migration assays were used to study the impact of galectin-targeted nanotherapeutics on the tumor growth processes.

Results: The exosome samples were characterized in terms of size, concentration, and protein content. We examined the protein expression levels of common exosomal markers (CD9, CD63, and CD81) and exhibited their abundant expression. We evaluated the expression of Galectin-1, Galectin-3, Galectin-8, Galectin-9 comparing a control group with glioblastoma patient groups. The findings align with published results on elevated galectin levels in the blood of tumor patients. Assay the migration and proliferation of glioblastoma primary tumor cells and commercial glioblastoma cell lines, under the influence of tailored glyconanotherapeutic itself or in combination with tumor exosomes, showed their impact on tumor progression.

Conclusion: The findings emphasize the dual role of exosomal galectins in glioblastoma as both biomarkers for monitoring therapeutic efficacy and as targets for novel therapeutic influence. Continued research in the area is essential for developing strategies against glioblastoma.

Funding information: Czech Health Research Agency, NU23-08-00307



P-BM-14

Umbilical Cord Blood Extracellular Vesicles (UCBEVs) study in Preterm Infants

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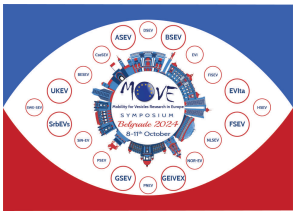
Introduction: Currently, umbilical cord blood (UCB) from term infants is used for cell therapy because it is rich in hematopoietic stem cells, immune cells, and growth factors, promoting better postnatal development. While the regenerative capacity of extracellular vesicles (EVs) has been studied [1], few studies have examined umbilical cord cell exosomes from preterm newborns, and the cellular characteristics of preterm UCB remain unknown. This explorative study aims to characterize EVs in UCB from preterm infants to evaluate their potential importance for future cell therapies, thereby expanding the applications of UCB in neonatal medicine.

Methods: UCB-EV concentrations and tetraspanin co-localization profiles were studied in 20 UCB samples from preterm infants. Clinical data, including maternal and perinatal history, gestational age, and comorbidities were collected. The Leprechaun® platform with the Human Plasma Tetraspanin Kit was used to characterize UCB-EVs, incubating diluted plasma (1:100), providing size distribution, particle concentration, and tetraspanin membrane composition using 1 µL of sample.

Results: The EV concentration from 20 UCB samples ranged from 3×10^5 to 2×10^6. Mean ± s expressions were 50 ± 20% for CD9, 24 ± 9% for CD81, and 30 ± 10% for CD63. A Wilcoxon rank-sum test (p-values < 0.05) was applied to compare tetraspanin profiles and UCBEVs concentrations between groups. Significant differences in CD81 expression (captured with CD9 and CD41a) between preterm infants with sepsis (n=6) vs. controls (n=14) were found. Furthermore, infants from multiple births (n=9) vs single births (n=11) were different in double positive expressions of CD81/CD9 (CD9), CD63/CD9 (CD41a), CD81/CD9 (CD81), and in UCB-EV concentrations captured by CD81. Significant differences were found between infants intubated (n=4) vs. non-intubated (n=16) upon admission in vesicles captured with CD41a, CD63, CD81, and CD9.

Conclusion: UCB-EVs have been characterized, revealing patterns that could be crucial for understanding health and disease conditions in preterm infants. Further studies with larger cohorts are needed to validate these preliminary findings.

Funding information: This work was supported by the AEPED Society; MCIN/AEI/10.13039/501100011033 and, as appropriate, by "European Union NextGenerationEU/PRTR" [grant number CNS2022-135398]; the Instituto de Salud Carlos III (ISCIII), Spain [grant numbers CD22/00081, CPII21/00003, PI23/00202] and co-funded by the European Union.



P-BM-15

Utilising extracellular vesicle glycoproteins for non-invasive identification of patients with clinically significant prostate cancer

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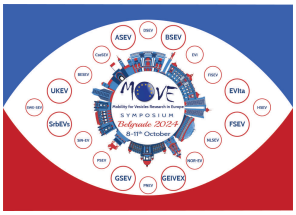
Introduction: Prostate cancer (PCa) accounts for >22% of all new cancer cases diagnosed within Europe. Many patients have slow growing tumours however, some will only present symptoms when the disease is at an aggressive, clinically significant, stage. Despite advancements in non-invasive diagnosis, early detection of clinically significant PCa is difficult, therefore better biomarkers are required. Previous work demonstrates glycoproteins and glycosaminoglycans, present on the surface of EVs, drive tumour growth *in vivo*. Here we show successful detection of glycoproteins, on EVs from patient serum, and their utility for early detection of patients with clinically significant PCa.

Methods: Assessment of EVs was performed on cell conditioned media samples, from PCa cells (LNCaP, Du145 and PC3), non-cancerous cells (PNT2 and BPH-1), prostate organoids derived from iPSCs, or serum samples from patients undergoing diagnostic testing for prostate cancer. EVs were isolated from patient serum by size exclusion chromatography. EVs were characterised based on size, by nanoparticle tracking analysis using the Zetaview PMX-130 platform (Particle Metrix), and immuno-affinity assessment of tetraspanins. The EV glycoprofile was assessed by immuno-affinity capture and lectin-based detection of glycoresidues.

Results: 30 lectins were tested initially, ensuring broad coverage of the glycome, to identify lectins that bound specifically to prostate cancer EVs. This resulted in a refined lectin panel that was taken forward. Prostate cancer organoids, with altered expression of *PTEN*, *TP53* and *MYC* were created to mimic characteristics of metastatic PCa. Several lectins were found to bind preferentially to EVs from metastatic PCa organoids compared to those from non-metastatic organoids. These lectins were capable of detecting EVs in serum from patients with clinically significant PCa.

Discussion: Analysis of the EV glycoprofile from cell and organoid conditioned media reveal distinct EV glycoprofiles, indicative of metastatic PCa. We have produced a proof-of-concept EV assay with potential for early detection of patients with clinically significant PCa.

PCUK funded



P-BM-16

Milk extracellular vesicles decipher the secrets of metabolic health status of lactating cows

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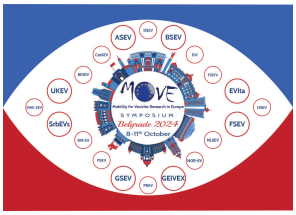
Introduction: High-yielding dairy cows partition more energy towards milk production leading to negative energy balance status and risk of metabolic diseases like insulin resistance (IR). The current diagnosis of IR in cows involves invasive sampling, advanced facilities and detects the disease at severe stages. Therefore, it is essential to identify novel methods to overcome these problems. Extracellular vesicles (EV) are nano-sized particles released by cells and their association in determining the pathophysiology of disease diagnosing has been widely appreciated. The present study was designed to explore potential application of cow milk EV (MEV) for diagnosis of the IR status in prepartum dairy cows.

Methods: Glucose tolerance test (GTT) was carried out on prepartum animals (n=12) and classified into three different IR groups (n=4 each) based on the Area Under the Curve (AUC) for blood insulin concentration. MEVs were enriched from the same animals' morning and afternoon milk using size exclusion chromatography and characterization was carried out. Fatty acids (FAs) profile of the MEV of different IR groups was analysed using gas chromatography.

Results: Physicochemical characterization of MEV revealed that the particle diameter was significantly higher ($p \leq 0.05$) in the high IR group compared to the low and moderate IR groups. No significant changes were observed in particle concentration. Twenty-seven FAs were detected in MEVs while Behenic acid (C22:0) was significantly high in abundance ($p \leq 0.05$) in the high IR group in both morning and evening milk-derived EVs. In addition, long-chain fatty acids such as Pentadecanoic Acid (C15:0) and Lignoceric acid (C24:0) were also significantly abundant ($p \leq 0.05$) in the high IR group of evening MEVs.

Conclusion: The MEV's long chain-FA profiles may potentially serve as promising bioindicators of cow metabolic status and can be used to develop IR-specific, non-invasive, early-stage biomarkers identification strategies in the future.

Funding information: European Union's Horizon 2020 research and innovation program, under grant agreement No. 857418 COMBIVET and The European Union through Horizon's coordination and support actions under grant agreement No. 101079349 OH-Boost.



Spectral signature of plasma-derived sEVs supports the classification of cancer patients

-**Tímea Böröczky**[1,2,3], Mátyás Bukva[1,2], Gabriella Dobra[1], Edina Sebestyén-Gyukity[1], Mária Harmati[1], Yasmin Ranjous[4], László Szívós[5], Katalin Hideghéty[6,7], Krisztina Budai[8], Judit Oláh[7], Péter Horváth[1], György Lázár[8], Zoltán Kónya[4], Pál Barzó[s], Álmos Klekner[9], Krisztina Buzás[1,2]

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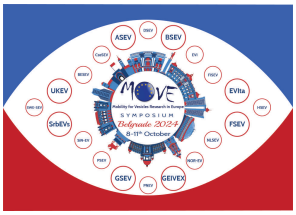
Introduction: Recent studies have shown that extracellular vesicles (EVs) could serve as innovative tools for various tumor diagnostic approaches due to their potential in liquid biopsy. In special cases, such as heterogeneous brain tumors, individual omics analysis is usually unsuccessful, but analyzing their entire molecular composition by Raman spectroscopy holds a promising novel solution for biomarker research. This approach is an underexplored area, therefore we used machine learning methods to create models based on the Raman spectra of small EVs isolated from patients' plasma to help the diagnosis of central nervous system (CNS) tumors.

Methods: The study was approved by the national ethics committee and conducted in accordance with the Declaration of Helsinki. An informed consent form was signed with all the participants. 532 plasma samples were collected from seven patient groups (glioblastoma multiforme, meningioma, brain metastasis, colorectal tumors, melanoma, and lumbar disc hernia patients as controls). Small EVs were isolated through differential centrifugation, then the isolates were characterized by Western Blot, transmission electron microscopy, and nanoparticle tracking analysis. For Raman spectra, the classification, Random Forest algorithm was performed. Classification accuracy, sensitivity, specificity, and the Area Under the Curve (AUC) value were used to evaluate the classification performance.

Results: According to these results, the patient groups are distinguishable with 74–92% sensitivity and 75–88% specificity based on the Raman spectra of small EVs. AUC scores of 0.79–0.92 suggest excellent classification performance.

Conclusion: Our findings indicate that Raman spectroscopic analysis of small EV isolated from plasma is a promising approach for developing noninvasive and cost-effective methods for diagnosing various cancers.

Funding information: OTKA-K143255; TKP2021-EGA09; GINOP-2.2.1–15-2017–00052



P-BM-18

Utilising machine learning for development of an EV-RNA assay for early detection of aggressive prostate cancer

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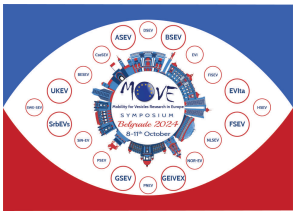
Introduction: Prostate cancer (PCa) is the second most common cancer diagnosed in men. Current diagnostic methods lack sensitivity and specificity for early detection of patients with high-risk PCa, likely to progress to aggressive disease. Despite advances in imaging technologies, accurate diagnosis can still require invasive biopsy. There remains a need for non-invasive biomarkers capable of early detection of high-risk PCa, with extracellular vesicles (EVs) receiving much interest. We have shown that the presence of tumour-reactive stromal cells is associated with disease progression and stromal EVs contain a unique RNA signature capable of identifying patients with aggressive PCa.

Methods: Stromal cells were isolated from needle biopsy specimens, from radical prostatectomies, from patients with PCa (n=17; Wales Cancer Biobank). EVs were isolated from cell conditioned media by serial centrifugation, or exoRNeasy Midi kits (Qiagen) for EV-RNA extraction. RNA libraries were prepared using the TruSeq Stranded Total RNA library prep kit (Illumina) and sequencing performed on the HiSeq2500 V4 platform (Illumina). Identified EV-RNAs were assessed in PCa patient serum by qPCR, comparing patients with low-risk or high-risk PCa (n=40/group; Wales cancer Biobank). EV-RNA candidates were streamlined using Boruta Feature Selection and a Random Forest machine learning model created to assess EV-RNAs in combination. Validation of our EV-RNA panel was performed in an independent cohort of patient samples (n=86).

Results: We identified 14 transcripts that effectively distinguished high-risk prostate cancer (PCa) patients from others. A Random Forest model incorporating all biomarkers achieved an AUC of 0.97 (95% CI: 4.32-7.26) for differentiating high- versus low-risk PCa. This was validated on an independent cohort, with results demonstrating that our EV assay could be used alongside the current blood PSA test.

Conclusion: This study highlights the value of serum EV RNA as a companion diagnostic test capable of early detection of patients with high-risk, aggressive, PCa.

Funding information: Cancer Research Wales (project grant ref 2531)



P-BM-19

Distinguishing Primary and Metastatic Clear Cell Sarcoma via Protein Signatures in Extracellular Vesicles

Djenana Vejzovic[1], Giorgia Magnarini[1], Christina Karner[1], Martin Wolf[2], Dirk Strunk[2], Barbara Darnhofer[3], Ruth Birner-Grünberger[4], Bernadette Liegl-Atzwanger[5] and Beate Rinner*[1,6]

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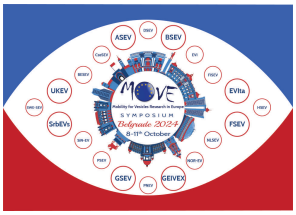
Introduction: Tumour-derived extracellular vesicles (EVs) have unique protein profiles, making them promising targets as disease biomarkers. Clear cell sarcoma (CCS) is a subtype of soft tissue sarcoma characterised by *the EWSR1::ATF1* gene fusion, which helps identify the malignancy but does not correlate with prognosis nor represents a drug target. In addition, CCS quickly disseminate with metastatic lesions often only being discovered when intervention is not possible anymore. As metastatic cells differ significantly in phenotype from their primary tumour, we aimed to identify multiomic based profiles on a cellular and extracellular level to cre ate a novel biomarker for advanced CCS.

Methods: CCS cell lines MUG Lucifer prim and MUG Lucifer met were established from matched primary and metastatic lesions and used for EV isolation from cell culture supernatants with tangential flow filtration (TFF). Size and concentration of EVs was determined by Nanoparticle Tracking Analysis (NTA). Tetraspanin expression and colocalization of single EVs were visualized by ExoView analysis. Label-free proteomics quantification was performed to generate EV proteomic profiles for each lesion.

Results: We observed distinct colocalization patterns of tetraspanins on the surface of tumor-derived EVs compared to non malignant cell derived EVs of the same patient, with triple positive ($CD9^+/CD63^+/CD81^+$) EVs being significantly more abundant in cancer-derived EVs. Preliminary proteomic results further revealed that EVs by metastatic cell showed a total of 69 unique proteins compared to primary tumour derived EVs, most notably the surface protein CSPG4 and the proto-oncogene SRC as potential candidates for distinction between EVs derived from different phenotypes.

Conclusion: In summary, the combination of several colocalized proteins creates a CCS metastasis specific EV marker and provides a promising platform as clinical biomarkers. Further validation steps involving liquid biopsies from mouse models and patient samples will provide more insight into the clinical relevance of the findings.



P-BM-20

Changes in the miRNA cargo of EV-mediated feto-maternal communication following light treatment

Bence Nagy[1], Zoltán Bognár[2,3,4,5], Timea Judit Csabai[2,3,4,5], Nóra Fekete[1], Edit Irén Buzás[1], Árpád Ferenc Kovács[6], Júlia Szekeres-Barthó[2,3,4,5 †], Éva Pállinger[1* †]

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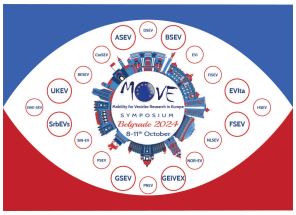
Introduction: Light exposure of embryos during assisted reproduction affects embryo quality and implantation capacity in a wavelength dependent manner. We investigated the molecular mechanism of light-induced changes through the comparative analysis of gene expression and regulatory miRNA profile of murine embryos cultured in dark environment or exposed to white / red filtered light. miRNA sequencing was used to assess the role of embryo-derived extracellular vesicles in the endometrium-embryo dialogue.

Methods: In vitro cultured mouse embryos (3.5 dpc) were exposed to white or red filtered light. After 24 hours, the miRNA content of embryo-derived extracellular vesicles were isolated and RNA-sequencing was performed. Differential expression analysis and functional enrichment analysis were used for evaluating the transcriptome results.

Results: Embryo-derived extracellular vesicles wavelength-dependently enclosed unique miRNA cargos the target genes of which play a role in embryo implantation. We have found many significantly changed miRNA within the embryo-derived extracellular vesicles, independent from the embryonic miRNA. Within the white light treated group, miRNA targeting genes in cell signaling and extracellular matrix reorganization were downregulated. Meanwhile following the treatment with filtered red light, there were significant elevations in levels of miRNA, targeting genes, that play a role in the implantation of embyros. Furthermore there were significant changes in miRNA targeting immunological signaling pathways, namely Fc gamma signaling, DAP12 signaling and inflammatory signalization.

Conclusion: Extracellular vesicles of light-exposed embryos play a role in blastocyst-decidua communication through the horizontal transfer of regulatory miRNAs. Our data prove that light exposure during in vitro fertilization modifies cell function that might affect the outcome of implantation.

This work was supported by OTKA K 147483, TKP-EGA10 and STIA-KF-17.



P-BM-21

Comparative Proteomics analysis of EVs Isolated by different methods from T. cruzi-Infected Cardiac Cells and Their Transcriptional Effects

Berta Barnadas-Carceller[1,2], Nuria Cortes-Serra[1], Nieves Martinez-Peinado[1], Albert Callizo-Armengol[1,2], Julio Alonso-Padilla[1,3], Joaquim Gascon[1,3], Maria Jesus Pinazo[3,4], Ana Paula Dantas[5,6], Carmen Fernandez-Becerra[1,2,3*]

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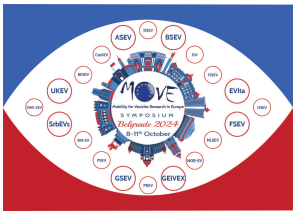
Introduction: Chagas disease (CD), caused by *Trypanosoma cruzi*, is a prevalent parasitic infection in Latin America, responsible for millions of cases annually. In recent years, migratory movements have facilitated the spread of this tropical disease into non-endemic areas, particularly in southern Europe, where it has emerged as a significant public health concern. One of the primary clinical challenges during chronic CD is the lack of effective biomarkers to monitor therapeutic response and disease progression. In this context, circulating extracellular vesicles (EVs) are emerging as promising sources of new biomarkers for the disease.

Methods: EVs derived from T. *cruzi*-infected human cardiac cells were isolated using two different methodologies. Supernatants from human cardiac fibroblasts (HCF) and myocytes (HCM), collected four days post-infection, underwent isolation via Size-Exclusion Chromatography (SEC) and direct CD9/CD81/CD63-Immunocapture (DIC). Mass-spectrometry analysis was subsequently conducted to identify parasitic and human proteins that could serve as potential biomarkers for *T. cruzi* infection, and to determine the most effective isolation method for detecting parasitic proteins. Additionally, transcriptional analysis of these EVs on uninfected cardiac cells is ongoing.

Results: Significant differences were observed in the proteomic profiles obtained from the two isolation methods, including variations in the number of human and parasitic proteins as well as protein abundance. Parasitic proteins were exclusively detected in EV samples isolated by DIC, indicating that immune-affinity capture is optimal for purifying EVs from infected cells harboring the amastigote stage of *T. cruzi*. Evaluation of the transcriptional impact of these EVs on uninfected cardiac cells is currently underway and will be presented accordingly.

Conclusion: This study underscores the critical importance of selecting an effective EV isolation method, thereby highlighting the potential utility of EVs as novel biomarkers for CD and other parasitic infections.

Funding information: This work was supported by Fundació La Marató de TV3 (reference 566/U/2018); CIBER - Consorcio Centro de. Investigación Biomédica en Red - (CB21/13/00112), Instituto de Salud Carlos III; AGAUR (2021 SGR 01554); Unión Europea - NextGenerationEU - Programa INVESTIGO 2022 (AGAUR- ref. BDNS 608313), 2022 INV-1 00046. **Acknowledgments:** We are particularly grateful to Hernando A del Portillo for helpful discussions and to Marc Nicolau and Paula Crego for technical assistant.



P-BM-22

Comprehensive Profiling of Endometriosis-Derived Extracellular Vesicles Unveils Novel Potential Biomarkers for Endometriosis

Karolina Soroczyńska[1], Tobias Tertel[2], Bernd Giebel[2], Piotr Laudański[3], Małgorzata Czystowska-Kuźmicz[3]

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Introduction: Endometriosis, an enigmatic gynecological disorder, poses therapeutic challenges with ineffective treatments and a scarcity of early diagnostic biomarkers. Addressing its multifaceted nature necessitates innovative approaches, focusing on diverse biomarkers for precise diagnostics and tailored therapies. EVs in bodily fluids emerge as promising liquid biopsy biomarkers due to their accessibility and diagnostic potential. A comprehensive strategy integrating advanced EV analysis techniques, such as proteomics and single-vesicle imaging flow cytometry (IFCM), holds promise for identifying new EV-based biomarkers or therapeutic targets for this heterogeneous condition.

Methods: EVs were isolated from plasma and peritoneal fluid (PF) of endometriosis and control patients using SEC and were verified by WB, F-NTA, IFCM and TMT-based quantitative proteomics analysis. Molecular profiling of EVs directly in plasma and PF samples was conducted using high-throughput IFCM with a specialized antibody panel. This panel included detection of antigens elevated during chronic inflammatory states (CD152), associated with early endometriotic lesions (CD82; CD44) and immune suppression (CD16; CD206).

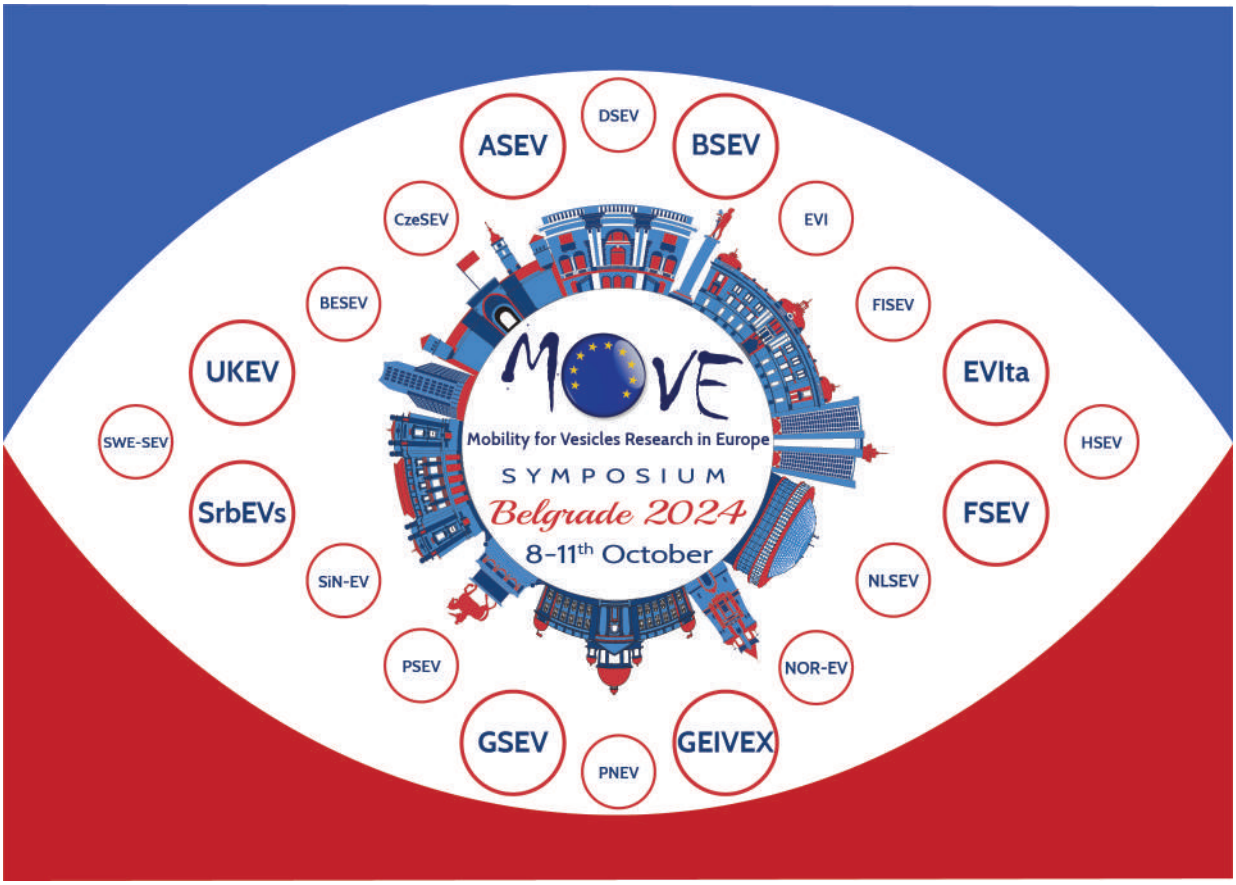
Results: A heterogeneous population of EVs, exhibiting typical characteristics of small EVs and containing bona fide EV markers, was identified in plasma and PF samples from both endometriosis patients and controls. Single EV analyses on the IFCM platform revealed that EV populations from endometriosis patients contain a wide range of molecules, some of which are associated with the pathogenesis of endometriosis. The proteomics analysis of these EVs further highlighted specific protein signatures unique to endometriosis patients, indicating distinct molecular profiles that could serve as potential biomarkers for the disease.

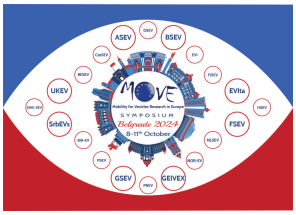
Conclusion: In summary, our study highlights the potential of EVs as promising liquid biopsy biomarkers for endometriosis. The presence of diverse EV populations, along with identified endometriosis-specific signatures, suggests promising applications in diagnostics, prognostics, and therapeutics. Further evaluation of these EV signatures is crucial for advancing non-invasive approaches in managing this complex gynecological disorder.

Funding: This research was funded by National Science Centre under grant 2021/41/B/NZ6/02291.

EVs in interspecies communication

- oral presentations -





O-I-1

Host Immune Cell Membrane Deformability Governs the Uptake Route of Malaria-Derived Extracellular Vesicles

Daniel Alfandari[1], Irit Rosenhek-Goldian[2], Ewa Kozela[1], Marcela Bahlsen[1], Anton Moisieiev[1], Noam Sogauker[1], Ido Azuri[3], Samuel Gelman[3], Edo Kiper[1], Daniel Ben-Hur[1], Raviv Dharan[4], Raya Sorkin[4], Ziv Porat[5], Mattia I. Morandi[6*] and Neta Regev-Rudzki[1*]

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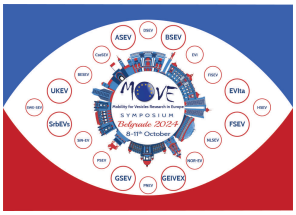
The malaria parasite, *Plasmodium falciparum* (*Pf*), utilizes extracellular vesicles (EVs) to facilitate its growth and communicate with the external microenvironment, primarily targeting the host's immune cells. However, how parasitic EVs are able to enter specific immune cell types within this highly heterogeneous pool of cells remains largely unknown. Using a combination of Imaging Flow Cytometry (IFC) and advanced fluorescence analysis, we here demonstrate that the uptake route for *Pf*-derived EVs is markedly different between host T cells and monocytes. While the adaptive system's T cells internalize *Pf*-derived EVs mainly through an interaction with the plasma membrane (‘capping pattern’), the innate system's monocytes uptake them via endocytosis. We further reveal that the membranal/endocytic balance of the EV internalization is driven mostly by the amount of endocytic incorporation. Integrating atomic force microscopy with data analysis methods revealed that this incorporation heavily depends on the cell's biophysical properties rather than solely on the molecular interaction. Moreover, we demonstrate that altering the cholesterol content in the cell membrane can tilt the balance in favor of one uptake route over another, thereby facilitating the "decision" for EV internalization path. Our results provide a novel mechanistic insight into how *Pf*-derived EVs ensure their entry into diverse host cells, by adjusting their entry mode to the recipient cell's specific mechanical properties. This study highlights the sophisticated cell-communication tactics evolved by the malaria parasite.



Therapeutic potential of extracellular vesicles of E. coli O83 in immunomodulation and allergic airway inflammation

Irma Schabussova

Institute of Specific Prophylaxis and Tropical Medicine, Centre for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

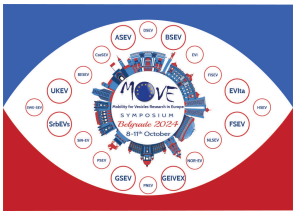
Introduction: The administration of live probiotics, such as those used to alleviate allergy symptoms carries certain risks. This study investigates the immunomodulatory capabilities of extracellular vesicles of probiotic EcO83 (EcO83-EVs) in experimental allergic airway inflammation and demonstrates their potential as postbiotic therapeutics.

Methods: EcO83-EVs were isolated by ultracentrifugation, optionally coupled with density gradient and were analysed for quantity, morphology, zeta potential and presence of DNA, RNA, proteins, carbohydrates or lipopolysaccharide. Using HEK293 cells expressing NOD1, NOD2, TLR2 and TLR4 and bone marrow-derived dendritic cells (BMDCs) from wild-type (WT) and TLR4 knockout (TLR4KO) mice, we investigated the activation of innate receptors and the immunomodulatory effects of EcO83-EVs. The immunotherapeutic efficacy of EcO83-EVs was investigated in a mouse model of ovalbumin-induced airway inflammation.

Results: EcO83-EVs elicited notable IL-8 production in NOD1-, NOD2-, TLR2- and TLR4-transfected HEK293 cells, highlighting the role of these receptors in EcO83-EV signalling. Stimulation with EcO83-EVs increased the levels of IL-23, IL-12, TNFα, IL-1β and IL-6 in WT BMDCs, while TLR4KO BMDCs exhibited reduced cytokine production. *Ex vivo* studies in human nasal epithelial cells showed that EcO83-EVs increased the expression of proteins associated with oxidative stress and inflammation. *In vivo* studies in mice showed that EcO83-EVs interact with nasal-associated lymphoid tissue, are internalised by airway macrophages, and stimulate the recruitment of neutrophil in the lungs. Mechanistically, EcO83-EVs activate the NF-κB signalling pathway, leading to the production of nitric oxide. Intranasal administration of EcO83-EVs reduced allergic airway hyperreactivity and lung eosinophilia but increased infiltration by pulmonary neutrophils, suggesting a possible shift from a Th2 to a Th1 immune response. Ongoing studies are investigating vesicle uptake and the impact of storage conditions on EV integrity.

Conclusion: Our results confirm that EcO83-EVs target NOD1, NOD2, TLR2 and TLR4 receptors and attenuate experimental allergic inflammation when administered intranasally, showing significant potential as a postbiotic alternative to live bacteria. Considering the limited effective treatment options for allergic diseases, our results position probiotic EVs as a promising, safe, and effective therapeutic strategy for the treatment of allergy and potentially other inflammatory diseases in humans.

Funding: The Government of Lower Austria project Danube-Allergy Research Cluster (P17); The FWF project P 34867; MSCA-PF project LactoVES (No. 101066450), OEAD: CZ 04/2024, CZ 07/2023, CZ 15/2023, RS 08/2022



O-I-3

Functional effects of cows' milk derived particles on human inflammatory responses in vitro

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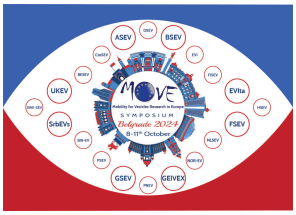
Introduction: Chronic inflammation is a feature of cardiovascular and obesity related disease and is a major cause of morbidity and mortality in developed countries. The consumption of milk and dairy has previously been highlighted as a component of the obesity crisis, however more recent research suggests that consumption of dairy may have positive health benefits. We have previously shown that as well as the milk fat globule (MFG), raw cows' milk contains extracellular vesicles (EVs).

Methods: We have collected milk from individual cows at different stages of lactation (Days in Milk, DIM). We measured EV in whole milk by flow cytometry (Annexin V+, MFG-E8+) and then isolated milk fat globule (MFG) and EV from raw milk using sequential ultracentrifugation. EV were applied to THP-1 monocytes or HMEC-1 endothelial cells. EV uptake, ROS production and cytokine production were assessed. RNA was isolated and sequenced.

Results: DIM correlated with total and annexin-V$^+$ EVs, and DIM negatively correlated with ROS stimulated by cows' milk derived-EV. There was no correlation with cytokine or chemokine production, and weak but not significant correlation with somatic cell count and EV-induced ROS and TNF in THP-1. Evaluation of uptake of different particles in combination showed that MFG inhibits EV uptake but not due to MFG-E8 protein. There was a weak correlation between MFG-E8 positive EV and DIM, suggesting incorporation of MFG-E8 into EV in later lactation. miRNA sequencing on EVs isolated from early, mid and late lactating cows demonstrated differences in the miRNA cargo at different stages that might explain the functional differences observed.

Conclusion: These findings suggest that the cargo of particles released in cows' milk should be examined to more fully understand the effects that dairy consumption has on vascular health in human chronic inflammatory conditions.



Exploring Extracellular Vesicle-Mediated Intercellular Communication in Malaria Cryptic Infections Using Organs-on-a-Chip

Nuria Sima Teruel[1], Paula Crego[1,2], Marc Nicolau[1,2], Hugo Caires[3], Sarinya Sirivisitakul[4], Oscar Castillo-Fernández[1,2], Wanlapa Roobsoong[4], Cristina Barrias[3], Aurora Hernández-Machado[5], Carmen Fernández-Becerra[1,2], Hernando del Portillo[1,2]

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Intercellular communication mediated by extracellular vesicles (EVs) plays an important role in the pathophysiology of human malaria. Recent evidence indicates that malaria parasites evolved cryptic niches in the bone marrow (BM), where sexual blood stages develop, and the spleen (SP), where most parasite biomass is found during chronic infections. Previous evidence demonstrated that EVs from the plasma of vivax malaria patients enhance parasite adhesion to spleen fibroblasts. We thus hypothesize that EVs from natural human malaria infections signal the BM and SP to promote, respectively, gametocyte differentiation and cytoadherence of asexual blood stages; thus, facilitating transmission and establishment of chronic infections.

To address the ethical and technical challenges of studying these erythropoietic tissues, we are using organs-on-a-chip mimicking 3D minimal functional units. These microfluidic devices feature a central channel to accommodate a fibrin-collagen matrix with tissue-specific human cells and two lateral channels that can be endothelialized to perfuse growth medium, EVs and parasites.

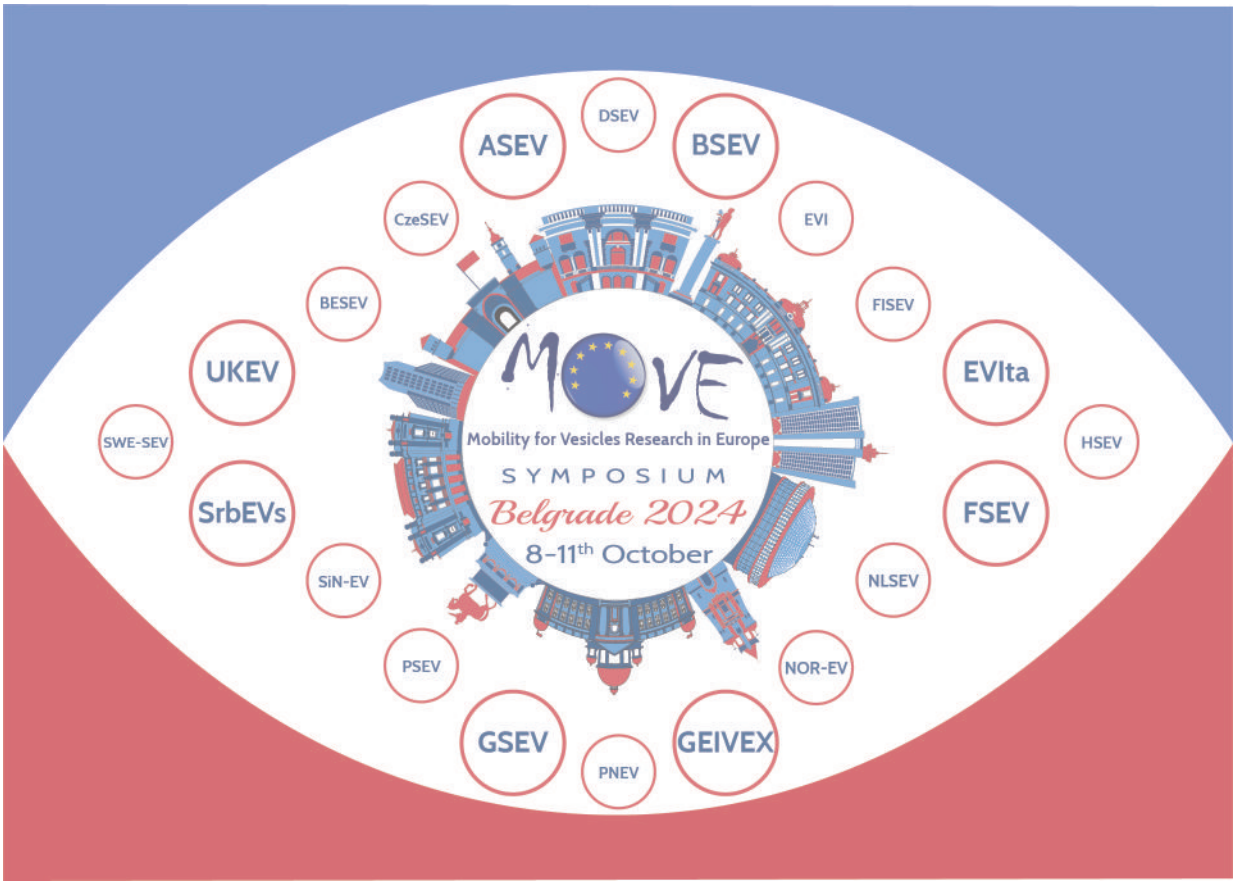
The BM-on-a-chip has been treated with EVs from P. vivax and P. falciparum patients, as well as from healthy donors as control, to investigate their effects on erythropoiesis and the homing of P. falciparum parasites into the BM. Results revealed a tendency to inhibit erythropoiesis, concomitant with specific signaling of PfEVs in inducing parasite migration towards the BM. The SP-on-a-chip successfully sustained the growing and expansion of human SP cell populations. EV uptake experiments and parasite passaging are presently being pursued.

These bioengineered devices will facilitate the study of EV roles at a spatial and temporal resolution that ensures interactions with all cells, providing molecular insights into parasite cryptic infections. Moreover, they will reduce the need for animal use in human experimentation. As the bone marrow and the spleen are immune-privileged sites, these studies will help in discovering alternative control strategies, ultimately contributing to malaria elimination.

Funded by *"la Caixa" Foundation (LCF/PR/HR21/52410021*

EVs in interspecies communication

- poster presentations -



P-I-1

Elucidating the mechanisms underlying the ability of pathogenic Acidovorax temperans and its derived outer membrane vesicles to promote lung cancer progression

Haneen Abu-Freih[1] Ishai Luz[1] Isana Veksler-Lublinsky[2] Dan Bar-Yaacov[1] Elena Voronov[1] Tomer Cooks[1]

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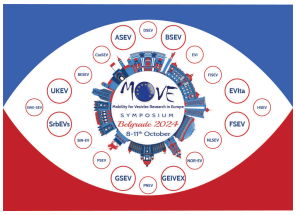
Introduction: Dysbiosis is common in solid tumors, but its direct contribution to tumor development is unclear. Our previous work identified the Gram-negative *Acidovorax temperans* as enriched in tumors of smokers and TP53 mutation patients, where it accelerated tumor growth through pro-inflammatory cell infiltration in the lungs. This study investigates the role of outer membrane vesicles (OMVs) shed by *A. temperans* in driving inflammatory dynamics and tumorigenesis.

Methods: OMVs were isolated using serial centrifugations, filtrations, and an OptiPrep density to remove flagella and non-vesicular elements. Purified samples were characterized using NTA, electron microscopy, and proteomics. Confocal microscopy assessed OMV cellular uptake. OMVs-treated cells were analyzed for the expression of cytokine genes using qRT-PCR. We optimized an in vivo protocol that introduced OMVs into the mice lungs by intranasal administration, confirmed by lung ex vivo imaging to monitor OMV delivery. Immune dynamics of OMV-treated lungs were examined through CyTOF analysis. OMV-RNA was isolated with the miRNeasy kit and prepared using NEBNext Multiplex Small RNA Library Prep.

Results: *A. temperans* OMVs are taken up by A549 lung cancer cells and induce a strong pro-inflammatory response in both A549 cells and THP-1 macrophages. In addition, macrophages exposed to *A. temperans* OMVs overexpressed SIRP-α, linked to tumor immune escape and progression. In vivo, intranasal administration delivers the OMVs directly to the lungs where they lead to an increased secretion of pro-inflammatory cytokines. Flow cytometry revealed selective uptake of OMVs by CD11b+ myeloid cells in mouse lungs. CyTOF analysis of OMVs-treated lungs revealed that OMVs increased infiltration of dendritic cells, macrophages, and T helper 17 cells. OMV sRNA-seq identified a unique RNA signature including various fragmented tRNAs that are selectively loaded in OMVs and are predicted to target host mRNAs.

Conclusions: Interactions between OMVs shed by *A. temperans* and lung cancer microenvironment lead to accelerated, inflammation-based tumorigenesis.



P-I-2

Characterization of extracellular vesicles in the interaction between the German cockroach, Blattella germanica, and its symbiotic partners

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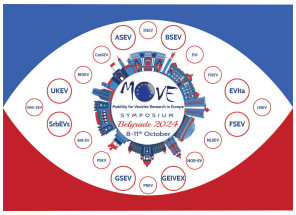
Introduction: Mutualistic symbiotic associations are among the evolutionary strategies that enabled organisms to adapt to new ecological niches. Eukaryotes have developed complex relationships with ecto- and endosymbionts, typically bacterial. These relationships go beyond exchanging primary or secondary metabolites; precise communication between organisms is essential. The host needs to regulate the symbiont population to prevent harmful effects, while the symbiont can induce metabolic and/or physiological changes in the host. The role of antimicrobial peptides (AMPs) or small non-coding RNAs (sRNAs) in these functions has been extensively studied. However, the involvement of extracellular vesicles (EVs) in insect-bacteria symbiotic relationships have been barely studied. The dual symbiotic system of *Blattella germanica* (including the endosymbiont *Blattabacterium cuenoti* and the gut microbiota) is an excellent model for this purpose.

Methods: We isolated EVs from the insect's hemolymph through size exclusion chromatography (SEC) using Sepharose CL2B. EV cargo and surface proteins, as well as EV-depleted samples were analyzed using LC-MS/MS. Bioinformatics analyses on clustering proteins included possible functions and cellular localization.

Results: Proteomics and Bioinformatics analyses revealed the presence of proteins involved either in the control of the endosymbiont population or in metabolic and genetic expression modifications. Our results identified proteins as possible markers of specific EVs.

Conclusion: EVs can be involved in key aspects of host-symbiont communications in insects. They provide new avenues for targeted studies and a better understanding of the molecular dialogue in symbiotic systems, which can be extended to further exploration of EV functions in other insect-bacteria symbioses.

Funding information: MCIN/AEI/10.13039/501100011033 and “ERDF A way of making Europe”, grant PID2021-128201NB-100. DSM is a recipient of a predoctoral fellowship from the University of Valencia. C.M.S-L is the recipient of a postdoctoral fellowship funded by MCIN/AEI/10.13039/501100011033.



P-I-3

Hepatoprotective effects of nanovesicles derived from lemon: an in vitro and in vivo investigation

Roberta Gasparro[1], Giulia Duca[1], Giuditta Gambino[2], Vincenza Tinnirello[1], Danila Di Maio[2], Nima Rabienezhad Ganji[1], Alice Conigliaro[1], Simona Fontana[1], Giuseppe Ferraro[2], Pierangelo Sardo[2], Riccardo Alessandro[1], Stefania Raimondo[1]

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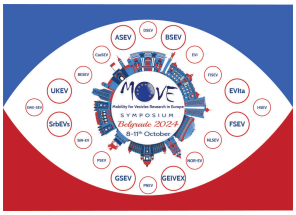
Introduction: Discoveries correlated to plant-derived nanovesicles (PDNVs) have emerged as a crucial starting point for investigating cross-kingdom communication. Numerous studies have been indeed carried out to examine the interaction between PDNVs and mammalian targets. The liver is the primary detoxifying organ that metabolizes compounds associated with the production of free radicals. Excessive ROS in the liver can induce hepatic structural and functional abnormalities that develop into diseases, such as Non-Alcoholic Fatty Liver Disease (NAFLD).

Methods: Lemon nanovesicles produced at laboratory scale (LNVs) were obtained from the juice, processed through differential centrifugations and ultracentrifugation. Industrial Lemon nanovesicles (iLNVs) were obtained using a patented process (IT patent n° 102019000005090). Human healthy hepatocytes (THLE-2) were pre-treated for 24h with LNVs (10 or 25µg/mL) or iLNVs (2.5µg/mL) and subjected to menadione (5 or 10µM), an inducer of oxidative stress. Rats were fed for 6 weeks with a High-fat diet (HFD) and orally administered for 4 weeks with iLNVs (1.2 mg/Kg).

Results: We screened for the first time the effect of LNVs and iLNVs in the reduction of oxidative stress in THLE-2 cells stimulated with menadione. Our findings demonstrate that LNVs and iLNVs reduce the level of ROS production and induce the upregulation of Nrf2 and HO1, as well as the increase of nuclear NRF2. The hepatoprotective effect of iLNVs was also evaluated in vivo, in HFD-fed rats. The oral administration of iLNVs improved glucose metabolism and showed a decrease in biometric parameters, Triglycerides, LDL, and systemic oxidants (d-ROM, LP-CHOLOX). Additionally, our results confirmed that iLNVs increase the level of HDL and systemic antioxidants (SHp and anti-d-ROM) in HFD-rats.

Conclusion: Although further research is required to gain a deeper understanding of the mechanisms involved in the Lemon nanovesicles' actions, this study supports their application for the management of metabolic syndrome disorders and hepatic oxidative stress.

Funding information: This work is funded by the University of Palermo (incentivi ad attività di ricerca interdisciplinare, delibera CdA 07/01 del 04.04.2023) —research project “Studio in vitro ed in vivo degli effetti epatoprotettivi delle vescicole extracellulari vegetali”, to Stefania Raimondo. RG, GD and V.T. and R.G. are PhD students in “Biomedicina, Neuroscienze e Diagnostica Avanzata” (XXXVII ciclo) at the University of Palermo.



P-I-4

Extracellular Vesicles from Lacticaseibacillus casei Modulate Interspecies Bacterial Interactions

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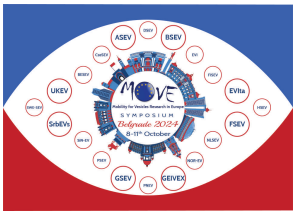
Introduction: Extracellular vesicles released by bacteria play a crucial role in interkingdom communication and can mediate interactions between different bacterial species. Understanding the role of EVs in interspecies communication is essential for unraveling the complex dynamics of polymicrobial communities within the human body and their impact on host health and disease.

Methods: In this study, EVs from Lacticaseibacillus casei (L. casei MVs) were isolated by ultracentrifugation and further purified through size exclusion chromatography (SEC). The purified SEC fractions enriched in MVs were characterized using the Bicinchoninic Acid Assay and Nanoparticle Tracking Analysis. The antimicrobial effect of L. casei MVs was assessed against Escherichia coli DH5α, Staphylococcus epidermidis DSM1798, and L. casei BL23 by monitoring bacterial growth through optical density measurements at 600 nm. To visualize interspecies interaction of L. casei EVs, DiI-labelled EVs were incubated with S. epidermidis DSM1798 for 16 h, followed by SYTO 9-labelling of bacteria. Images were taken using a FLUOVIEW FV3000 confocal microscope.

Results: The SEC fraction enriched in EVs had a protein content of 271±89 µg/ml. The EVs had a mean size of 173±9 nm and a particle concentration of 2.53×1011 particles/ml. The ultracentrifugation pellet (≥1.6×1012 particles/ml) and the EV pellet (5×1011 particles/ml) inhibited the growth of E. coli and S. epidermidis to a greater extent than L. casei growth. Interestingly, lower concentrations of the ultracentrifugation pellet (8×1011 particles/ml) stimulated the growth of E. coli and S. epidermidis. After 16 h incubation, we observed attachment of DiI-labelled EVs to SYTO 9-labelled bacteria.

Conclusion: This study demonstrates the potential role of L. casei EVs in interspecies communication, exhibiting both antimicrobial and growth-promoting effects on different bacterial species. The findings highlight the complex nature of EV-mediated interactions and their potential implications in modulating polymicrobial communities and host-microbe interactions.

Funding information: Country-related cooperation program with Argentina - ALEARG Promotion, 2023, funding program number 57660890.



P-I-5

Exploring biomarkers in major depressive disorder: a multi-OMICs approach on stool samples

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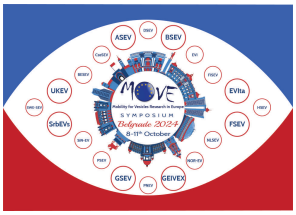
Introduction: Major depressive disorder (MDD) is a prevalent and debilitating mental disorder affecting millions of people worldwide. Despite its high prevalence, diagnosis of MDD is complex and the identification of diagnostic, prognostic and predictive biomarkers is an unmet clinical need. Recent research highlights the role of gut microbiota alteration (i.e. dysbiosis) in several diseases, including MDD. Gut bacteria, as well as eukaryotic cells, release extracellular vesicles (EVs), that are nanoparticles that closely resembling the features of their parental cells. Thus, content analysis of bacterial EVs (bEVs), as well as eukaryotic ones (eEVs) released from all cells, may represent an innovative and non-invasive approach to study microbiota changes and influences. The aim of this study was to evaluate eEVs and bEVs in stool samples from MDD patients, which could serve as biomarkers in the disease onset and progression.

Methods: Stool samples from 47 MDD patients were collected and fecal EVs were isolated by a method established in the laboratory. Western blot and scanning electron microscopy (SEM) analysis were employed for their further characterization. A metaproteomic analysis was conducted to characterize bEVs, whereas the MACSPlex Exosome Kit was used to characterize eEVs.

Results: Metaproteomic analysis on bEVs revealed that B. Uniformis was the most active species. A linear regression model indicated that increased activation of B. Uniformis on the bEVs side, along with high levels of $CD1c^+$ and $CD11c^+$ on the eEVs side at the time of enrolment, were associated to clinical outcomes including a less improvement in antidepressive symptoms, social and work functioning, and reduced subjective quality of life.

Conclusion: This work provides evidence that bEVs and eEVs might represent predictive biomarkers for MDD.

Funding information: Microvesicles at the INtersection between Dysbiosis and Major dEpression disorder: an OMIC approach (MIND-ME) - 2019-3277



P-I-6

Intranasal application of Trichinella spiralis muscle larvae extracellular vesicles alleviate inflammation in mouse model of respiratory allergy

Sofija Glamočlija[1], Anna Schmid[2], Nataša Radulović[3], Alisa Gruden-Movsesijan[1], Ljiljana Sabljić[1], Irma Schabussova[2], Maja Kosanović[1]

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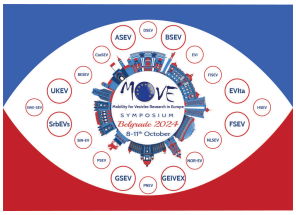
Introduction: The parasitic helminth, the nematode Trichinella spiralis, affects the host immune system through its excretory-secretory products (ES L1), which contain extracellular vesicles (EVs) that prevent host immune response to itself and also the development of chronic inflammatory diseases in the host. EVs from T. spiralis muscle larvae (TsEVs) have been shown to exert immunomodulatory properties on human dendritic cells, inducing a stable tolerogenic phenotype and eliciting regulatory T cells (Treg). Furthermore, it has been shown that infection with T. spiralis can alleviate allergic airway inflammation in mice. Therefore, the aim of this work was to investigate whether TsEVs alone have the same effect.

Methods: Allergic airway inflammation was induced in BALB/c mice by intraperitoneal injection of ovalbumin (OVA) in alum followed by intranasal application of OVA. TsEVs were isolated from ES L1 by differential ultracentrifugation and purified by ultrafiltration. TsEVs were administered intranasally on the days of sensitisation and challenge.

Results: Administration of TsEVs resulted in a decrease in the number of eosinophils in the bronchoalveolar lavage, a decrease in the percentage of eosinophils, macrophages and NK cells in the lungs and in a significant decrease in OVA-specific IgE in the sera compared to the untreated mice. In addition, the percent of CD103+ dendritic cells in the lungs of the treated mice was increased and the percent of CD11b+Ly6C+ cells was reduced. Notably, TsEVs led to a significant increase in CD4+Foxp3+ Tregs and IL-10-producing Tregs. Treated mice had significantly lower production of the Th2 cytokines IL-4, IL-5 and IL-13 and increased production of IL-10 from immune cells isolated from the lung and spleen.

Conclusion: These results suggest that TsEVs could be utilised for the development of new therapeutics to alleviate allergic airway inflammation due to their potent immunomodulatory properties.

Funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, Co. No. 451-03-66/2024-03/200019 and No. 337-00-577/2021-09/41; the Inovation Fund of the Republic of Serbia Grant No. 5906; the Government of Lower Austria project Danube-Allergy Research Cluster (P17); the FWF project P 34867, and the OEAD projects CZ 04/2024, CZ 07/2023, CZ 15/2023, and RS 08/2022.



P-I-7

Interactions of Trichinells spiralis mucle larvae extracellular vesicles with target cells and their mechanisms of action

Sofija Glamočlija[1], Anna Schmid[2], Alisa Gruden-Movsesijan[1], Jelena Đokić[3], Irma Schabussova[2], Maja Kosanović[1]

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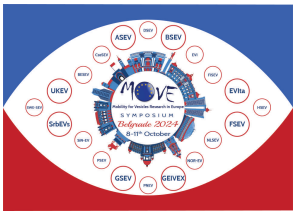
Introduction: The parasite Trichinella spiralis induces a tolerogenic immune microenvironment in its host, which also leads to the alleviation of chronic inflammatory diseases. This effect is exerted by excretory–secretory products of the muscle larvae (ES L1), with extracellular vesicles (TsEVs) being one of their most active components. TsEVs have been shown to induce a stable tolerogenic phenotype of human monocyte-derived dendritic cells (hDC), which are then able to induce regulatory T cells (Treg). It has also been shown that TsEVs can alleviate allergic airway inflammation in a mouse model. However, the mechanisms of action TsEVs underlying the observed effects are still unknown. Therefore, the aim of this study was to investigate how TsEVs interact with human cells and which signalling pathways they employ.

Methods: TsEVs were isolated from ES L1 by differential ultracentrifugation and purified by ultrafiltration. The glycosylation of TsEVs was analysed with lectins in the ELLA assay. Uptake of TsEVs by hDC was observed with or without inhibitory sugars. The interaction of TsEVs with innate pattern recognition receptors was analysed using transfected HEK293 cells. The signaling pathways employed by TsEVs were investigated in TsEVs-treated hDC by qPCR.

Results: Glycosylation analysis revealed that TsEVs have a glycosylation pattern characteristic of helminths and uptake experiments showed that the interaction of TsEVs with hDC is partially dependent on glyco-interactions. However, TsEVs also activate the innate pattern recognition receptor TLR2. PCR results suggest that TsEVs, probably due to having multiple active molecules, activate multiple signaling pathways, such as mTOR, NF-kB2 and IDO.

Conclusion: The results obtained in this work represent the first steps towards elucidating the mechanisms of action of TsEVs as potent immunomodulators and facilitate the future development of TsEVs-based therapeutics.

Funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, Co. No. 451-03-66/2024-03/200019 and No. 337-00-577/2021-09/41; the Inovation Fund of the Republic of Serbia Grant No. 5906; the Government of Lower Austria project Danube-Allergy Research Cluster (P17); the FWF project P 34867, and the OEAD projects CZ 04/2024, CZ 07/2023, CZ 15/2023, and RS 08/2022.



P-I-8

Interkingdom communication between oral pathogenic bacteria and Candida species at the level of extracellular vesicles

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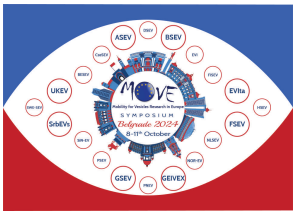
Introduction: The human oral cavity is colonized by more than 700 microbes, such as bacteria, viruses, fungi, known as the oral microbiota. As a result of environmental effects, such as smoking or infections, the microbial composition may change, which can result in dysbiosis that may lead to diseases, such as oral candidiasis. Oral candidiasis is most commonly caused by *Candida albicans*, which can alter the bacterial diversity. To examine the nature of such fungal-bacterial interactions, we aim to investigate the interaction between *Candida* species- and oral pathogenic bacteria at the level of extracellular vesicles (EV).

Methods: For our experiments we used the *C. albicans* SC5314 and C. parapsilosis CLIB214 strains, along with *Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeruginosa* as pathogenic bacterial counterparts. We optimized the fungal and bacterial EV isolation protocol from solid media. The characterisation of the EVs by transmission electron microscopy and NanoSight showed round shaped particles with diameters between 50 and 250 nm. We examined the effects of EVs released by *C. parapsilosis* and the yeast and hyphae form of *C. albicans* on the growth and biofilm formation efficiency of *S. aureus, P. aeruginosa* and *E. faecalis* and vica versa.

Results: Regarding the effect of bacteria, the bacterial EV treatment reduced the number of CFUs of *C. albicans* cells. Bacterial EVs also altered the biofilm formation efficiency of the fungal species in a species dependent manner. *Staphylococcus aureus* derived EV treatment significantly decreased the efficiency of *Candida albicans* biofilm formation, while it increased it in the case of *Candida parapsilosis*.

Conclusion: Altogether these results suggest the presence of an active interaction between fungal and bacterial cells at the level of EVs.

Funding information: This research was supported by the grant HUN-REN 2001007, Centre of Excellence for Interdisciplinary Research, Development and Innovation of the University of Szeged, Competence Centre for Molecular Biology, Bionics and Biotechnology and by the Louis-Jeantet Foundation. Project no TKP2021-EGA-28 has been implemented with the support provided by the Ministry of Culture and Innovation of Hungary from the National Research, Development and Innovation Fund, financed under the TKP2021-EGA funding scheme." EU's Horizon 2020 research and innovation program under grant agreement No. 739593 Hungarian Scientific Research Fund (NKFIH K 147510).



P-I-9

The role of extracellular vesicles in oral squamous cell carcinoma-Candida interaction

Éva Veres[1], Zóra Szilovics[1], Dóra Adamecz[2], Gergő Svorenj[1], Krisztina Buzás[3], Mónika Kiricsi[2], Attila Gácser[4]

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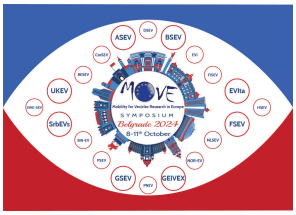
Introduction: Oral squamous cell carcinoma (OSCC) accounts for 90% of oral cancer episodes worldwide. In the context of OSCC, the normal oral microbiota is often altered, which may predispose to local infections or conditions such as oral candidiasis. A previous study from our laboratory showed that the presence of *Candida albicans* enhances the progression of OSCC *in vitro* and *in vivo* by enhancing the activity of genes and signaling pathways involved in tumor progression, oncometabolite production and matrix metalloproteinase (MMP) activity. However, the main component causing the changes has not yet been identified. Therefore, in our work, we investigate the effect of *Candida*-derived extracellular vesicles (EV) on the progression of OSCC. As we hypothesize that EVs play a crucial role in cell-cell communication, they may also play an important role in host-pathogen interaction.

Methods: During our experiments, we isolated vesicles from *Candida albicans* and *Candida parapsilosis*. In the case of *C. albicans*, EVs was isolated from both yeast and hyphal forms. The uptake and mechanism of *Candida*-derived EVs were investigated by flow cytometry and confocal microscopy. We also investigated the effect of *Candida*-derived EV treatment on various processes involved in the epithelial-mesenchymal transition of the tumor, such as the migration, MMP activity and gene expression profile of the HSC-2 human OSCC cells used.

Results: As a result of the experiments, we found that EV treatment affects the migration and morphology of tumor cells. In addition, *C. albicans*-derived EVs significantly increase the MMP activity and expression of the tumor cells. Gene expression changes were also detected after *Candida* EV treatment.

Conclusion: These results suggest that *Candida* EVs play a role in promoting OSCC epithelial-mesenchymal transition and thus in tumor progression.

Funding information: This research was supported by the grant HUN-REN 2001007, Centre of Excellence for Interdisciplinary Research, Development and Innovation of the University of Szeged, Competence Centre for Molecular Biology, Bionics and Biotechnology and by the Louis-Jeantet Foundation. Project no TKP2021-EGA-28 has been implemented with the support provided by the Ministry of Culture and Innovation of Hungary from the National Research, Development and Innovation Fund, financed under the TKP2021-EGA funding scheme." EU's Horizon 2020 research and innovation program under grant agreement No. 739593 Hungarian Scientific Research Fund (NKFIH K 147510)



P-I-10

Probiotic EVs binding to mucin and an in vitro model of intestinal epithelial barrier

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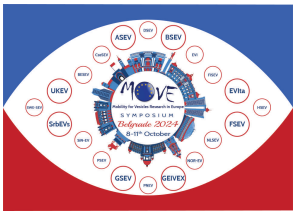
Introduction: Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”. The mechanisms involved in the health benefits of probiotics are not completely understood, but these effects can be mediated, at least in part, by probiotic-derived extracellular vesicles (EVs). In this line, EVs from Lacticaseibacillus casei BL23 contain proteins associated with probiotic effects including proteins related to the adhesion to the intestinal mucus and epithelium.

Methods: To study the adhesion capacity of L. casei BL23 EVs, we carried out 2 adhesion assays: EVs binding to mucin (the main structural component of mucus) and EVs binding to an in vitro model of intestinal epithelial barrier. Bacteria were grown for 48h and EVs were isolated by ultracentrifugation. Purified EVs were labelled with CFSE (ex498nm/em517nm) and washed. In both assays, the concentrations of EVs incubated were 8×108, 6×108, 4×108, 2×108 and 0 EVs/ml. For the adhesion assay to mucin, an ELISA plate was coated with porcine stomach type III mucin, followed by incubation with different concentrations of labelled EVs for 2h at 37ºC. After successive washes, the mucin-bound EVs were collected to measure fluorescence in a plate reader. For the other adhesion assay, human intestinal Caco-2 cell line was differentiated 15-21 days in a 24-well plate and were incubated with different concentrations of labelled EVs as mentioned before.

Results: L. casei BL23 EVs were able to bind mucin in a dose-dependent manner (8.108 EVs/ml ≠4.108 EVs/ml). The same effect was observed in the ability of EVs to bind an intestinal epithelial barrier (8.108 EVs/ml≠2.108 EVs/ml). Two-way ANOVA (concentration of EVs and block), n=3, $P<0.05$, was performed.

Conclusion: L. casei BL23 EVs binding to mucin and intestinal epithelial cells could be a relevant step before being transported into the bloodstream to exert their probiotic action.

Funding information: National Scientific and Technical Research Council (PICT 2020-00150 and PICT 2021-00347) and Universidad de Buenos Aires (UBACYT Nº 20020190100297BA).



P-I-11

Nanovesicles from frozen homogenized Fasciola hepatica adults exhibit similar properties to extracellular vesicles from parasitic cultures

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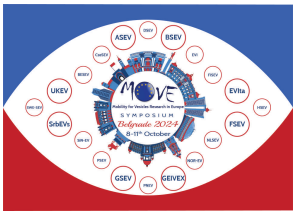
Introduction: Helminth extracellular vesicles (EVs) play a crucial role in modulating the host immune response, specially involving macrophages. However, the research on helminth EVs is largely dependent on obtaining worms from infested hosts. This process is highly variable and affected by environmental factors, leading to important time delays, which can hinder research methodologies and impede further studies. In this study we evaluate the possibility of employing nanovesicles derived from homogenized Fasciola hepatica (Fh-hNVs) as a comparable source to EVs naturally secreted in culture by the parasite (FhEVs).

Methods: Parasites were collected from local slaughterhouses and cultured for 4 h to obtain the FhEVs. Frozen adult worms were thawed, washed, and homogenized in PBS with protease inhibitors to obtain Fh-hNVs. Both types of vesicles were extracted using differential centrifugation and size exclusion chromatography, and characterized by NTA, TEM, LC-MS/MS, and immunogold labeling for CD63 and for FhEVs-specific polyclonal antibodies (FhEVs-PAb). Identified proteins were classified by gene ontology analyses. THP1-XBLUE ™-CD14 macrophages were exposed to FhEVs or Fh-hNVs and cell viability was monitored using MTT and trypan blue assays, whereas NF-κB activation was quantified with Quanti-Blue™ reagent (Invivogen).

Results: Both Fh-hNVs and FhEVs showed round-shaped morphology and similar size distribution, although FhEVs displayed more variability in shape. 120 proteins were identified in FhEVs and 178 in Fh-hNVs, with 67 proteins common to both, including key EV markers like Alix and CD63. α-CD63 and FhEVs-PAb antibodies recognized surface proteins on both FhEVs and Fh-hNVs. Both preparations reduced NF-κB activation in THP1 cells and reduced their viability in a dose-dependent manner.

Conclusion: Fh-hNVs show similar shape and functional properties to FhEVs. They include a common core of proteins, including typical EV markers, but also contain a plethora of other proteins with potentially interesting applications in parasitic studies and disease control.

Funding information: Supported by the Agencia Estatal de Investigación, Ministerio de Ciencia, Innovación y Universidades, Spain (Grants PID2019-105713GB-I00 and PID2023-146116NB-I00 and ERDF/EU), and Conselleria d'Educació, Cultura i Esports, Generalitat Valenciana, Valencia, Spain (Grants PROMETEO/2020/071 and CIPROM/2023/54). C.M.S-L is the recipient of a Postdoctoral fellowship funded by AEI/10.13039/501100011033.



P-I-12

Establishing an extracellular vesicle isolation protocol for a beneficial plant fungus

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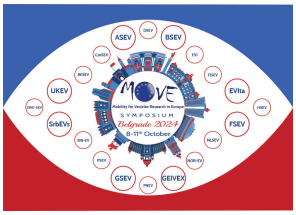
Introduction: *Fusarium solani* strain K (FsK) is a beneficial fungal endophyte which can help tomato plants resist different biotic (root and foliar pathogens) and abiotic stressors (drought, salinity). We have recently established that FsK is able to transmit sRNAs to *Nicotiana benthamiana*, triggering systemic RNA silencing and DNA methylation of a reporter gene. The mechanistic details of how the sRNAs are being transmitted still remain elusive, but extracellular vesicles (EVs) are an emerging carrier of sRNAs.

Methods: During this study we aim to establish an effective extracellular vesicle isolation protocol for FsK. To isolate the EVs using as starting material fungal liquid culture we applied differential centrifugation combined with density gradient purification method. For characterization of the isolated vesicles, the highly used NTA method was performed. Additionally, a lipid characterization assay was also applied.

Results: After ultracentrifugation (100.000 g) the resulting pellet was separated in iodixanol density gradient, and 10 fractions were washed and analyzed. NTA analysis revealed the presence of two populations of particles. Starting from 250 mL of 4 days old liquid culture of FsK, the concentration of particles was 10^{10} particles/ml and 10^{12} particles/ml in less dense and denser fractions, respectively. The less dense fraction contained particles of 150 nm average size, while in denser fractions the average particle size was 115 nm. Lipid content of isolated particles was also confirmed.

Conclusion: Using differential centrifugation combined with density gradient purification method, high yield of particles was obtained from *F.solani* liquid culture. Two populations of vesicles of different average sizes and densities were detected. Both populations had the size range expected for EVs. Future experiments can entail the investigation of EV-associated sRNAs and their functionality during cross-kingdom RNAi phenomena.



P-I-13

Arabidopsis thaliana root cells interact with outer membrane vesicles (OMVs) produced by plant beneficial bacterial strain Paraburkholderia phytofirmans PsJN

Dragana Nikolić[1], Sofija Nešić[1], Aleksandra Divac Rankov[1], Dragana Bosnić[1], Vesna Spasovski[1], Maja Kosanović[2], Jelena Samardžić[1]

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Introduction: Plant growth-promoting bacteria (PGPB) play an essential role in the growth and development of plants by facilitating the uptake of nutrients and increasing stress resistance. The interaction between plants and their beneficial bacteria is multifaceted, but the role of extracellular vesicles (EVs) in this interkingdom communication is poorly understood. To investigate the interaction of outer membrane vesicles (OMVs) with plant cells, EVs produced by Gram-negative bacteria, we isolated and characterized OMVs produced by Paraburkholderia phytofirmans PsJN, a PGPB strain known for its plant-protective abilities.

Methods: The OMVs of P. phytofirmans PsJN were isolated using an affinity-based chromatography system and characterized by Nanoparticle tracking analysis (NTA). To monitor the interaction of OMVs with plant cells, the vesicles were labelled with the lipid-binding dye Vybrant™ DiD. To ensure the specificity of the signals, different methods to remove the unbound dye were tested, namely dialysis, ultracentrifugation, ultrafiltration and iodixanol density gradient purification. Arabidopsis thaliana roots were incubated with DiD-labelled OMVs and observed by confocal laser scanning microscopy (CLMS).

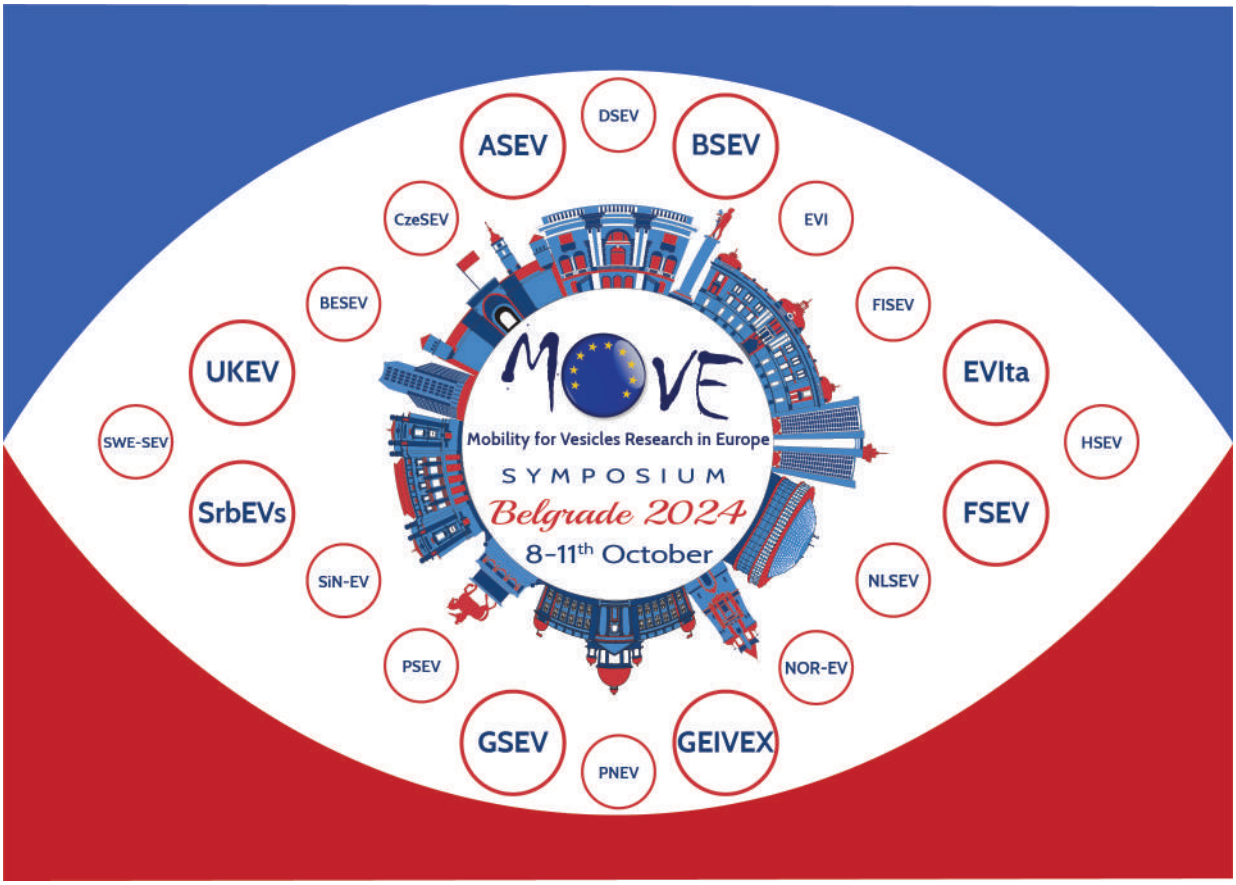
Results: NTA revealed 70nm to 180 nm size range of isolated OMVs. Regarding preparation of OMVs for monitoring their interaction with plant cells, the most satisfactory removal of unbound dye was obtained after the separation of DiD-OMVs in the density gradient. A. thaliana roots treated with DiD-OMVs showed specific red signals in the root hairs and epidermal cells, while the signals were absent in the control-treated roots.

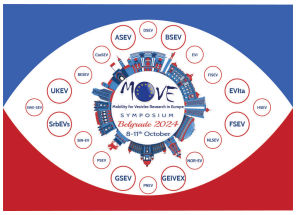
Conclusions: The results suggest that PsJN OMVs directly interact with the root hairs and epidermal cells of A. thaliana. As root hairs play a crucial role in plant nutrient uptake and interactions with microbes, further investigation is needed to determine the potential contribution of PsJN OMVs to plant recognition of beneficial bacteria, establishment of mutual interactions and the exertion of a protective effect by PGPB.

Funding information: The Science Fund of the Republic of Serbia, grant number 7744906 .

Novel EV preparation/analysis techniques

- oral presentations -





O-N-1

NANOSPACER: Nanofluidic sizing of extracellular vesicles and biomacromolecules in solution

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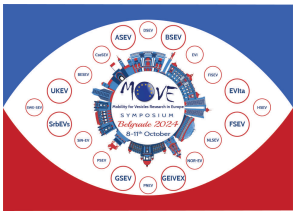
Introduction: Nanofluidic devices have great potential for applications ranging from human health to single molecule metrology. However, their broad application potential remains largely untouched as microfluidic equipment with pumps, setups and complex chip handling protocols limit their usability – while the research community remains in urgent need of a standardized way of sizing biomacromolecules and vesicles in an easy manner. A method for the detection of nanoscopic specimen and rapid size-exclusion of larger particles such as blood cells/platelets/cells without time-consuming steps (e.g. ultra centrifugation) would be of great benefit to the field.

Methods: Here we demonstrate the usage of disposable nanofluidic capillaries (NANOSPACERS) for single particle tracking (NTA) of body fluid-derived exosomes and other biomolecules in solution using fluorescence and label-free methods (Raman, phase-contrast, dark-field).

Results: Within seconds we measure the size of up to 1000's of exosomes in parallel using conventional microscopy equipment within a single pipetting step. EVs are classified into individual sub populations upon hydrodynamic size and scattering intensity using open-source software (FIJI, ImageJ). The method is demonstrated on various biomolecules in the micro-, to nano regime: from highly motile bacteria (~1µm) to nanocolloids (44-500nm), exosomes (40-120nm), viruses (40-45nm), DNA (100bp-1.5kbp) down to the single molecule level (0.5nm).

Conclusion: We showcase with this approach a versatile method for exosome researchers to use their existing equipment for rapid exosome size analysis, spectroscopy, and classification. Nanospacers allow biological laboratories with limited funds to implement cutting-edge single molecule research in their facilities – enhancing various sectors of life science and biotechnology.

Funding information HORIZON-MSCA-2021-PF-01-01, 101064246; UiT Talent Innovation Grant 2024



O-N-2

Achieving Ultra-Pure EVs: Combining Charge-Based Filtration, Tangential Flow, and Lipoprotein Adsorption

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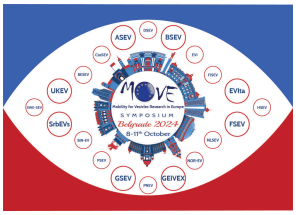
Introduction: Introduction: This study introduces an innovative hybrid EV extraction technology by combining the charge-based exosome separation technology, ExoFilter, with lipoprotein-specific adsorption filter technology and integrating it with Tangential Flow Filtration (TFF), a technology that excludes nanoparticles below a certain size. Traditional technologies often face significant challenges in effectively removing impurities and processing large sample volumes. The need to overcome these limitations has driven the development of this advanced methodology, which addresses these constraints by offering improved purity and scalability.

Methods: ExoFilter, an ion-exchange based technology, was recently developed to process negatively charged EVs from small sample volumes to liter-scale samples. TFF, widely used for its unique ability to concentrate samples by excluding nanoparticles below a specific size, when combined with charge-based ExoFilter technology, achieved unexpectedly high recovery rates and high purity through iterative flow processes. Additionally, by incorporating a filter process with an Apop-B-specific aptamer, a surface marker of lipoproteins, the previously obtained high-purity extracts were further refined to ultra-high purity without reducing recovery rates.

Results: This series of integrated processes retained the individual technical advantages, resulting in remarkable purity improvement and a synergistic effect that more than doubled the existing recovery rates. This represents a significant technological advancement in the extraction technology by efficiently removing lipoproteins, which are difficult to eliminate with conventional methods.

Conclusion: The combined technology of each filter, scalable from small blood samples to hundreds of liters of culture medium, presents a new method for efficiently obtaining high-quality exosomes for various biological and medical applications.

Funding information: Korea Evaluation Institute of Industrial Technology (KEIT 20018560/NTIS 1415184668)



O-N-3

Inline Raman spectroscopy provides versatile molecular monitoring for platelet extracellular vesicle purification

Heikki Saari[1], Heli Marttila[2], Jacopo Zini[3,4], Saara Laitinen[1]

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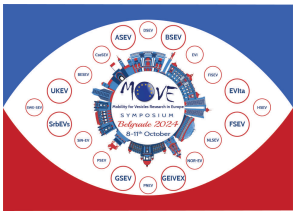
Introduction: Due to their small size and heterogeneous structure, the development of new methods for isolating and characterizing extracellular vesicles (EVs) is critical. Conventional EV isolation methods are poorly scalable and require a lot of hands-on work for both the isolation itself and monitoring the process. As downstream analysis methods for EVs can be very laborious and do not provide real-time information, a reliable inline detection method for EV manufacturing process is needed. For this purpose, Raman spectroscopy is a strong candidate as it can be used to identify functional groups in a sample, allowing the identification of EVs or impurities in different stages of the process. In our study, we demonstrate the use of an inline Raman detector in conjunction with anion exchange chromatography for the isolation of EVs from human platelets.

Methods: EVs were produced with human platelets acquired from donated blood through Finnish Red Cross, Blood Service. EVs were purified with monolithic anion exchange chromatography, using an inline Raman detector to record Raman spectra during the different phases of the purification. Analysis of the spectra was compared to conventional EV characterization methods: western blotting and Nanoparticle Tracking Analysis.

Results: Our results indicate a good separation of impurities from the EVs during the anion exchange chromatography. The collected Raman spectra could be used to separate non-EV flowthrough and EV-enriched elution fractions based on differences in composition. Additionally, it was able to detect variations in the process, when challenged with higher sample load or an additional non-EV contaminant, consistent with the conventional downstream analysis methods.

Conclusion: Inline Raman spectroscopy can provide detailed molecular insight, when monitoring EV purification processes. This method shows the results in real time and does not require any hands-on lab work, in contrast to conventional EV analyses.

Funding information: Instruct-ERIC R&D pilot project award (APPID 2729), Academy of Finland GCN Flagship 1-year grant (Novel Raman spectroscopy methods for characterizing biological nanoparticles, Sigrid Juselius Foundation, The Research Fund of the Finnish Red Cross Blood Service (Chromatographic purification of platelet-derived extracellular vesicles – from side streams towards high quality products and Jenny and Antti Wihuri Foundation (Cell eye - Timegate Raman as next generation advanced process control platform for biopharmaceutical production).



O-N-4

Isolation of extracellular vesicles from resistant tumor cells using nanobodies-based immunoaffinity approach

Lidija Filipović[1], Marija Tursunović[1], Maja Kosanović[2], Marija Grozdanić[3], Ana Podolski-Renić[3], Milica Pešić[3], Milica Popović [4]

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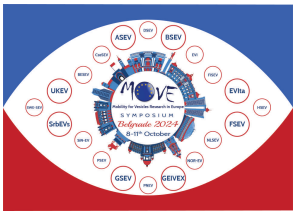
Introduction: Extracellular vesicles (EVs) are an important contributing factor to drug resistance in cancer. In order to study their features and elucidate their molecular composition. To that extent, we have decided to use two pairs of multi-drug resistant (MDR) cancer cell lines (non-small cell lung carcinoma NCI-H460/R and glioblastoma U87-TxR) and their sensitive counterparts (H460 and U87, respectively ion and contribution in drug resistance, EVs need to isolated in an efficient manner and sufficient quantities. Broadening new possibilities in EV-based diagnostics requires innovative, adaptable, and affordable methods for the scalable isolation of high-purity EVs from different sources. This study aims to adapt high-performance immune capture chromatography based on nanobody technology for EVs isolation from cell culture media of MDR cancer cells and their sensitive cells.

Methods: The nanobodies utilized in this study were selected from a heavy-chain only-VHH library by direct panning against EVs and generated in *E. coli* with eGFP and a 6xHis tag. To isolate EVs, purified VHHs-GFP were immobilized on polymethacrylate polymer to create immunoaffinity capture. Isolated vesicles have been characterized by a set of biochemical and instrumental techniques (colorimetric sulfophosphovanilin-SPV assay, BCA assay, Flow cytometry, and Nanoparticle tracking analysis).

Results: The combined analysis of proteins, lipids, and flow cytometry analysis of three tested biomarkers (CD9, CD63, and CD81) showed that we successfully isolated EVs from both pairs of cancer cell lines. The detergent control (TRITON X-100) for biomarkers analysis showed reduced signal, thus confirming the presence of lipid-origin structures. The NTA analysis showed that MDR cancer cells produced EVs with a bigger diameter.

Conclusion: This study demonstrates the application of spherical porous methacrylate-based polymer coupled with VHHs for the purification of EVs from MDR cancer multi-drug-resistant cells. This inexpensive, relatively fast, and easy-to-perform method has great potential for the isolation of different classes of EVs from various biological sources.

Funding information: This research was supported by the Science Fund of the Republic of Serbia, Grant PRISMA No. 4747, Project title: Advancing REversible immunocapture toward SCALablE EV purification—RESCALE-EV.



Plant Extracellular Vesicles as Novel Drug-delivery Systems

Kocholata Michaela[1], Janouskova Olga[1], Maly Jan[1]

[1]Centre for Nanomaterials and Biotechnologies, Faculty of Science, Jan Evangelista Purkyně University in Ústí nad Labem, Ústí nad Labem, Czech Republic

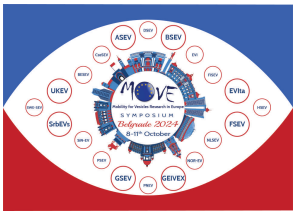
Exosomes and extracellular vesicles, nanometer-sized vesicles secreted by cells, have garnered significant attention in mammalian systems for their roles in intercellular communication and disease processes. However, in recent years, there has been a growing interest in plant extracellular vesicles (pEVs) due to their immense potential for medical applications, particularly as carriers for drug delivery. As the ultimate goal of our research is the application of plant extracellular vesicles in human and veterinary medicine, our initial step was to identify optimal high-yield plant sources of EVs and the most effective methods for their high-quality isolation.

We selected Nicotiana tabacum and Arabidopsis thaliana as model organisms, alongside several medicinal plants, and successfully established in vitro plant explants. To achieve high-yield production of plant exosomes, we use tangential filtration as our isolation method, ensuring the efficient and high-quality extraction of pEVs. The primary focus of our research centered on tobacco plants.

We detected the exosomal marker HSP70 using western blotting, we detected RNA within tobacco EVs, and we identified two secondary metabolites, nicotine and anabasine, using liquid chromatography-mass spectrometry. Additionally, we evaluated the ability of plant exosomes to be loaded with various molecules, including Cy5-siRNA, YOYO-pDNA, molybdenum cluster compounds, and doxorubicin. The efficiently loaded pEVs successfully transported these molecules into various cell types, including tobacco cells, rat mesenchymal stem cells, and larvae of Arion vulgaris.

Our findings highlight the significant potential of plant extracellular vesicles (pEVs) in medical applications, particularly as efficient drug delivery systems.

Funding information: The research was supported by the Research Infrastructure NanoEnviCz LM2023066 and by The Johannes Amos Comenius Programme, project No. CZ.02.01.01/00/22_008/0004562.



O-N-6

Time-resolved surface-sensitive waveguide scattering microscopy of single extracellular vesicles reveals content and biomarker heterogeneity

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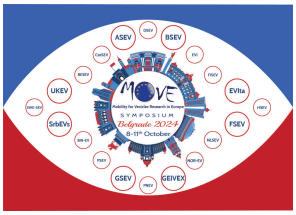
Introduction: Deciphering EV heterogeneity is essential for understanding their biological functions and enhancing their use in drug development and medical diagnostics. We present label-free waveguide scattering microscopy (WGSM), coupled with simultaneous fluorescence readout, for multiparametric EV profiling.

Methods: The WGSM setup combines an upright microscope with a planar waveguide chip for time-resolved surface-sensitive nanoparticle imaging. Our surface functionalization approach consists of capturing with cholesterol self-insertion into the EV membrane. The optical contrast between blood-derived EVs and the surrounding medium is varied using membrane-permeable and -impermeable solutes, allowing to resolve EV size and refractive index (RI), distinguish between vesicles and non-vesicular particles using the glycerol-available volume, and calculate the concentration of biomolecules in the EV lumen. In addition, dual-color time-resolved staining against CD markers is performed.

Results: RIs for platelet EVs (PEVs) and red blood cell (RBC) EVs were similar (1.41) but RI distribution was wider for PEVs, and the presence of low-RI subpopulations (RI < 1.38) was found for both EV types. Concentration of cargo biomolecules was high (~0.4 g/mL) for both EV types. Monitoring time-resolved antibody binding allowed to calculate number of CD markers per EV and rates of antibody binding to individual EVs. Median numbers of CD63s per PEV (~70) are lower than number of CD9s (~100), and wide distribution in binding rates for anti-CD63 suggest heterogeneity in antibody-CD63 protein interaction. Fractions of CD9-positive and CD63-positive PEVs were measured as 48% and 23%, respectively.

Conclusion: WGSM provides low sample consumption (~100 µL at a concentration of ~$1x10^{10}$ particles/mL) and moderate measurement times (40 to 80 minutes) for analysis of a few thousand EVs in a single experiment. Therefore, we see potential for the method to aid in assay standardization, liquid biopsy analysis, and other tasks that benefit from multiparametric EV assays beyond size and surface marker profiling.

Funding information: Knut and Alice Wallenberg Foundation, #2019-0577, Swedish Research Council, #2018-04900, The Research Fund of the Finnish Red Cross Blood Service, NordForsk Nordic University Hub project Nordic POP, #85352



O-N-7

Isolating and Detecting Extracellular Vesicles on Microfluidic Chips and Metamaterial Sensors

Eylul Gulsen Yilmaz[1,2], Esma Derin[1,2], **Fatih Inci**[1,2]

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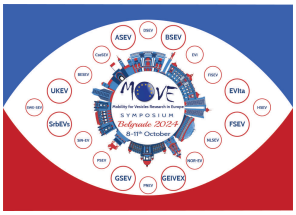
Introduction: Acute kidney injury (AKI) affects 13 million people globally—causing 1.7 million deaths annually, and impacts 20–40% of COVID-19 ICU patients. AKI poses a significant health concern now and in the future due to the lack of efficient biomarkers. Improved strategies involve new-generation biomarkers, with extracellular vesicles (EVs) offering new opportunities for diagnosis and drug delivery. However, isolating and determining EVs from complex fluids is challenging into clinical settings. This study aims to both isolate and determine surface markers of EVs released from AKI patient urine samples with facile (5-steps in total for the chip and sensor), rapid (1.5-hours), and inexpensive ($5) assets. Accordingly, we develop an ultrafiltration-chip for isolating EVs from culture samples and real urine samples (healthy and AKI patients). We repropose nanograting plastic surfaces into sensitive metamaterial sensors, which are further modified with EV-related antibodies for detecting EVs.

Methods: By using an ultrafiltration chip and metamaterial sensor, we isolated and detected EVs from culture samples and real urine samples (healthy controls and AKI patients). Isolated EVs were further confirmed by NTA, fNTA, Western Blot, SEM, and XPS. As comparisons, we employed ultracentrifuge and commercial ELISA for assessing the performance of our platforms, subsequently followed by statistical assessments for comparisons.

Results: Ultrafiltration chip provided comparable results with ultracentrifuge, isolating ~10^9 vesicles/mL. In comparison to ELISA, we achieved ~100-time more sensitivity along with larger dynamic range (4 orders of magnitude). Using the metamaterial sensor, we further validated patient and control samples with a broad AKI panel (ATF-3, NGAL, THP, AQP-1, CD133, and Fetuin A) in addition to tetraspanins.

Conclusion: Our metamaterial sensor demonstrated superior sensitivity, and we introduced an easy-to-use, cost-effective, and rapid ultrafiltration chip. In near future, both platforms can be seamlessly integrated into clinical settings, thereby enhancing the utility of EV markers in point-of-care diagnostics.

Funding information: The authors gratefully acknowledge support from the Scientific and Technological Research Council of Turkey (TÜBİTAK) 2232 International Fellowship for Outstanding Researchers (Project No: 118C254), Outstanding Young Scientists Award (GEBİP) from Turkish Academy of Sciences, Young Scientist Awards Program (BAGEP) form Science Academy, and the exRNA-PATH COST Action CA20110.



O-N-8

Optimization of Extracellular Vesicles Isolation Protocol for Proteomics Analysis from Limited Cell Media Samples

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Introduction: Extracellular vesicles (EVs) are lipid bilayer-enclosed particles secreted by cells, containing proteins, lipids, and nucleic acids reflective of their origin. These vesicles, including exosomes, microvesicles, and apoptotic bodies, are pivotal in cell communication and have potential applications in liquid biopsies and therapeutic delivery. For proteomics analysis, isolating EVs from limited biological samples poses a challenge due to their low abundance and the complexity of media contaminants.

Methods: Here we aimed to optimize an EV isolation workflow tailored for proteomics from small volumes (15 mL) of cell culture media. We compared two workflows: a combination of polymer-based precipitation followed by size exclusion chromatography (PPT+ExoSpin) and an ultrahigh-performance liquid chromatography-size exclusion chromatography (UHPLC-SEC) method.

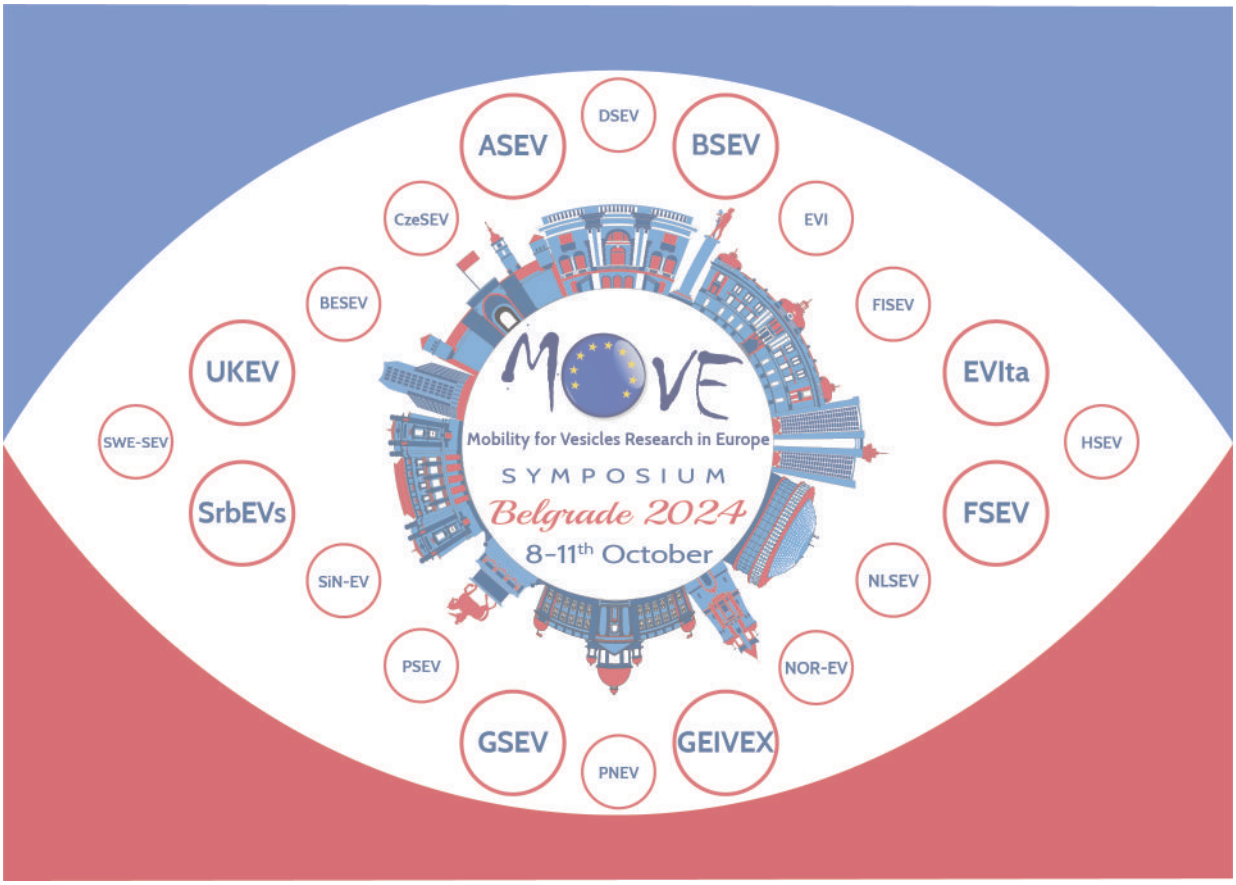
Results: Cell media from Suit2-028 cells was processed, and EVs were isolated using both workflows. The PPT+ExoSpin method eliminated the need for a concentration step of the EVs fractions after SEC separation, significantly reducing EV loss. Isolated EVs were characterized via transmission electron microscopy (TEM) and western blot. The PPT+ExoSpin workflow demonstrated superior sensitivity, identifying a greater number of EV-associated proteins compared to the UHPLC-SEC method. Proteomics analysis revealed that the PPT+ExoSpin method identified 97 out of the top 100 proteins listed in the ExoCarta database, compared to 68 for the UHPLC-SEC method.

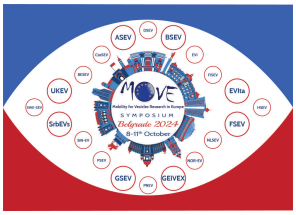
Conclusion: Our optimized PPT+ExoSpin workflow provides a reproducible and efficient approach for isolating EVs suitable for proteomics, enabling more comprehensive protein profiling from limited cell culture media volumes. This advancement is crucial for biomarker discovery and the broader application of EVs in clinical diagnostics and therapeutic development.

Funding information: FPS grant 2020

Novel EV preparation/analysis techniques

- poster presentations -





P-N-1

Effective enrichment of CD9 positive population of extracellular vesicles from human cerebrospinal fluid in asymmetric flow field flow fractionation and size exclusion chromatography fractions

Hrvoje Križan[1], Junjie Wang[2], Anna Placci[2], Mario Kurtjak[3], Pietro Parisse[4,5] Loredana Casalis[4], Barbara Roda[2], Andrea Zattoni[2], Pierluigi Reschiglian[2], Valentina Marassi[2]*, Mladenka Malenica[1]*

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*corresponding authors

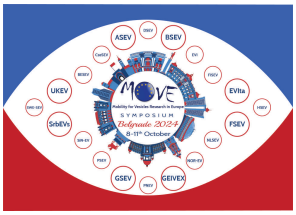
Introduction: Isolation and sample enrichment are major challenges in extracellular vesicles (EVs) research. To achieve the highest possible yield of EVs, various conventional methods are used for their purification. In this work, gravity-driven size exclusion chromatography (SEC) and asymmetric flow field flow fractionation (AF4) are used as methods in which the physiological state of EVs is well preserved. Further detailed analysis of the enriched fractions of EVs revealed a higher efficiency of the AF4 method compared to SEC, characterizing this method as very effective and fast.

Methods: The native sample of human cerebrospinal fluid (CSF) from patients with traumatic brain injury (TBI) is obtained by ventriculostomy drainage and subjected to purification and enrichment by SEC and AF4 techniques. Immunoblot on specific protein markers (CD9) and non-vesicular extracellular particles (NVEPs; albumin and APO-A1) is performed. Dynamic light scattering (DLS) is used to determine the size distribution of particles. In the CD9 positive EV fractions, atomic force microscopy (AFM) is performed to reveal the size and different morphology of EVs.

Results: Enrichment of EVs was confirmed in SEC and AF4 fractions, and efficient elimination of NVEPs with immunoblots for albumin and APO-A1. The diameter of EVs shown by DLS is between 60 and 110 nm. Different morphologies of EVs were determined by AFM.

Conclusion: The obtained results show the AF4 as a very efficient, fast and reliable method in the enrichment of EVs from CSF. Unlike SEC, AF4 uses a smaller sample input volume and ensures less sample dilution in the fractionation process, and thus higher molecular signal and yield of EVs. AF4 fractionation shows a more efficient separation of protein from EV fractions with simultaneous monitoring of protein concentration, size distribution and molecular weight of particles in the sample. High-quality fractionation and enrichment are a prerequisite for further analyzes of the morphology and biological functions of individual subpopulations of EVs.

Funding information: This work has been financed by the European Union – NextGeneration EU through the Italian Ministry of University and Research under PRIN 2022 RESOLVE, 202233FTW8 CUP B53D23013360006; and the University of Rijeka grant under the project number: uniri-biomed-18-279 (grant to M.M.) and NFFA-Europe-Pilot (n. 101007417 from 1/03/2021 to 28/02/2026) having benefitted from the access provided by CNR-IOM in Trieste (Italy) within the framework of the NFFA-Europe Transnational Access Activity, proposals ID 390 to M.M.



P-N-2

Glycan mapping of tumor-derived extracellular vesicles: a multiplex lectin bead approach

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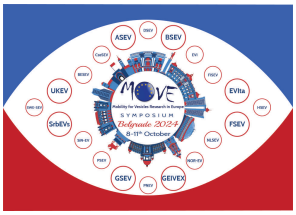
Introduction: The glycosylation profiles of lipids and proteins play crucial roles in cell signaling and immune response. In cancer, altered glycosylation patterns enhance tumor progression, metastasis, and immune escape. Tumor cells release extracellular vesicles (EVs) containing various biomolecules that can serve as liquid biopsy biomarkers. However, the variation in glycan profiles of tumor-derived EVs (tEVs) across different cancer stages remains unclear. Currently, there are limited quick and affordable technologies for glycan profiling. Our goal is to develop a rapid and cost-effective technique to characterize these glycan profiles.

Methods: We isolated HEK EVs using size exclusion chromatography and quantified particle numbers with a Zetaview analyzer. To profile glycans, we developed an in-house bead-based protocol utilizing lectins. EVs were pulled down using lectin-conjugated beads and analyzed via flow cytometry.

Results: We successfully standardized our lectin profiling protocol. PNGase F-treated EVs served as a negative control. Our findings indicate differential glycan expression on HEK EV surfaces. Using lectins such as ConA, WGA, and UEA, which recognize mannose, sialic acid, and fucose residues respectively, we observed higher sialic acid levels on HEK EV surfaces compared to other glycans, with negligible mannose detection. Fluorescent-tagged tetraspanin antibodies confirmed EV binding to lectins.

Conclusion: Our in-house lectin-based EV profiling technique is faster (<24 hours) and more cost-effective than traditional glycan profiling methods like microarrays and mass spectrometry. We aim to develop a multiplex lectin-based bead panel to fingerprint cancer cell glycan profiles and identify potential glycan-based biomarkers for future research.

Funding information: This project is funded by Cancerfonden, the Swedish Research Council fund (VR grant), and the CIMED grant.



P-N-3

Development of an analytical reference material for the study of extracellular vesicles

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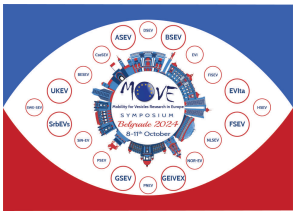
Introduction: Extracellular vesicles (EV) are emerging as a promising biomarker source for various diseases, due to their cell-specific content and presence in bodily fluids. Despite their potential, a significant challenge hindering the translation of EV-based biomarkers to clinic setting is the limited availability of analytical standards necessary for the development of reproducible and robust assays. Although significant progress has been made on this front with the introduction of reference materials for EV research, the biomarkers of interest might not always be expressed on them. Current reference material, derived from either artificial sources or healthy conditions, fails to accurately represent the molecular content of EV, affecting biomarker detection accuracy. To address these challenges, we have developed an analytical reference material derived from Du145 prostate cancer cell lines to standardize EV research.

Methods: EVs were separated from Du145 cancer cell line using ultracentrifugation and size exclusion chromatography (SEC). The isolated Du145-EV were characterized for their tetraspanin expression, concentration and particle size using nanoparticles tracking analysis (NTA), protein content by western blotting (WB), and morphology through transmission electron microscopy (TEM). The Du145-EV were subsequently used to standardize time-resolved-fluorometry-immunoassay (TRFIA) and lateral flow immunoassay (LFIA).

Results: The TRFIA and LFIA techniques can measure Du145-EV with high sensitivity and linearity in comparison with commercial artificial EV. The Du145-EV showed differential expressions of various proteins and altered glycans on their surface, which is detected by using different antibodies, and lectins.

Conclusion: This study introduces a novel Du145-EV reference material characterized with disease-specific proteins and altered surface glycans, aimed at enhancing EV research and diagnostics by closely replicating EV biological properties. This Du145-EV could serve as a reference for method development, data normalization, and assessment of analytical variables in EV studies. Nevertheless, to validate its utility, further comparative assessments with existing reference materials are essential, employing a diverse array of EV-detection methodologies.



P-N-4

Isolation of spontaneously-released brain extracellular vesicles: implications for stress-driven brain pathologies

Ioannis Sotiropoulos [1,2] Patrícia A. Gomes[2], Cristian Bodo[3], Filippos Katsaitis[1], Carlos Noguera-Ortiz[4], Martina Samiotaki[5], Minghao Chen[6,7], Carina Soares-Cunha[2], Joana M. Silva[2], Bárbara Coimbra[2], George Stamatakis[5], Liliana Santos[2], George Panayotou[5], Clarissa L. Waites[8], Christos Gatsogiannis[6,7], Nuno Sousa[2], Dimitrios Kapogiannis[4], Bruno Costa-Silva[3]

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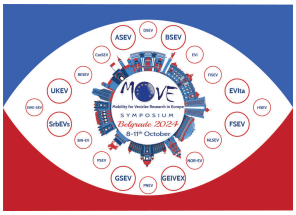
Introduction: In the era of Precision Medicine, extracellular vesicles (EVs) exhibit great potential for the theragnostic of brain disorders such as Alzheimer's disease (AD), a complex disease with no effective treatment and poorly understood risk factors, where pathological heterogeneity and diverse clinical presentations complicate the development of precise patient-tailored therapies. Thus, the collection and characterization of physiologically relevant EVs, as well as the study of its precipitating/risk factors, are of the utmost importance. However, standard brain EV isolation approaches rely on tissue dissociation, which can contaminate EV preparations with intracellular vesicles.

Methods: Based on a multiscale analysis, including cryo-EM, label-free proteomics, and ExoView, we hereby present a novel isolation method of small EVs (sEVs), named “release method”, based on their spontaneous release from the human and mouse brain tissue. Moreover, we have also tested the release method under conditions of chronic psychological stress, a known risk factor of AD.

Results: Our advanced EVs analysis demonstrated that the release method represents an efficient method that captures a small EV-enriched population spontaneously released by brain tissue. In addition, we tested the significance of the release method under conditions where biogenesis/secretion of sEVs was pharmacologically manipulated and under exposure to chronic stress, a clinically-relevant precipitant of AD. Here we found that the release method monitors the drug-evoked inhibition or enhancement of sEVs secretion in a very sensitive manner, while chronic stress induced the secretion of Tau-carrying brain-derived EVs accompanied by memory loss and mood deficits suggesting a potential role of sEVs in the brain response to stress and progression of related stress-driven brain pathologies, such as AD.

Summary/Conclusion: This spontaneous release method may contribute to the characterization and biomarker profile of physiologically relevant brain-derived exosomes in brain function and pathologies such as chronic stress and its molecular “footprints” of brain EVs.



P-N-5

Isolating Outer Membrane Vesicles of Patient-derived Microbiota Pathogens on 3D-printed Ultrafiltration Platforms for Nucleic Acid-based Diagnostics

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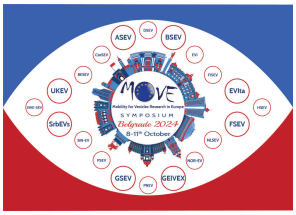
Introduction: Bacterial Membrane Vesicles (bMVs)—membrane-originated, nanometer-sized (50-150 nm) lipid shuttles released from bacterial cells, carry cellular information including pathogenicity-physiological states and resistance signals. Clinical application bMVs as diagnostic markers are limited with labor-intensive and expensive isolation strategies. We develop a combinatorial strategy to isolate bMVs with a 3D printed ultrafiltration microfluidic chip, and test nucleic acid targets from isolated bMVs through an isothermal nucleic acid test system. We hence aim to both isolate and diagnose the patient derived bMVs through a cost-effective (single assay: $1 for the chip and $4 for the test) and easy-to-operate (2-steps) protocol as a new generation of diagnostic strategy.

Methods: 3D printed ultrafiltration chip includes both dead-end and cross-flow strategies on the same device, containing of 200 nm and 50 nm filter set for isolating bMVs from common urinary track pathogens including *Acinetobacter baumannii*, *Clostridium difficile*, *Helicobacter pylori*, and *Pseudomonas aeruginosa*. Isolated bMVs are analyzed with Scanning Electron Microscope (SEM), Nanoparticle Tracking Analysis (NTA), X-Ray Photoelectron Spectroscopy (XPS) and Transmission Electron Microscopy (TEM), and also, their target nucleic acids are detected with an in-house isothermal assay.

Results: We demonstrate that bMVs could be isolated from both bacterial growths and patient urine samples (up to 10^9 vesicles/mL), and target regions from different pathogenic strains could be detected down to 100 vesicles/mL. We validated size range of the vesicles with SEM and TEM, and our XPS analyses concluded presence of membrane specific organic bonds from the samples.

Conclusion: Overall, bMVs from urinary track pathogens are isolated on an ultrafiltration chip, and isolated bMVs are used to assess pathogen markers with isothermal nucleic acid amplification tests by a minimal setup. Future studies will be focusing on transferring this strategy to the clinical settings and establishing an advanced detection system based on bMV isolation from larger cohorts.

Funding information: This study is supported by Health Institutes of Türkiye (TÜSEB) (Project No: 16913).



P-N-6

Extracellular Vesicle-Imprinted Optic Biosensors for Breast Cancer Detection

Eylul Gulsen Yilmaz[1,2], Fatih Inci[1,2], **Yeşeren Saylan**[3]

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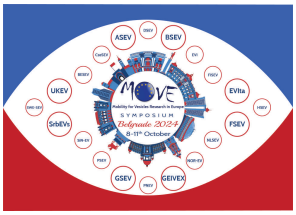
Introduction: Cancer is a leading cause of death worldwide, with 2.3 million new breast cancer cases in 2020. Liquid biopsy techniques are vital for early detection, yet detecting circulating tumor cells is challenging due to their rarity. Extracellular vesicles (EVs) shuttles crucial information in normal and diseased conditions. In this study, On the other hand, due to fluid shear stress (FSS) on breast cancer cells, we need to adapt conventional culture systems. In this study, we recapitulate the tumor microenvironment using biomaterials and controlling FSS, then dynamically isolate the released EVs. These EVs serve as templates for imprinting molecular fingerprints on polymeric nanoparticles as artificial antibodies for specific EV detection on an optical biosensor.

Methods: A microfluidic chip was fabricated to simulate continuous flow as FSS conditions for dynamic cell culture of MCF-7 (breast cancer cells) and MCF-12A (non-cancerous breast cells). EVs were isolated from MCF-7 cells using ultrafiltration with 200 nm and 50 nm filters, and characterized by Nanoparticle Tracking Analysis (NTA), Scanning Electron Microscopy (SEM), and Western Blot. EV-imprinted nanoparticles were created using methacrylic acid-based micro-emulsion polymerization, with their size and morphology analyzed by a Nano Zetasizer and Transmission Electron Microscopy (TEM).

Results: EVs isolated from a collagen-coated microfluidic chip were measured 129.2 ± 4.5 nm in size and had a concentration >10^9 particles/mL. SEM verified the removal of larger particles, isolating particles within the 50–150 nm range, which were spherical in shape. Western Blot analysis confirmed the presence of CD63 and CD81. EV-imprinted nanoparticles were measured at 62.78 nm with low polydispersity (PdI=0.116), also supported by TEM.

Conclusion: This study aims to develop an EV-imprinted biosensing platform for sensitive, specific, and real-time detection of EVs. In the future, EVs will be analyzed to comprehend their impact on MCF-12A, providing insights into changes in tumor microenvironment.

Funding information: All authors gratefully acknowledge the support from The Scientific and Technological Research Council of Turkiye (TÜBİTAK) (Project Numbers: 121C226).



P-N-7

Microfluidic Chip-Based Systems For Monitoring Cancer Therapy Via Extracellular Vesicles

Eylul Gulsen Yilmaz[1,2], Gamze Kara-Magden[3], Yeseren Saylan[4], Fatih Inci[1,2]

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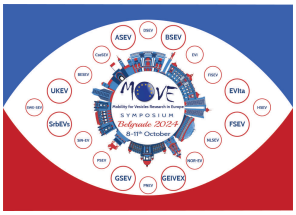
Introduction: Cancer cells experience various fluid shear stresses (FSS) in the tumor microenvironment, but current in vitro 3D models are inadequate for studying these effects on cancer progression and chemoresistance. The need to explore mechanical forces has led to novel methods, including microfluidic systems. These chips are popular for their low cost, high throughput, minimal material use, and ease of manipulation. They also create extracellular matrix-like environments with biomaterials like silk fibroin (SF), which is ideal for 3D cell culture due to its biocompatibility, strength and biodegradability. Research also shows that tumor-derived extracellular vesicles (EVs) influence cancer development, chemoresistance and spread.

Methods: In this study, MCF-7 breast cancer cells were cultured under static 2D, static 3D, and dynamic conditions. EVs from these cultures were collected and analyzed using Western Blot, Nanoparticle Tracking Analysis (NTA), and Scanning Electron Microscopy (SEM). EVs were isolated using an ultrafiltration-based microfluidics device with 200 nm and 50 nm filters. In the second part, MCF-7 cells will be treated with the anticancer drugs Doxorubicin and Docetaxel to determine if these drugs cause any changes in EVs under FSS.

Results: EVs from control groups averaged 177 nm (static 2D), 91 nm (static 3D), and 128 nm (dynamic conditions). EVs from static environments were more clumped compared to those from dynamic ones. Doxorubicin treatment reduced EV size as the drug dose increased. SEM images showed similar results with both doxorubicin and docetaxel. Western blot analysis confirmed EV isolation. CD63 had a strong band between 30-80 kDa, especially at 70 kDa, while CD81 showed a band around 60 kDa. Also, microRNA expression for MCF-7 originated EVs were analyzed.

Conclusion: In conclusion, we successfully isolated and characterized EVs from different conditions. EVs would be a vital biomarker for cancer and they could be a promising alternative for early diagnosis of cancer.

Funding information: The authors gratefully acknowledge support from the Scientific and Technological Research Council of Turkey (TÜBİTAK) 2232 International Fellowship for Outstanding Researchers (Project No: 118C254).



P-N-8

Development of nanobody-based sandwich ELISA for sensitive detection of HIV-1 protein Nef in extracellular vesicles

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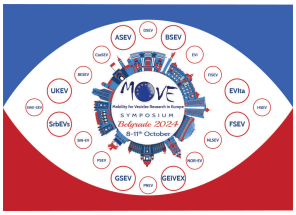
Introduction: Viral protein Nef is released from HIV-1-infected cells in extracellular vesicles (EVs). EVs containing Nef are implicated in various HIV-1-associated chronic pathologies, affecting people living with HIV-1 even in the era of highly active antiretroviral therapy. There is a clear need for sensitive tests to detect extracellular Nef in biological samples. Classical immunoassays provide high-throughput and robust quantification but are challenged by high background and detection limit. Alternative approaches to overcome these drawbacks are essential. Due to their stability, high affinity, and specificity, nanobodies offer a novel immunodetection tool with potential advantages over classical antibodies.

Methods: The anti-Nef nanobody sdAb19 was biotinylated and immobilized on a neutravidin-coated surface as a capture antibody. Upon Nef binding, the signal was detected using an anti-Nef monoclonal antibody and an HRP-conjugated secondary antibody. Absorbance was measured to generate the standard curve, from which the limit of blank (LoB) and the limit of detection (LoD) were determined. Assay performance was further evaluated on detergent-treated pellets obtained by differential ultracentrifugation (2K, 10K, and 100K) of cell culture-conditioned media from human microglia cells expressing Nef.GFP.

Results: We developed a nanobody-based sandwich ELISA protocol for viral protein Nef detection. We optimized surface coating, sdAb19 immobilization, sample preparation, detection antibody binding, and signal detection. The established assay reached a LoB of 2.5 ng/mL and a LoD of 2.7 ng/mL. The highest mean Nef concentration was detected in 2K pellets (268.2 ng/mL), followed by small EVs-enriched 100K pellets (211.6 ng/mL), with large-EVs enriched 10K pellets containing the lowest Nef-concentration (121.0 ng/mL).

Conclusion: The developed nanobody-based Nef sandwich ELISA outperforms the current commercial ELISA kit *in vitro* and efficiently detects EV-bound Nef. With further optimizations, the assay could prove valuable in detecting extracellular Nef from various clinical samples, thus enhancing our understanding of the role of Nef in HIV-1-associated pathologies.

Funding information: The study was supported by research grant P1-0170 funded by Slovenian Research and Innovation Agency. We thank Prof. Serge Benichou, PhD, from the Institut Cochin, Inserm, France, for kindly providing the plasmid encoding the sdAb19 nanobody.



P-N-9

Implementation of Single-domain antibodies-based approach for isolation of extracellular vesicles from human plasma

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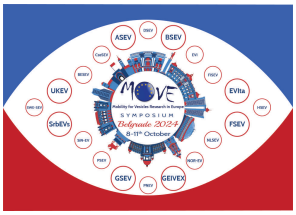
Introduction: Extracellular vesicles (EVs) have important roles in physiological and pathological conditions, thus could be used as promising biomarkers in clinical practice. There is a need for appropriate methods for EVs isolation that bypass major challenges of current methods such as obtaining low-yield and impurity of obtained vesicles. Therefore, it is crucial to establish methods for isolating that are economical, easy-to-operate and reproducible. The aim of this work was to utilize nanobodies, obtained from a naïve library by direct panning on EVs from human cell lines, for immunocapturing of exosomes from healthy-donor plasma.

Methods: Nanobodies (NA8, $ND10_1$, $ND10_2$) were fused with green fluorescent protein and 6XHis tag, then produced in *E. Coli* cells and purified using metal-affinity chromatography. By immobilizing each nanobody to methacrylate polymer, exosomes from human plasma were isolated and their biochemical traits were determined with various methods: SDS PAGE, Bradford assay, sulfophosphovanilin assay, flow cytometry and nanoparticle tracking analysis - NTA.

Results: Nanobodies are produced in soluble form and resulted in high yield (14-32 miligrams per litre of bacterial medium). Protein and lipid contents are determined in obtained preparation and the combined analyses of biomarkers (CD9, CD63, CD81) and morphological traits confirmed presence of EVs with appropriate diameter. Treatment of isolated EVs with detergent showed reduced signal on flow cytometry which indicates the presence of lipid membrane structures.

Conclusion: Results obtained in this work show that immuno-affinity system based on nanobodies can be successfully used for isolation of EVs from human plasma. Due to its efficacy, simplicity and the low costs of nanobody production, this method has a great potential for future implementation in isolating EVs from human plasma in research and clinical settings for various diagnostic and investigative purposes.

Funding information: This research was supported by the Science Fund of the Republic of Serbia, Grant PRISMA No. 4747, Project title: Advancing REversible immunocapture toward SCALablE EV purification—RESCALE-EV.



P-N-10

Isolation and Characterization of Citrus-Derived Nanovesicles Through Three Different Approaches

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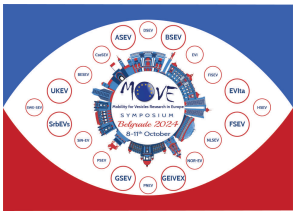
Introduction: Plant-derived nanovesicles (PDNVs) have received great attention in recent years, and with the scientific community calling for rigor and standardization, many efforts are being made to identify the most advantageous methods of isolation, in terms of yield, purity, reproducibility, scalability potential, and cost-effectiveness. Currently, there is no single 'gold standard' for the isolation of PDNVs and several techniques are employed, each with their advantages and disadvantages.

Methods: Therefore, in this study, we compare the characteristics of nanovesicles from lemon (LNVs) and tangerine (TNVs) isolated via three different methods, including one on an industrial scale.

Results: Our findings show that differential centrifuges and ultracentrifugation (UC) of lemon juice produce the smallest LNVs, with a homogeneous size distribution of less than 100 nm, and a particle number of $3.13x10^{11}$ particles/ml, with a characteristic morphology. Larger LNVs (150- 200 nm), with the same homogeneous size distribution, were obtained through the size exclusion chromatography (SEC) and the industrial microfiltration-based method, showing similar particle number yields, respectively of 9.67×10^{10} and 3.55×10^{10} particles/ml, and the classic round or slightly cupped shape. Minor size differences were found between TNVs isolated via UC and SEC. Both showed heterogeneous size distribution, with size <200 nm for those isolated via UC and >200 nm for those isolated via SEC with a particle number yield of $2.36x10^{10}$ and 3.22×10^{11} particles/ml, respectively. In addition, the analysis of the active compounds present in our differentially isolated LNVs and TNVs reveals an overlapping compound profile that includes flavonoids, limonoids, and organic acids, giving them strong anti-inflammatory and antioxidant properties, proven both in vitro and in vivo.

Conclusion: Overall, our findings show that although different isolation methods may impact the mean dimension of citrus-derived nanovesicles, they do not seem to affect their content and biological properties.

Funding information: FFR2024 from the University of Palermo to Stefania Raimondo



P-N-11

Optimizing buffer composition for cryoprotection of red blood cell-derived extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are lipid membrane-enclosed spherical structures essential for biomass transport and cell-to-cell communication, serving as potential drug carriers and biomarkers that reflect the physiological and pathological states of their originating cells. Despite extensive research, isolation and storage of EVs still face many challenges and lack standardization. This study aims to develop a standardized cryopreservation protocol to preserve the structural and biochemical integrity of red blood cell-derived EVs (REVs) by optimizing freezing conditions, such as buffer composition and concentration.

Methods: To identify optimal cryoprotective conditions, REVs were stored at -80°C for 1 day and 1 week in different dilution in PBS and PBS supplemented with cryoprotective additives, albumin and trehalose. After thawing, to investigate the effects, concentration and diameter was measured by microfluidic resistive pulse sensing (MRPS) and dynamic light scattering (DLS). Additionally, REVs were immunofluorescently stained with erythrocyte-specific (CD235a) antigens and analyzed by flow cytometry (FCM).

Results: Our results demonstrate that storing REVs in PBS without any cryoprotectant additives and in a less concentrated form reduces the recovery rate. FCM results also indicate a change in vesicle composition, showing reduced complexity and the appearance of a new disintegrated population. The best preservation results were achieved using PBS supplemented with albumin and trehalose.

Conclusion: The optimal conditions for preserving the structural and biochemical integrity of REVs while storing at -80°C were identified as freezing concentrated samples in PBS supplemented with albumin and trehalose.

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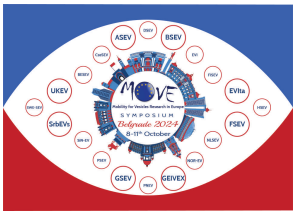

P-N-12

Evaluating Nano-Flow Cytometry for Detecting HER2-Positive Extracellular Vesicles in Breast Cancer Patients

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Introduction: Breast cancer is the most common cancer and leading cause of cancer-related deaths among females globally. HER2 is overexpressed in approximately 20% of breast cancers, making its detection crucial for targeted therapy. Recent findings suggest that HER2-positive extracellular vesicles (EVs) are present in the serum of HER2-positive breast cancer patients. This study evaluates the use of nano-flow cytometry (nFCM) to detect HER2 on individual EVs in plasma from these patients.

Methods: We established a non-washing nFCM staining protocol using EVs from HER2-positive (BT-474) and HER2-negative (TF-1) cell lines, including appropriate controls. The detection limit of HER2 was assessed by spiking BT-474 EVs into TF-1 EVs and healthy plasma. EVs were analyzed directly or following isolation using IZON qEV1 70 size exclusion chromatography (SEC). Finally, EVs were isolated from the plasma of patients with HER2-positive and triple-negative breast cancer (TNBC) for HER2 analysis.

Results: Nano-FCM detected HER2 on 20-30% of BT-474 EVs and <1% of TF-1 EVs. HER2-positive EVs were detected in spike-in samples in a dose-dependent manner. The detection limit of HER2 was around 1%, which was consistent with the positivity found in healthy control plasma and TNBC samples, suggesting this 1% positivity is due to non-specific binding of the antibody. HER2-positive EVs were observed in HER2-positive breast cancer samples (0.7-1.7%), but the difference was not statistically significant compared to TNBC (0.5-1.5%) or healthy controls (0.5-1%).

Conclusion: HER2 can be detected on individual EVs from the BT-474 cell line using nFCM. While this method shows potential for detecting HER2-positive EVs in breast cancer patients, further optimization is necessary to improve detection sensitivity and specificity.

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P-N-13

Evaluating the influence of different serum-free culture conditions on the production and function of Natural Killer cell-derived extracellular vesicles

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Introduction: Natural Killer (NK) cells are exploited in cellular therapies for cancer. While NK cell therapies are efficient against hematological cancers, it has been difficult to target solid tumors due to low tumor infiltration and a hostile tumor microenvironment. NK-cell derived extracellular vesicles (NK-EVs) target and kill cancer cells in vitro, and represent an alternative treatment strategy for solid tumors. To exploit their potential, it is necessary to standardize NK-EV production protocols.

Materials and Methods: Here, we have performed a comparative analysis of EVs from the human NK-92 cell line cultured in five serum-free commercial media optimized for growth of human NK cells and one serum-free medium for growth of lymphocytes. The effect of growing the NK-92 cells in static cell cultures versus shaking flasks was compared. EVs were purified via ultracentrifugation followed by size-exclusion chromatography.

Results: We found that there were no significant differences in EV yield from NK-92 cells grown under static or dynamic conditions. However, we found clear differences between the different culture media in terms of EV purity as assessed by enrichment of the CD63 and CD81 markers in the isolates, that translated into their capacity to induce apoptosis of the colon cancer cell line HCT 116.

Conclusions: These findings will be instructive for design of future production protocols for therapeutic NK-cell derived EVs.

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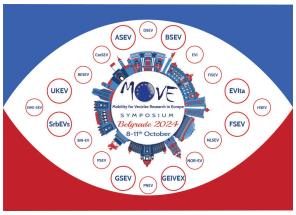

P-N-14

EV-Imprinted Nanoparticles for Real-Time Detection of Kidney-derived EVs Using Nanoplasmonic Sensor

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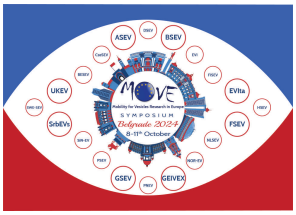
Introduction: Extracellular vesicles (EVs) are vital biomarkers for understanding disease progression and diagnosis. In particular, current biomarkers for kidney diseases (*e.g.,* serum creatinine) have impediments in sensitivity and specificity, leading to delayed diagnoses and no insights into diseased conditions. Due to their unique biogenesis and composition, EVs offer more disease- and site-specific biomarkers for accurate diagnosis. Herein, we aim to develop EV-imprinted nanoparticles for real-time kidney-EVs detection using molecularly imprinted polymers (MIPs) and nanoplasmonic sensors. MIPs provide high specificity and affinity by creating selective binding sites, while nanoplasmonic sensor allows real-time and label-free detection. Combining MIP's selectivity and multi-time usability along with nanoplasmonic sensor's sensitivity offers a novel, rapid, and accurate kidney-EV detection.

Methods: An ultrafiltration system with 200 nm and 50 nm filters was used to isolate EVs from HEK-293 cells. EVs were characterized using Nanoparticle Tracking Analysis (NTA) and Scanning Electron Microscopy (SEM). Characterized EVs were used as targets for EV-MIPs synthesis. A nanoplasmonic sensor was modified with EV-MIPs, and the surface chemistry was confirmed with X-ray photoelectron spectroscopy (XPS). EV solution was introduced to the sensor and wavelength shift was recorded with the sensors.

Results: Isolated EVs were obtained with 143.8±16.2 nm in size and 5.99×10^9 particles/mL of concentration. Their spherical morphology was confirmed with SEM. On the sensor, when EVs were introduced, the baseline signal shifted 1.34 nm, pointing out EV binding to EV-MIPs on the surface.

Conclusion: This study successfully exhibits that EV-imprinted MIPs could be used instead of antibodies to detect HEK-293 cell line-derived EVs. The binding of EV and EV-MIPs was confirmed with nanoplasmonic sensors, and this study hold potential to use MIPs in kidney-EV diagnosis in the future.

Funding information: The authors gratefully acknowledge support from the Scientific and Technological Research Council of Turkey (TÜBİTAK) 2232 International Fellowship for Outstanding Researchers (Project No: 118C254), Outstanding Young Scientists Award (GEBİP) from Turkish Academy of Sciences, and Young Scientist Awards Program (BAGEP) form Science Academy.



P-N-15

Double size exclusion chromatography enriches high-quality extracellular vesicles from calf and lamb feces

Chanaka Premathilaka[1], Suranga Kodithuwakku[1,2], Kasun Godakumara[1], Aneta Andronowska[3], Marina Loch[1], Toomas Orro[1], Alireza Fazeli[1,4,5*]

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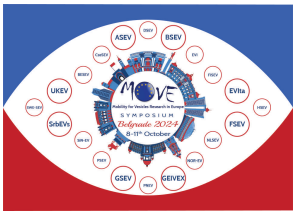
Introduction: Gastrointestinal (GI) health is critical for well-being and productivity of farm animals. GI disorders like infections, inflammations, and dysbiosis can obstruct their growth and productivity. Developing diagnostic and therapeutic strategies requires understanding the physiological and pathological processes of GI tract. Faecal extracellular vesicles (fEVs) have emerged as a promising non-invasive tool for studying gut physiology and pathophysiology. This study aimed to optimize a method for fEV enrichment from calf and lamb faeces and compare their basic characteristics.

Methods: Faecal samples were directly collected from the rectum of the two weeks old calves and lambs. Fecal samples weighing 0.3 g were suspended in PBS, sequentially centrifuged to remove debris, and subjected to dual size exclusion chromatography to enrich fEVs. Nanoparticle tracking analysis (NTA), protein quantity assay, and transmission electron microscopy (TEM) were employed to characterize the enriched particles.

Results: NTA analysis revealed that fractions 5 to 9 are enriched with particles. The total particle concentration was 1.4 ± 0.17 × 10^{10} particles/mL in calves and 7.5 ± 1.93 × 10^{10} particles/mL in lambs. The nanoparticles from calf feces ranged in size from 50 to 450 nm, while those from lamb feces ranged from 50 to 350 nm. Average particle sizes were 226.76 ± 4.26 and 157.82 ± 3.56 respectively. TEM analysis confirmed the presence of typical EV-like particles. Additionally, the Bradford protein assays showed that the fEV-enriched fractions had minimal soluble protein contamination.

Conclusion: The described protocol can enrich fEVs of sufficient purity from calf and lamb feces. Thus, further research is needed to evaluate the effectiveness of using these fEVs as a non-invasive means of studying ruminant gut health.

Funding information: European Union's Horizon 2020 research and innovation program, under grant agreement No. 857418 COMBIVET and The European Union through Horizon's coordination and support actions under grant agreement No. 101079349 OH-Boost.



P-N-16

Detection of organelle-specific dyes labelled extracellular vesicles with colocalization-fluorescence Nanoparticle Tracking Analysis

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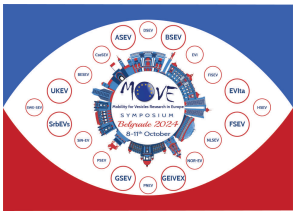
Introduction: Most cell types release a diverse array of extracellular vesicles (EVs) that contribute to intercellular communication. In particular, considering the heterogeneity of EVs, methods capable of identifying and measuring individual vesicles are limited.

Methods: Here, we used fluorescence and colocalization Nanoparticle Tracking Analysis (NTA) to identify the subcellular origin of vesicles and determine their physical characteristics as well as colocalization ratios of endoplasmic (ER) and Mitochondria (Mito) positive EVs in human choriocarcinoma cells (JAr) and bovine follicular fluids (BFF).

Results: The labelling efficiency for ER- labelled JAr EVs purified in SEC was 67.11 ± 25.40 % , compared to 96.27% ± 13.72 % of BFF EVs. Regarding Mito dye labelling efficiency, SEC-purified BFF EVs (14.21 ± 7.45) provided lower Mito-positive fluorescent particles than JAr EVs (25.74 ± 4.46). The proportion of CellMask™Deep Red (CMDR) membrane labelling of nanoparticles varied across JAr and BFF EVs. Furthermore, colocalization analysis of ER and Mito dye-labeled JAr and BFF revealed potential intracellular interactions/crosstalk between organelles and the EVs biogenesis pathways.

Conclusion: The integration of colocalization technology into fluorescence-NTA represents a significant advancement in the field of single EV particle analysis in deepening our understanding of EV biogenesis/biology.

Funding information: European Union's Horizon 2020 research and innovation programme under COMBIVET ERA Chair grant agreement no. 857418, the European Union through Horizon coordination and support actions under grant agreement No. 101079349 OH-Boost and a research grant by Particle Metrix GmbH.



P-N-17

Multi-Parametric Surface Plasmon Resonance - powerful new technology to measure small EV size and concentration and more

Abhishek Sharma, Matias Jokinen, and Annika Järvinen

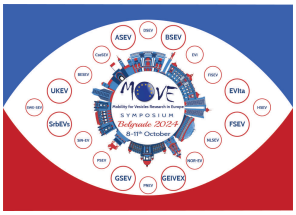
BioNavis Ltd, Tampere, Finland

Surface Plasmon Resonance (SPR) has been used already a few decades for label-free detection and quantitation of biochemical kinetics and affinities for many different types of analytes. SPR is a well-established technique to measure biomolecular interactions, such as drug-protein binding kinetics and affinity, in real-time, without labels and with high-purity analytes.

The new Multi-Parametric Surface Plasmon Resonance (MP-SPR) technology is a technique utilizing complete SPR angular spectra in the interaction measurements, allowing for the characterization of several parameters from the SPR curve in real-time. Uniquely MP-SPR with multiple wavelength setup allows a unique feature to measure the small EV (<100nm) in high resolution. BioNavis's instruments with multiple wavelengths allow EV size and concentration measurement[1,2,3]. For EV size and concentration measurements, technique utilizes differing evanescent fields caused multiple wavelengths[1,2,3]. Measurements do not require any fluorescent labels which can interfere with EV-antibody binding[4].

MP-SPR can also be used for EV diagnostic/therapy development. MP-SPR has been widely used for protein-protein kinetic measurements. The binding kinetics between EVs with capturing antibodies or aptamers can be easily studied with MP-SPR[5]. EV protein corona can have effects on the interaction between EVs and cells. MP-SPR can be used to distinguish between hard and soft corona[6]. Components of the BioNavis's EV size and concentration kit can be used to measure EV-antibody/aptamer kinetics. Being a label-free technique, MP-SPR has also been used to study interaction between EVs and adherent cells[7].

Beyond these MP-SPR is an excellent tool for biomolecular interaction studies (protein-protein, protein-drug) protein-lipid). Apart from biomolecular interaction studies, MP-SPR has been used to study complex biological such as, cell-drug interactions and T-cell-antibody interactions. We also offer a new regenerable avidin for surface modification [8,9]. Regenerable avidin is an ideal reagent for reversible surface immobilization with high precision. In conclusion, MP-SPR is a powerful label-free technique for studying EVs whether its EV size and concentration, EV corona or EV therapeutics.



P-N-18

Specific extracellular vesicle detection and isolation in complex samples

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Introduction: Specific detection, quantification and isolation of extracellular vesicles (EV) is a particularly challenging task due to the heterogeneity of EVs and the complexity of the matrices in which they are typically found, such as culture medium or blood plasma. Extracellular vesicles analytics combined with liquid biopsy could lead to earlier diagnosis and stratified treatment that will save lives (e.g. cancer mortality reduction 20-25%) and lower the healthcare burden cost (>5% reduction). However, current methods to purify and quantify Evs take a long time, introduce a bias, are costly and imprecise, thereby hampering progress in EV R&D, while the lack of methods to characterize the whole EV directly in crude samples restricts clinical sensitivity in diagnostics.

Methods: We have developed a breakthrough label-free fiber-optic method in an automated platform for biomolecular sensing, directly in the unpurified sample. This method combines affinity based quantification with simultaneous affinity isolation or downstream analysis.

Results: This integrated solution using a sensor dip-in probe is a new, selective, automated (scalable), fast (hours) and specific (purity up to 95%) method to accurately quantify and isolate EV directly from cell culture supernatant and plasma samples. Proof of concept data will be presented to demonstrate yield for downstream analysis and for specific capture of circulating neuronal tissue EVs from human plasma.

Conclusion: We have established a one-step automated method for affinity specific detection, quantification and isolation of extracellular vesicles for downstream analysis, to lead to unbiased and reproducible development of EV based biomarkers in disease and therapy research.

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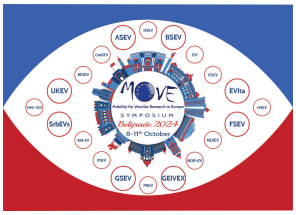

Quantification of EV surface proteins with fluorescent labelling and single EV detection

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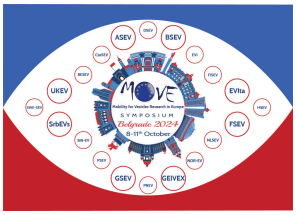
Single particle measurement platforms allow for the potential to quantify not only different particle sub-populations, but the surface proteins, lipids or cargo that make them unique. Fluorescent labelling is a well utilized tool, in both EV and whole cell characterization, and in this body of work we draw from quantitative flow cytometry to quantify proteins presented on EV surfaces.

Equivalent Reference Fluorophores (ERF) beads were used to demonstrate the relationship between designated number of fluorophores fluorescence intensity measurements and by nFCM and NTA. Distinct populations were observed with linear relationship between fluorescence and number of fluorophores allowing fluorophore quantification and for lower limit of detection (LLoD) to be described.

EVs from different sources were labelled with AF488-conjugated antibodies with average epitope presentation calculated and epitope distribution presented in histograms. EVs were then decorated with different quantities of protein target using commercially available kits. Subsequent labelling, detection and epitope quantification demonstrated a strong correlation between expected epitope presentation ratios and measurements by nFCM.

The materials, protocols and technology presented here demonstrate highly versatile approaches to quantifying epitopes on the surface of EVs and other nanoparticles.



P-N-20

Size-based extracellular particle sorting: methods and media effects on breast cancer cells

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Introduction: Extracellular particle (EP) populations of different sizes are known to have unique molecular profiles and can act as disease biomarkers. Isolating a single population can enhance diagnostic sensitivity, making accessible sorting methods essential for advancing EP clinical research. Biomarker discovery often starts from cell culture lines, however, the impact of culture conditions on EP characteristics needs to be explored. This study evaluates standard lab techniques for fractionating EPs from MCF7 cells and compares the impact of commonly used culture media on EP subpopulation profiles.

Methods: MCF7 cells were grown in EMEM+insulin or DMEM supplemented with 10% FBS. EPs were isolated from conditioned media using differential centrifugation, two-step PEG precipitation or inverted syringe filtration. EP fractions were characterized using Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA). Protein content in each fraction was quantified using a BCA assay, while TRIzol purified RNA using 260nm absorbance.

Results: Cells grown in EMEM had visually different morphology from DMEM grown and exhibited different EP population profiles. Each separation method led to successful EP fractionation as assessed by DLS and NTA. Higher protein content was found in large extracellular vesicles (EVs) (>200nm) fractions compared to small EVs (<200nm), while RNA quantification revealed that the smallest EPs had higher RNA content per particle than small or large EVs.

Conclusion: The study highlights the importance of culture conditions on extracellular particle populations, which can have significant implications in their role in disease studies or biomarker research. Crude size-based fractionation of EPs was achieved with basic equipment and led to similar trends where the smallest EPs are enriched in RNA, while larger EVs contain higher protein content, correlating with previous findings in the literature. These results demonstrate the utility of accessible fractionation techniques for exploring EP subpopulations and their molecular profiles.

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EVs in therapy
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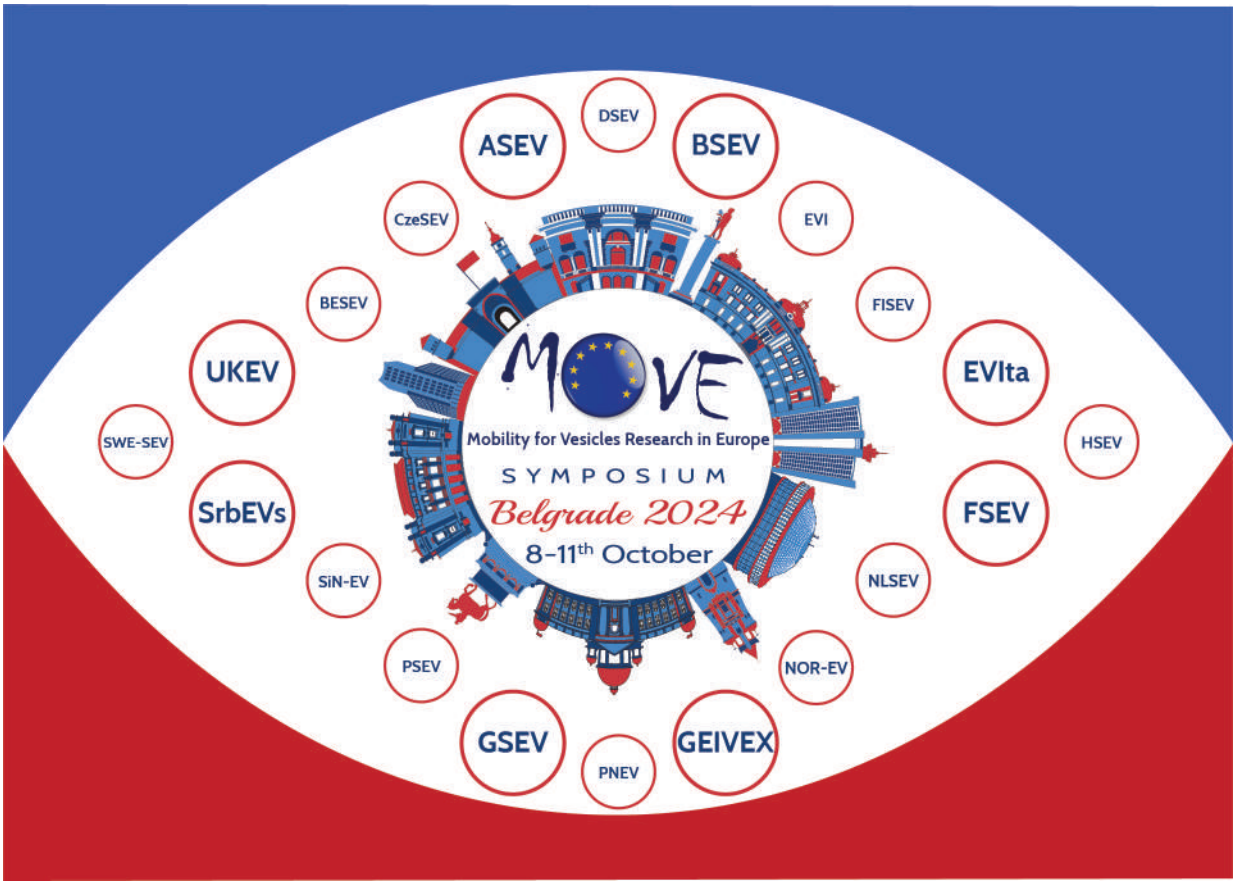

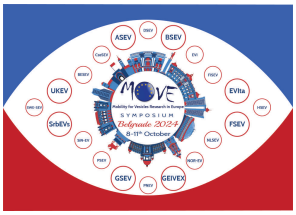

O-T-1

Extracellular vesicles powered cancer therapy: targeted delivery and enhanced anti-neoplastic effect of adenovirus-based cancer vaccine in humanized melanoma model

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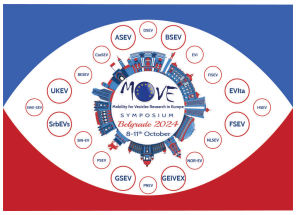
Introduction: Malignant melanoma, a rapidly spreading form of skin cancer, is on the rise globally. While surgery can effectively treat early-stage melanoma, patients with advanced disease have a low chance of survival. Melanomas with mutations in the NRAS gene are known for being more aggressive and having a poor prognosis. As a result, new treatment options are needed, particularly for this specific group of patients. One promising approach is using cancer vaccines to boost the body's immune response against the tumor. However, current vaccines have limited effectiveness and more potent methods are required.

Methods: In this study, we propose the systemic administration of a novel adenovirus-based cancer vaccine complexed in extracellular vesicles (EVs) with the aim of achieving a targeted therapeutic effect. This vaccine, consisting of a combination of an oncolytic adenovirus, Ad5/3-D24-ICOSL-CD40L, with clinically relevant melanoma antigens, targets NRAS mutations to enhance its anti-cancer effects. Here, we first tested the antineoplastic effect in two-dimensional (2D) and three-dimensional (3D) co-culture model based on NRAS mutated melanoma cells with peripheral blood mononuclear cells (PBMCs).Then we explored the systemic delivery of the vaccine in EV-formulations in a humanized NRAS mutated melanoma mouse model.

Results: *In vivo* and *ex vivo* imaging analysis conducted in humanized mice allowed to demonstrate the selective ability of EVs to deliver the oncolytic vaccine to both primary and metastatic tumor. Moreover, the observed anticancer efficacy was attributed to reduced tumour volume and increased infiltration of tumour infiltrating lymphocytes, including activated cytotoxic T-cells (GrB+CD8+). These findings are also in line with the observed synergistic anti-tumor effect. Additionally, a correlation between tumour volume and activated CD8+ tumour infiltrating lymphocytes was observed.

Conclusions: Collectively, this research suggests EVs as a safe and effective tool for personalized cancer treatment paving the way for a systemic administration of immunogenic vaccines in hard-to-inject melanoma.

Funding information: STARS Starting Grant, Grant Number: GAR- O_STARS_MUR22_01, EU funding within the MUR PNRR "National Center for Gene Therapy and Drugs based on RNA Technology", Project no. CN00000041, CN3 – Spoke #8 "Platform for DNA/RNA delivery".



O-T-2

Extracellular vesicles from clonally expanded immortalized mesenchymal stromal cells protect against ischemia-reperfusion-induced kidney injury

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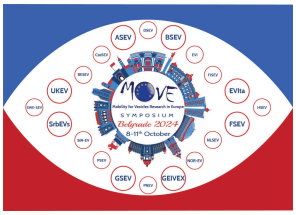
Introduction: Ischemic kidney injury is a severe clinical syndrome associated with high morbidity and mortality. Mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) hold promise as a therapeutic option in various organ injury models. The challenges posed by the donor heterogeneity and senescence of MSCs can be addressed by using EVs derived from clonally expanded immortalized MSCs (ciMSCs). The aim of this study was to evaluate the effect of ciMSC-EVs on ischemic kidney injury.

Methods: Eight-week-old C57BL/6 mice were subjected to unilateral ischemia-reperfusion (uni-IRI) kidney injury. ciMSC-EVs or vehicle were administered intravenously immediately before and 24 hours after surgery. Mice were sacrificed 48 hours post-surgery. Serum creatinine (Cr) and blood urea nitrogen (BUN) levels were measured using standard clinical methods, while kidney injury was assessed after Periodic Acid-Schiff staining. qPCR and Western blotting were employed to analyze inflammatory, apoptotic, autophagy and regenerative processes. Immunohistochemistry was performed to evaluate macrophage infiltration in the kidney tissue.

Results: In vehicle treated animals, uni-IRI led to a significant increase of serum Cr and BUN levels, marked morphological damage of kidney parenchyma, and formation of abundant tubular casts. ciMSC-EVs treatment significantly decreased impaired renal function and ameliorated morphological damage in the kidney. Furthermore, ciMSC-EV administration decreased the expression of IL6, MCP1, IL1β, TNFα, RANTES mRNA in affected kidneys, while no effect on apoptotic nor autophagy markers was recognized. Kim-1 and NGAL, known markers of tubular damage, showed significant decrease after the administration of ciMSC-EVs. Importantly, ciMSC-EV treatment significantly recovered the expression of antiaging and antifibrotic protein Klotho in the damaged kidney, and decreased macrophage infiltration (F4/80) in the renal parenchyma.

Conclusion: The present study demonstrates that ciMSC-EV administration ammeliorate IRI and mediate anti-inflammatory effects in the kidney. Upon modulating immune response, the ciMSC-EV treatment appears as a promising intervention to promote recovery of kidney after ischemic injury.

Funding information: Instituto de Salud Carlos III (ISCIII), CP19/00027 and PI21/00204.



O-T-3

Erythrocyte membrane-based vesicles as siRNA carriers for safe and efficient gene silencing therapy

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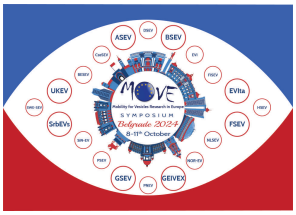
Introduction: Among different circulatory cells, erythrocytes are the most abundant and thus can be isolated in sufficiently large quantities to decrease complexity and cost of the treatment compared to other cell-based vaccines. In the present work, we used fresh red blood cells as starting material for a neutral therapeutic nucleic acid delivery system. Our therapeutic nucleic acid of choice is siRNA, for its compactness and already well-established clinical applications. As proof-of-concept, we loaded an anti-TdTomato siRNA in erythrocytes membrane vesicles (EMVs). EMVs were proven to be a successful, cheap and safe delivery system for siRNA in vitro and in vivo, on a melanoma cell-line and on tumor-induced mice.

Methods: We optimized purification strategy and long-term storage techniques of EMVs. siRNA-loaded EMVs were evaluated for nuclease resistance. Vesicles were analysed via TEM and cryo-TEM. siRNA release and RNA interference ability was evaluated in vitro. Finally,siRNA-EMVs, containing Cy5-labeled anti-tdtomato siRNA, were labeled with Vybrant-DiO dye and a total of 30 µg of Cy5-siRNA (or 1.5 mg/kg of weight) loaded EMVs was injected via caudal vein in each mouse, for a total of n=19, divided in control group (n=4), 2 hours after injection (n=5), 24 hours after injection (n=5) and 48 hours after injection (n=5). Blood was collected ten minutes after EMVs injection and right before sacrifice. Spleen, lungs, brain, kidneys, tumor, heart and liver were imaged using confocal microscopy and processed for RNA extraction (real-time PCR).

Results: We confirmed the occurred loading with siRNA in our EMVs via freeze-fracture TEM and STED-laser equipped confocal microscope, effectively visualizing siRNA within the lumen of vesicles. siRNA-EMVs showed a high level of protection against RNAse A. In vitro, silencing ability of siRNA-EMVs was found to be around 80%, at a final well concentration of 0.3 nM, a remarkable lower dose when compared to HEK293-derived EV and Neuro2a-EV loaded with lipidic siRNA. As previously stated, the key for efficacy in neutral carrier-based delivery of therapeutic RNAs is likely to be the number of copies per each single object rather than the number of objects. Such mechanism seems to be shared among the cell lines we evaluated (CT26, B16F10 and NHLF) with no preferred target. In vivo, on melanoma bearing mice, at 2.5 mg/kg of body weight, DCC-siRNA-EMVs were capable, upon systemic administration, to elicit a 60% of Tdtomato gene silencing, while intact EMVs, containing anti TdTomato siRNA lead strand, were still present in the blood flux after 48 hours from administration.

Conclusion: We were therefore able to develop a simple, cost-effective, therapeutic nucleic acid carrier, that is also prone to easy scaling-up. Future developments for these carriers will include active targeting to tumor sites and the simultaneous loading to a reporter agent, in order to obtain an all-in-one theranostic agent.

Funding information: The study was supported by the Slovenian Research Agency ARIS (program numbers P2-0084, P3-0003, project numbers PR-10482, J3-14531 and J4-50150).



O-T-4

Effect of blood centrifugation on the platelet, extracellular particle and molecular content of plasma

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Introduction: Plasma is a yellowish liquid that presents a platform for blood cells, nanoparticles and molecules circulating throughout the organism. Autologous plasma is prepared from the patient's own blood following procedures that are easy to perform. With autologous application there is low risk of infections or immune rejection. Moreover the procedures are already being used in clinical practice in many fields of medicine, however, the healing mechanisms are not yet completely understood. Recent developments have outlined nano-sized (ranging from 20 nm–1000 nm) cellular particles in preparations from blood.

Methods: We have prepared more than 200 plasma samples from canine, equine and human blood by applying different centrifuge settings to one-step centrifugation of blood. In plasma, we have determined platelet number density by flow cytometry and extracellular particle number density by interferometric light microscopy. In human plasma, we have assessed inflammation markers and growth factors by ELISA tests. We have assessed oxidative stress markers by flow cytometry. We have constructed a mathematical model of erythrocyte sedimentation and estimated optimal time and centripetal acceleration of the centrifuge for an individual sample. We have assessed lipid content in equine blood by mass spectrometry.

Results: We outlined a quantity τ that is proportional to the product of the time of centrifugation and centripetal acceleration of the centrifuge rotor as an unifying parameter for all blood samples analyzed. We found that platelet yield, growth factors and lipid content were sensitive to τ. Too large τ rendered plasma poor with platelets and growth factors. Extracellular particle yield was in the range of 10^{10}/mL and the sizes were insensitive to τ.

Conclusion: Plasma preparation significantly affects its composition. Using the same protocol for all patients may result in considerably different contents, therefore methods for individual estimation of the centrifuge setting should be further developed.

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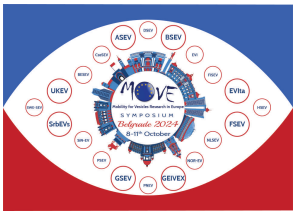

O-T-5

Potential anti-senescence effect of Naringin and Hesperidin-loaded extracellular vesicles in intervertebral disc degeneration

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Introduction: Low Back pain (LBP) affects more than 80% of the world population associated to intervertebral disc degeneration (IDD). Cell senescence contributes to this age-related condition through the release of phenotypic proteins associated with cell senescence, leading to inflammation, tissue degradation, aberrant cell differentiation and senescence in neighboring cells. No therapeutic approach was demonstrated to revert or arrest IDD, even if several treatments are available.

Naringenin and Hesperidin are flavonoids found in citrus fruits, which possesses a wide range of biological and pharmacological activities, including antioxidant, anti-inflammatory, cytoprotective and antitumor actions. Our aim is to study the senolytic and anti-inflammatory effect of natural molecules-loaded EVs in IDD.

Methods: Human nucleus pulposus cells (hNPCs) were isolated from surgical specimens of patients suffering of LBP and culture expanded *in vitro.* Drugs cytotoxicity was assessed by MTT assay to establish best concentration for EVs loading. EVs were isolated by differential (ultra)-centrifugation, from immortalized bone marrow mesenchymal stem/stromal cells (iMSC) and characterized by TEM, WB, NTA. For hesperidin or naringin iMSCs-EVs loading, three different methods were tested: incubation, electroporation and sonication. EV integrity was evaluated by TEM. Drug loading capacity (LC) and encapsulation efficiency (EE) for each method were evaluated by Fluorescence/by Spectrophotometry and HPLC analysis.

Results: A dose dependent increase in hNPCs cytotoxicity was noticed after treatment with either drug or a combination of both drugs. Best therapeutic doses for hNPCs were determined as 5ug/mL and 1ug/mL for Hesperidin and Naringin, respectively. TEM images confirmed that loading methods do not affect EVs morphology. LC and EE percentages are being evaluated for the different methods.

Conclusion: EVs may represent a natural "bio-nanomedicine" with drug delivery potential, high chemical stability, low immunogenicity and toxicity.

Funding information: MOVE EUROPEAN MOBILITY FELLOWSHIPS 2023; EORS EXCHANGE TRAVEL GRANT 2023

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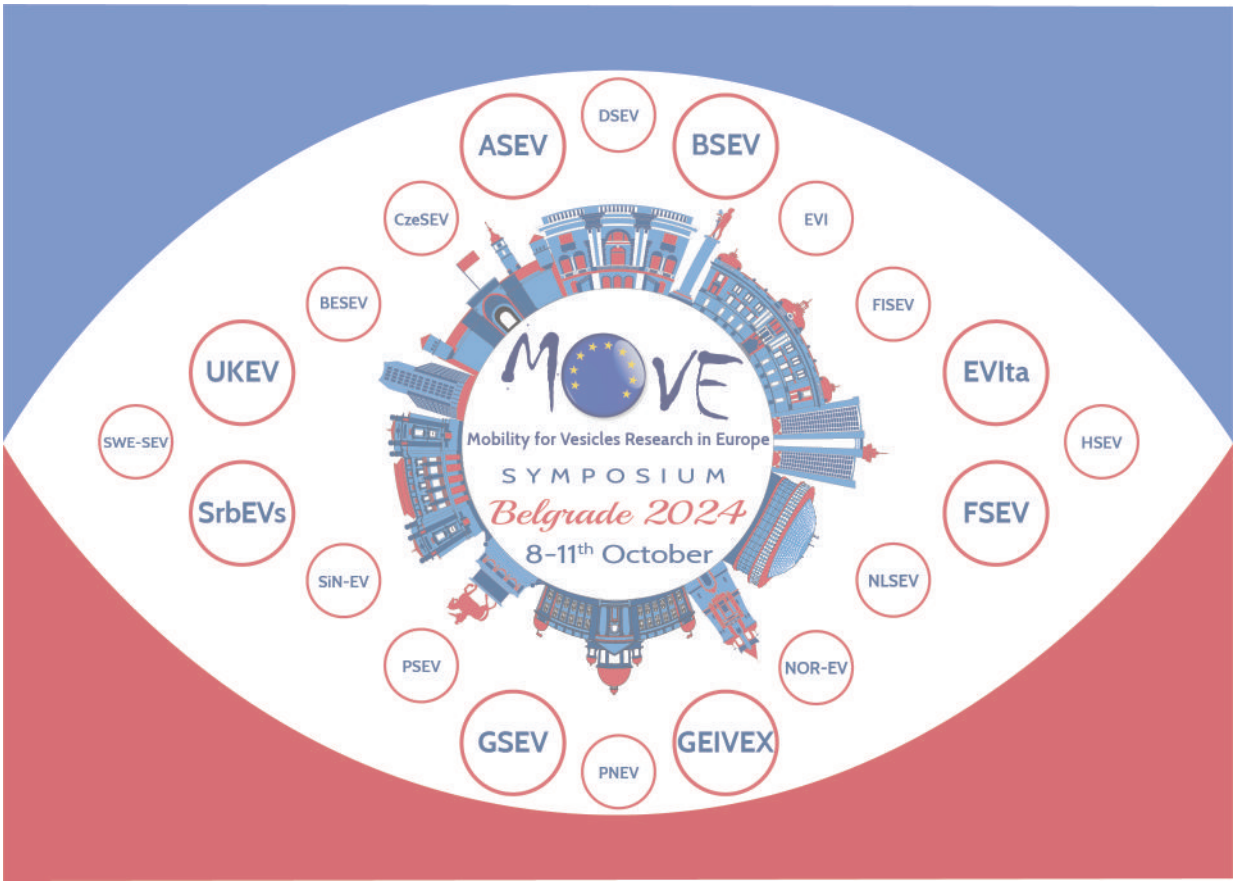

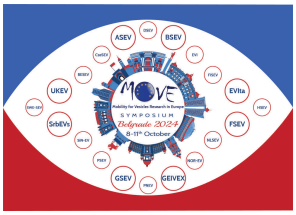

P-T-1

Exploring Dendritic Cells-Derived Extracellular Vesicles As An Immunotherapeutic Tool In Triple-Negative Breast Cancer

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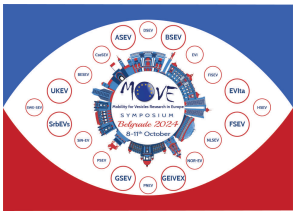
Introduction: Dendritic cells (DCs) are antigen-presenting cells involved in tumor antigen presentation to naive T cells. DC-derived extracellular vesicles (DC-EVs) promote tumor antigen-specific responses, have 10–100 times more MHC complexes/surface area than DCs and resist to immunosuppression. Nevertheless, DC-EVs immunotherapeutic potential is not fully explored. We propose to unveil the DCs-EVs role as an immunotherapeutic approach for triple-negative breast cancer, orphan of effective therapies.

Methods: Two strategies were developed to do MDA-MB-231 lysates: sonication (S) and freeze/thaw plus sonication (FT+S). Monocytes from healthy blood donors were cultured in DC differentiation media (FBS EV-depleted) with IL-4 and GM-CSF. FT+S and S lysates, combined with MPLA, were added to induce DCs activation. DCs-derived EVs were isolated from media by ultracentrifugation, Optiprep density gradient, SEC and characterized by Western Blot, NTA and TEM. DC-EVs potential to activate T cells was evaluated by flow cytometry for specific T cells lineage and activation markers.

Results: Protein with integrity compared to the standard RIPPA method was obtained with FT+S and S lysates. DCs phenotype was confirmed by flow cytometry (CD11c, CD86, CD40 and HLA-DR), suggesting that DCs are activated with FT+S cell lysates combined with MPLA, according to increased expression of CD86 and HLA-DR, compared to control group. ELISA of DCs conditioned media showed an increase of IL-6, IL-12p40 and TNF-**α** expression. We observe a peak corresponding to EVs size (~130nm) by NTA, and cup/dish shaped particles by TEM, as well as expression of EVs markers (CD9, CD81, CD63, Alix), suggesting successful EVs isolation. The impact on T cell proliferation and activation is under analysis.

Conclusion: Our preliminary data shows that tumor cell lysates, in the presence of MPLA, activate DCs. The isolation of DC-EVs was successful and further proteomic studies will dissect the immunomodulatory potential of DC-derived EVs, opening a new door for immunotherapy.

Funding Information: This work was supported by scientific project "RADIORESISTANCE: Dissect the tumor microenvironment to battle cancer radioresistance and immune escape" reference: PTDC/MED-ONC/4165/2021, financed by the Portuguese Science and Technology Foundation (FCT). Sofia T. Quintas (2022.11948.BD) and F. Castro (2021.01773.CEECIND) acknowledge PhD fellowship and CEEC contract, respectively.



P-T-2

Pooling of umbilical cord mesenchymal stromal cells improves extracellular vesicle yield and therapeutic benefit in inflammatory arthritis

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Introduction: Novel biological therapies have revolutionised the management of Rheumatoid Arthritis (RA), but no cure currently exists. Human umbilical cord mesenchymal stromal cells (UC-MSCs) present a promising therapeutic alternative due to their innate anti-inflammatory properties. However, problems have arisen with MSC heterogenicity, practicalities of transport and storage, and their response to an inflammatory microenvironment. One potential solution is the use of their extracellular vesicles (EVs). However, EV production suffers from low yields and must be scaled without compromising therapeutic potential. Using an antigen-induced arthritis model (AIA), we sought to determine the effectiveness of UC-MSCs and their derived EVs from single and pooled donors in combatting RA-like pathology.

Methods: Single and pooled MSC EVs were enriched using a sucrose cushion, characterised and used to treat an AIA model, along with their parental MSCs. Joint swelling was assessed over 72 hours, and histological analysis used to determine arthritis index.

Results: EVs from pooled donor yielded significantly more particles than from single donor, with most particles displaying an EV-like morphology and expression of EV and MSC associated markers.

Joint swelling decreased for MSC and EV treatments, in comparison to a media control, but only MSC-EV treated mice achieved significance for single and pooled donors at the 48- and 72-hour timepoints. Pooled EVs surpassed pooled MSCs and single donor EVs in alleviating RA pathophysiology as indicated by the significantly lower arthritis index at 72 hours post arthritis induction.

Conclusion: Whilst UC-MSCs and single donor EVs improved joint swelling and histopathological signs of inflammatory arthritis, pooled EVs surpass them. This is backed by the significant reduction in arthritis index suggesting that pooled EVs can not only mimic their cellular counterparts, but may be a superior treatment for RA.

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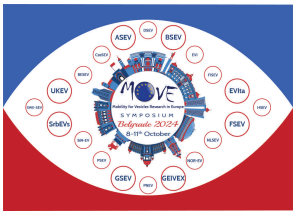

P-T-3

Inhibitory Effects of Chlorella vulgaris Algasomes on Listeria species Growth and Biofilm Eradication

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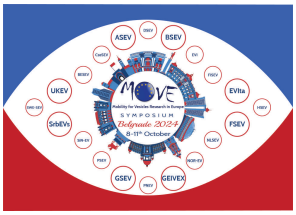
Introduction: Antimicrobial resistance is rising, diminishing the efficacy of synthetic antimicrobials. Natural alternatives, like extracellular vesicles (EVs), show promise against pathogens. This study investigates EVs from *Chlorella vulgaris* spent medium (algasomes) on *Listeria monocytogenes*, focusing on growth, biofilm formation, and eradication.

Methods: *Listeria monocytogenes* ATCC 19115, ATCC 13932, and *Listeria innocua* ATCC 33090 were cultured at 1x10^7 CFU/ml and co-cultured with algasomes at 1x10^9 EVs/µl in Brain Heart Infusion broth for 24 hours at 37°C. Bacterial growth was measured by absorbance at 620 nm. For biofilm studies, bacterial cultures were standardized to McFarland 1.0. Biofilm inhibition was tested by co-culturing bacteria with algasomes on a 96-well plate for 24 hours at 37°C. For biofilm eradication, bacteria were cultured for 24 hours, washed, exposed to algasomes (1x10^9 particles/mL), and incubated for another 24 hours at 37°C. Biofilm biomass was assessed via crystal violet staining at 620 nm, compared with PBS-negative controls and gentamicin (10 µg/mL) as the positive control.

Results: Algasomes significantly inhibited (p<0.05) *Listeria* growth at 13 hours: 20.2% ± 0.38 for *L. monocytogenes* ATCC 13932, 11.2% ±1.07 for *L. monocytogenes* ATCC 19115, and 8.0% ± 2.77 for *L. innocua* ATCC 33090. While algasomes did not significantly inhibit biofilm formation, they showed considerable eradication potential: 18.2% ± 2.09 for *L. monocytogenes* ATCC 19115, 14.6% ±1.40 for *L. monocytogenes* ATCC 13932, and 14% ±1.11 for *L. innocua* ATCC 33090, compared to the antibiotic's 3% ±3.64, 0.3% ±2.97, and 0.7% ±3.16, respectively.

Conclusion: The study demonstrates that algasomes from *Chlorella vulgaris* effectively inhibit *Listeria* spp. growth and eradicate biofilms, suggesting algasomes as a promising natural antimicrobial strategy.

Funding: The study was supported by the COMBIVET ERA Chair initiative, backed by the European Union's Horizon 2020 research and innovation program, under grant agreement No. 857418, the OH-BOOST grant provided by the European Union via HORIZON Coordination and Support Actions, under grant agreement No. 101079349, as well as the Estonian Research Council project grant PRG1441.



P-T-4

Autologous plasma-derived EVs for chemotherapy delivery to human tissues

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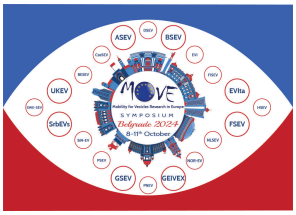
Colorectal Cancer (CRC) has the third highest incidence rate and second highest cancer-related mortality worldwide, demonstrating the need for better treatment approaches. Extracellular vesicles (EVs) are small, membrane enclosed particles produced by all cells that are readily taken up by cancer cells, meaning they have potential as a natural drug delivery vehicle, however little is known about how EVs retain within tissue, nor how systemic administration of allogeneic EVs might impact on biocompatibility. In this study, we aimed to establish the efficiency of autologous EV drug loading, and interaction with extracellular matrix substrates.

EVs were isolated from healthy volunteers, early stage (T1/T2, no nodal involvement of evidence of metastasis) or later stage (T3/4, N1/2 or M1) CRC under REC approval. Isolation was performed by size-exclusion chromatography (SEC) before loading with doxorubicin. EV properties and loading efficiency were assessed by size/particle count (nano flow cytometry), marker profile (DELFIA ELISA) and transmission electron microscopy (TEM). Drug uptake by the primary SW480 and isogenic metastatic SW620 CRC cell lines was assessed using flow cytometry and immunofluorescence. Binding to ECM substrates was assessed using ECM-coated plates.

EVs were successfully isolated by SEC, loaded with doxorubicin at efficiencies of 10-20%, and were rapidly uptaken by CRC cells. Tissue-derived EVs delivered drug more efficiently than plasma EVs ($p < 0.0001$), and EVs derived from CRC patients more efficiently than healthy controls ($p < 0.05$). However, EVs from late-stage patients were less effective at delivery than early-stage patients ($p < 0.05$), and delivery was lower to metastatic CRC cells ($p < 0.05$). Finally, EVs from CRC patients bound less efficiently to collagen than healthy controls, with differences observed in binding profile when comparing human to mouse collagen and under hyperglycaemic conditions, suggesting that EV drug loading and matrix retention are disease and species specific.



P-T-5

Local delivery of Dendritic Cell-secreted Extracellular Vesicles using 3D biomaterial scaffolds to promote bone tissue formation and spine fusion

Maria Cardona-Timoner[1, 2], Joana Coelho[1, 2], Mafalda Bessa-Gonçalves[1,2], Andreia M Silva[1, 2], Mário A Barbosa[1, 2], Raquel Gonçalves[1, 2], **Susana G Santos**[1,*]

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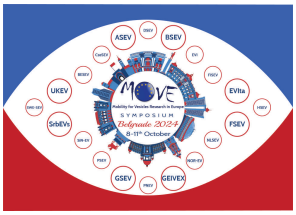
Introduction: Communication between immune and tissue cells is paramount for a timely resolution of inflammation and the progress of tissue repair. As part of their intercellular communication toolbox, cells secrete Extracellular Vesicles (EV), with important roles ascribed for inflammation and immunity, cell proliferation and differentiation and matrix deposition and remodelling (1,2). Our work has been addressing the communication between immune cells and Mesenchymal Stem/Stromal Cells (MSC), in the context of bone repair. Herein, we will discuss our results and approaches to harness the potential of immune cell-secreted EV for promoting MSC recruitment and differentiation, enhancing new bone tissue formation.

Methods: Ethical permission for human and animal studies was obtained. Macrophages(Mac)/Dendritic cells(DC) were differentiated from rat bone marrow or human peripheral blood. EV were obtained by differential (ultra)-centrifugation and characterized by NTA, TEM and Western blot (3). Chitosan (Ch), Fibrinogen (Fg) and Magnesium (Mg)-modified Fg scaffolds, were produced by freeze-drying (4). EV were incorporated in Ch scaffolds using a layer-by-layer method and onto Fg-based scaffolds by adsorption, and analyzed by microscopy. Recruitment of MSC and fibroblasts, and MSC differentiation, were evaluated. EV-enabled 3D scaffolds were implanted in a spine fusion mouse model.

Results: Our previous results showed that DC-secreted EV were the main secretome component mediating MSC recruitment(3). DC-EV were more efficient at promoting MSC recruitment than Mac-EV, did not impact MSC proliferation/differentiation, and did not promote fibroblast recruitment. EV-loaded scaffolds retained the ability to promote MSC motility/recruitment. EV-enabled FgMg scaffolds were most efficient at combining enhanced MSC recruitment and osteogenic differentiation, and their potential to promote bone tissue formation is being evaluated in vivo, in a spine fusion model.

Conclusion: Our results support that DC-EV act specifically on MSC recruitment. DC-EV enabled FgMg scaffolds combine increased MSC recruitment with osteogenic differentiation, rendering them promising new therapeutics to promote spine fusion.

Funding information: Work funded by Orthoregneration Network (ON Pilot Grant Spine 2021, EVS4Fusion). MCT supported by ERASMUS+ program.



P-T-6

Bone marrow stromal cell derived extracellular vesicles for bone regeneration

Evangelia Bochti[1], Monica Tsimbouri[1], Biswajoy Bagchi[2], Xin Li[2], Matthew Dalby[1], Catherine Berry[1]

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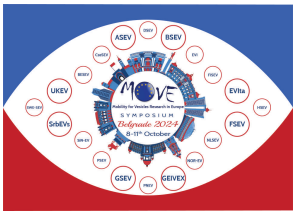
Introduction: Bone tissue is the second most transplanted tissue in the body, worldwide, with over 4 million annual transplants. Bone marrow-derived stromal cells (BMSCs) are extensively used in bone tissue engineering (BTE) as components for bone constructs due to their osteoblastic lineage. The beneficial effects of BMSCs are in part due to their paracrine action, potentially mediated through extracellular vesicles (EVs). In collaboration with Sphere Fluidics Limited, this project aims to manufacture a novel BMSC-EV microenvironment, encapsulating osteogenic-primed EVs within hydrogel beads for cell-free bone regeneration and repair therapies.

Methods: BMSCs were osteogenically primed for 28 days using two distinct approaches: (i) chemical induction, using osteogenic media and (ii) mechanical induction, via nanoscale vibrational displacements, termed nanokicking. Osteogenic differentiation of BMSCs was verified via protein analysis (western blotting) and histological staining (alizarin red). BMSC-derived EVs were isolated using differential centrifugation, ultrafiltration, and size exclusion chromatography, and characterised by size and concentration (microBCA, dynamic light scattering, tunable resistive pulse sensing, nanoparticle tracking analysis, nano flowcytometry, and transmission electron microscopy). EVs' bioactive cargo is currently assessed at a cytokine level with further evaluations at metabolomic, proteomic and RNA levels, planned. Osteogenic-derived EVs are currently co-cultured with naïve BMSCs, where their osteogenic potency will be assessed.

Results: Both chemical and mechanical inductions successfully induced osteogenesis in BMSCs. Successful EV isolation was verified and EVs were consistently sized (60-250nm) across both induction methods and the non-treated (negative control) derived BMSCs. Differences in EV cargo are noted in the cytokine profile, with ongoing studies at a metabolomic, proteomic and RNA level, between the chemically and mechanically induced osteogenic-derived EVs. Both types of osteogenic-derived EVs are currently being assessed for promotion of osteogenesis in naïve BMSCs.

Conclusion: BMSC analysis indicated osteogenesis occurring under both chemical and mechanical stimulation, with mechanical induction being a slightly slower process. Ongoing characterisation of BMSC-EVs aims to determine any differences in osteogenic-derived EVs cargo, both compared to the negative control and between the induction approaches.

Funding information: This PhD research project is funded by Medical Research Scotland (MRS) and Sphere Fluidics Limited, studentship ref. number: PHD-50388-2021



P-T-7

Exploring the anti-inflammatory response of pomegranate-derived extracellular vesicles in an in vitro model of osteoarthritis

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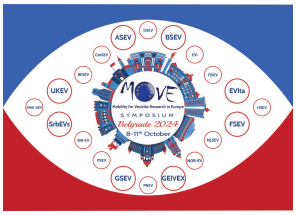
Introduction: Osteoarthritis (OA) is a chronic degenerative disease of the joint, characterised by cartilaginous degradation due to an imbalance in anabolic and catabolic processes. As a result, the patient experiences pain, reduced mobility and diminished quality of life. Currently there are no curative treatments for osteoarthritis, instead available treatments aim to improve symptoms. Recent studies have focused on the use of extracellular vesicles (EVs) to treat OA. In this regard, we have described the anti-inflammatory effect of pomegranate EVs.

Methods: Pomegranate extracellular vesicles (EVs) were isolated using size exclusion chromatography and characterised using nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). SW1353 cells (technical triplicates; n=3) were expanded to 70% confluency. Cells were then serum starved, and treated with pomegranate extracellular vesicles at different concentrations (2.5µg, 5µg, and 7.5µg) prior to or post cytokine (IL-1β and TNF-α 10ng/ml) stimulation. Cells were harvested and targeted gene expression analysis undertaken using reverse transcription quantitative polymerase chain reaction on a panel of chondrogenic, inflammatory and hypertrophic protein coding genes. Statistical analysis was undertaken in GraphPad prism, using analysis of variance.

Results: Prior treatment of 7.5µg of pomegranate EVs significantly reduced the expression ($p<0.0001$) of interleukin 6 (IL-6) and matrix metalloproteinase 3 (MMP3) following cytokine stimulation. Pomegranate EV treatment at all concentrations post cytokine stimulation significantly reduced ($p<0.0001$) IL-6 expression, returning to baseline control expression levels. Post cytokine stimulation treatment of pomegranate EVs resulted in a significant ($p<0.0001$) decrease in MMP3 expression at specific concentrations. Prior-treatment with pomegranate EVs at 5µg and 7.5µg decreased SRY-box transcription factor 9 (SOX9), All post cytokine treatment concentrations significantly ($p<0.0001$) decreased SOX9 expression.

Conclusion: This study identified the potential for pomegranate EVs to reduce osteoarthritis associated inflammation *in vitro.* This has potential implications for their use to promote joint health across human and veterinary medical applications.

Funding information: This work was funded by the Institute of Life Course and Medical Sciences, University of Liverpool.



P-T-8

Cytokine-primed umbilical cord mesenchymal stem cells enhanced therapeutic effects of extracellular vesicles on osteoarthritic chondrocytes

Thu Huyen Nguyen[1], Huy Hoang Dao [1,2], Chau Minh Duong[1,3], Xuan-Hung Nguyen[1,4], Diem Huong Hoang[1], Xuan-Hai Do[5], Trung Quang Truong[6], Tu Dac Nguyen[1], Liem Thanh Nguyen[4,7], and Uyen Thi Trang Than[1]

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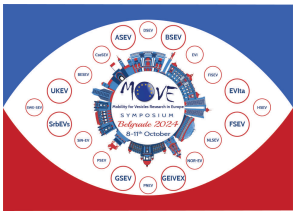
Introduction: In recent years, extracellular vesicles (EVs) secreted by mesenchymal stem cells (MSCs) have emerged as a potential cell-free therapy for osteoarthritis (OA). This study investigates the therapeutic effects of EVs released by cytokine-primed umbilical cord-derived MSCs (UCMSCs) on osteoarthritic chondrocyte physiology.

Methods: UCMSCs were pre-conditioned with anti-inflammatory transforming growth factor beta (TGFβ), interferon-alpha (IFNα), or inflammatory tumor necrosis factor-alpha (TNFα), all linked to OA pathogenesis. We then assessed the therapeutic efficacy of their derived EVs on chondrocytes in vitro, focusing on proliferation, migration, and marker expression.

Results: Priming UCMSCs individually with cytokines significantly reduced the sorting of miR-181b-3p but not miR-320a-3p; two negative regulators of chondrocyte regeneration, into EVs. However, the EV treatment did not show any significant effect on chondrocyte proliferation. Meanwhile, EVs from both non-priming and cytokine-primed UCMSCs induced migration at later time points of measurement. Moreover, TGFβ-primed UCMSCs secreted EVs that could upregulate the expression of chondrogenesis markers (COL2 and ACAN) and downregulate fibrotic markers (COL1 and RUNX2) in chondrocytes.

Conclusion: Priming UCMSCs with cytokines can deliver selective therapeutic effects of EV treatment in OA and chondrocyte-related disorders.

Funding information: Vietnam Ministry of Health, 2575/QĐ-BYT (20/06/2019) & Vinmec Joint Stock Company (ISC.18.07)



P-T-9

Advanced Technologies for Regenerative Medicine: From Tissue Engineering to Extracellular Vesicles

Moran Yadid

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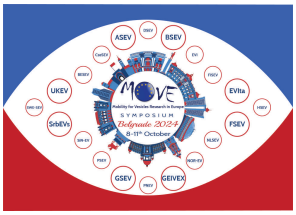
Introduction: Regenerative medicine offers different approaches for treating damaged organs. Specifically, in ischemic heart disease (IHD), while current treatments only help with managing symptoms of the disease, regenerative medicine approaches aim to minimize infarct size and even regenerate the failing heart. Cellular and acellular therapies present such regenerative medicine approaches. Extracellular vesicles (EVs) derived from various stem cell sources can induce cardioprotective effects during ischemia-reperfusion injury (IRI) and reduce infarct size via their anti-apoptotic, pro-angiogenic, miRNA cargo. However, endothelial cells, which are naturally abundant in the heart and are sensitive to oxygen deprivation, may hold therapeutic potential toward ischemic myocardial injury via EV-mediated signaling. We explore acellular approaches for organ regeneration as routes to induce self-repair.

Methods: We developed advanced in vitro assays, termed 'heart-on-a-chip' (hoc), to model and assess the function of the human myocardium. The hoc was used to simulate IRI, to assess the protective effects of endothelial EVs (EEVs) on human myocardium, and to screen proteome data to shed light on the possible mechanisms of action.

Results: EEVs alleviated cardiac cell death, preserved contractile capacity, and increased the respiratory capacity of cardiomyocytes, in an uptake- and dose-dependent manner. Moreover, EEVs partially restored protein profiles of the injured myocytes towards healthy profiles, while presenting enrichment of various metabolic processes related to cellular respiration and modulation of the cellular response to stress. EEV protein cargo was characterized and lead cardioactive proteins were identified. Specifically, EEVs contained proteins associated with cellular metabolism, redox state, and calcium handling, among other processes, corresponding with the modifications EEVs induced to the myocytes' proteome

Conclusion: The combination of the functional data derived from the hHOC system, and the deep proteome analyses suggest that EEVs target multiple pathophysiological routes related to cellular stress, as well as metabolic routs, and thereby induce an innate protective mechanism.

Funding information: This project is funded by the Israeli Science Foundation, grant number 1802/23, and supported by the Zuckerman STEM Leadership Program.



P-T-10

The role of extracellular vesicles (EVs) in chronic graft vs. host disease, and the potential function of placental cell-derived EVs as a therapeutic tool

Mor Zavaro [1, 2], Ayelet Dangot [1,2], Tali Hana Bar-Lev [1], Odelia Amit [3], Irit Avivi [2,4], Ron Ram [2, 3], **Anat Aharon** [1, 2]

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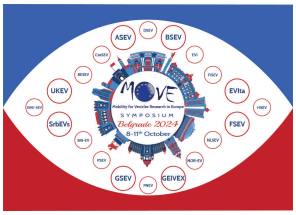
Introduction: Graft-versus-host disease (GVHD) is the most serious complication following allogeneic hematopoietic cell transplant (HCT). Chronic GVHD (cGVHD) is a multi-organ, immune-mediated, life- threatening disorder, occurs in 30–70% of patients. Mesenchymal stromal cells (MSCs) derived from different tissues include placenta or their derived extracellular vesicles (EVs) may use for treatment of cGVHD. The current study aimed to characterized cGVHD patients' EVs compared to those of heathy controls and analyzed the anti-inflammation and anti fibrosis potential of placental EVs.

Methods: EVs obtained from fourteen cGVHD patients were characterized compared to EVs obtained from match healthy controls using Nano-tracing analysis, western blot, flow cytometry and RT-PCR. In addition, the anti-inflammatory and anti-fibrotic effects of placental EVs on Human keratinocyte cell line (HACAT) and Normal Human Dermal Fibroblasts (NHDF) were explored.

Results: EVs obtained from cGVHD patients, compared to healthy subjects EVs, contained higher levels of fibrosis-related proteins, TGFβ and α-smooth muscle actin (αSMA), and display changes in miRNAs related to TGF-beta signaling pathway. The exposure of NHDF cells to the patients' EVs increased the NHDF cells' TGFβ and αSMA expressions. Placental EVs derived from placental-expanded cells (PLX) (Pluri Inc.) and human villous trophoblast (HVT) cells expressing the mesenchymal markers CD29, CD73, and CD105, penetrated into both the epidermal keratinocytes (HACATs) and NHDF cells. Stimulation of the HACAT cells with cytokine TNFα/INFγ (0.01-0.1 ng/µL) reduced cell proliferation, while the addition of placental EVs attenuated this effect, increasing and normalizing cell proliferation. The treatment of NHDF cells with a combination of TGFβ and placental EVs reduced the stimulatory effects of TGFβ on αSMA production by over 40% ($p = 0.0286$).

Conclusion: EVs from patients with cGVHD can serve as a biomarker for the cGVHD state. Placental EVs may be used to regulate dermal inflammation and fibrosis, warranting further investigation of their therapeutic potential.

Funding information: Ministry of Health, Estates Fund, grant number 20200575



P-T-11

Neuropeptide receptor Y as potential target for specific cancer treatment

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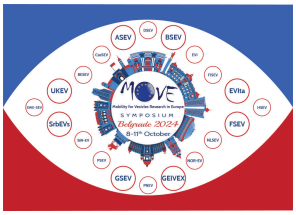
Introduction: Neuropeptide Y (NPY) and its analogs show promise for cancer therapy by targeting NPY receptors (NPYRs) overexpressed in various tumors. By attaching NPY analogs to erythrocyte membrane vesicles (EMVs), we aim to enhance targeted drug delivery to cancer cells and minimize damage to healthy tissues. Peptide stapling techniques are used to improve the stability and bioactive conformation of NPY analogs. This study highlights the potential of NPY-EMV conjugates for selective and effective cancer treatment.

Methods: Erythrocytes were washed with PBS and treated with a hypotonic solution to release intracellular components. Erythrocyte membrane ghosts were sonicated and extruded to obtain EMVs. DLS and NTA measured EMV size and distribution, while zeta potential measurements assessed surface charge. In silico techniques designed high-affinity NPY analogs. SPPS synthesized the peptides, which were purified using HPLC. Peptides were then conjugated to EMVs using click chemistry or maleimide-thiol coupling.

Results: EMVs were examined for size and morphology using TEM, cryo-TEM, freeze-fracture EM, DLS, and zeta potential measurements. The majority of EMVs (78.7%) fell within the 150-200 nm size range, with a mean zeta potential of -14.59 mV. SDS-PAGE effectively detected small peptides (1-2 kDa). SL15 and SL23 peptides were visible in monomeric form around 2 kDa. The conjugation of peptides to EMVs showed promising initial results, although the process requires further optimization.

Conclusion: We successfully prepared and characterized EMVs, demonstrating their suitability as carriers for targeted drug delivery. The use of SDS-PAGE confirmed the presence of small peptides, and we developed an effective conjugation protocol. The ongoing characterization and optimization of NPY-EMV conjugates indicate a strong potential for selective and effective cancer therapy. This approach could significantly enhance the precision and efficacy of cancer treatments, reducing side effects by targeting only cancer cells.

Funding information: ARIS, P2-0084, J4-50150, PR-12900



P-T-12

Advanced 3D In Vitro Cultures as Novel Models for Screening Extracellular Vesicle-based Therapies

Gabriele Vella[1], Martin P. Barr[2], Anne-Marie Baird[3], Adriele Prina-Mello[1], Dania Movia[4,5]

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Introduction: Lung cancer remains a leading cause of cancer-related deaths worldwide, necessitating innovative research models to advance therapeutic strategies. This study focuses on establishing 3D multilayered cultures grown at the air-liquid interface (ALI-MCCs) as models for evaluating Extracellular Vesicle (EV)-based therapies against lung cancer.

Methods: ALI-MCCs were formed using A549 cells (human lung adenocarcinoma cell line) either alone (monocultures) or in co-culture with MRC-5 cells (healthy human lung fibroblast cell line). Viability and culture morphology/thickness were assessed over 14 days using live/dead assay and confocal imaging, providing a baseline for subsequent experiments. In parallel, the penetration of CD63-GFP-tagged EVs was evaluated to investigate the models' suitability for EV research. Furthermore, ALI-MCCs were exposed to in-house isolated EVs to obtain preliminary cytotoxicity responses. These EVs were isolated through size exclusion chromatography (SEC) from A549 conditioned cell culture media (CCM), characterized for particle concentration and protein impurities using nanoparticle tracking analysis (NTA) and BCA assay, respectively, and tested for their cytotoxicity (lactate dehydrogenase (LDH) assay).

Results: At 14 days, monocultures showed sustained viability and thickness of around 15 µm. Conversely, co-cultures exhibited declined viability after 3 days and reduced thickness of around 9.5 µm at 14 days. Confocal microscopy demonstrated penetration of CD63-GFP-tagged EVs into ALI MCCs. We identified SEC fractions of the CCM with high A549-derived EV count and low protein impurities, while no significant cytotoxicity was detectable in ALI-MCCs exposed to such SEC fractions.

Conclusions: Our data support the potential of ALI-MCCs as preclinical testing platforms for EV-based therapies, allowing for EV penetration and toxicity screening. These findings indicate mono- and co-cultures grown for 14 and 3 days, respectively, as the most appropriate *in vitro* testing platforms. Future work will use ALI-MCCs to test genetically manipulated in-house A549-isolated EVs, laying a foundation for novel-engineered EV-based lung cancer therapies.

Funding information: EC-funded Horizon Europe INSPIRE project (Grant agreement ID: 101057777); Horizon Europe (EXPERT) (Grant agreement ID: 8258280).

Influence of Trophoblast-Derived Extracellular Vesicles on Atopic Dermatitis-like Keratinocyte Phenotype

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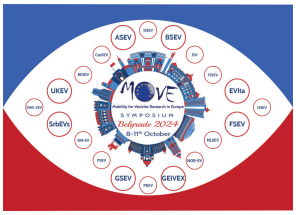
Introduction: Atopic dermatitis (AD) is a chronic inflammatory skin condition characterized by intense itching, erythema, and eczema. The pathogenesis of AD involves a complex interplay between genetic, environmental, and immunological factors, leading to skin barrier dysfunction and immune dysregulation. Recent studies suggest that extracellular vesicles derived from placental trophoblast cells (TEVs), may have immunomodulatory properties. This study investigates the effects of TEVs on keratinocytes treated with a cocktail of pro-inflammatory cytokines (TNF-α, IFN-γ, and IL-4) implicated in the pathogenesis of AD.

Methodology: TEVs were isolated from culture medium conditioned by immortalized first-trimester extravillous trophoblast HTR-8/SVneo cells and cultured for 24 h with immortalized human keratinocyte HaCaT cells. TEV-pretreated HaCaT cells were then exposed for 5 h to AD-like cytokine environment. Finally, cell viability (MTT assay), reactive oxygen species (ROS) production , pro-inflammatory cytokines' and filaggrin expression levels (cell-based ELISA assay) were determined.

Results: The MTT assay indicated that both AD-like cytokine coctail (TNF-α, IFN-γ, and IL-4) and TEVs significantly stimulated the metabolic activity of HaCat cells and, consistently, production of ROS. Additionally, protein expression of IL-6, IL-8, and IL-1β (cytokines released by keratinocytes in AD) and filaggrin (a key molecule implicated in skin barrier function and pathogenesis of AD) also altered upon both AD-like cytokines' and TEV stimulation.

Conclusion: The findings indicate that TEVs may not be suitable for mitigating AD-like phenotype in keratinocytes and potentially other inflammatory skin diseases. On the other hand, the results suggest TEVs' potential regenerative properties, which could be harnessed for therapeutic purposes in wound healing or aging-related skin conditions. Future studies should focus on elucidating the range of immunomodulatory and/or regenerative effects of TEVs and their efficacy in models of skin disorders.

Funding: Ministry of Science, Technological Development and Innovation, Republic of Serbia (Grant No. 451-03-66/2024-03/200019).



P-T-14

Extracellular Vesicles from Extravillous Trophoblast Modulate D-galactosis-Induced Keratinocyte Senescence

Milica Jovanović-Krivokuća[1], Aleksandra Vilotić[1], Andrea Pirković[1], Janko Legner[1], Mirjana Nacka-Aleksić[1]

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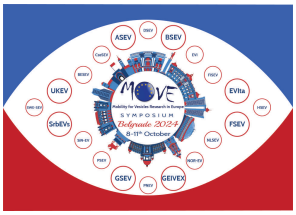
Introduction: Placenta-based skincare products have gained popularity and are advertised for their potential benefits in modern skincare. Previous work has shown that placenta-derived products can effectively reduce skin senescence, but few studies have specifically focused on the role of trophoblast-derived extracellular vesicles (TEVs) in that context.

Methodology: In this study, extracellular vesicles derived from immortalized first-trimester extravillous trophoblast HTR-8/SVneo cells were isolated from conditioned cell culture medium, and their ability to modulate D-galactose-induced senescence of HaCaT keratinocytes was assessed. Key analyses included keratinocyte proliferation and the expression of mammalian target of rapamycin (mTOR), β1 integrin subunit and involucrin.

Results: Keratinocytes treated with D-galactose exhibited upregulated mTOR, marker of senescence, indicating a shift towards a senescent phenotype. Additionally, the effects of D-galactose treatment on the β1 integrin subunit, which plays a vital role in cell adhesion and signal transduction, and involucrin, a marker of keratinocyte differentiation, further affirmed the impact on cellular senescence and differentiation pathways. Pretreatment of keratinocytes with TEVs revealed their modulating effect on senescence-associated markers.

Conclusions: Our results suggest that TEVs may possess anti-aging properties, potentially through the delivery of bioactive molecules that counteract the effects of D-galactose. These findings open avenues for further research into putative TEV regenerative action, which could be harnessed for therapeutic purposes in wound healing and senescence-related skin conditions.

Funding: Ministry of Science, Technological Development and Innovation, Republic of Serbia (Grant No. 451-03-66/2024-03/200019).



P-T-15

The Effects of Extravillous Trophoblast Cell-Derived Extracellular Vesicles on Cell Viability and Cisplatin Response in Choriocarcinoma Cells

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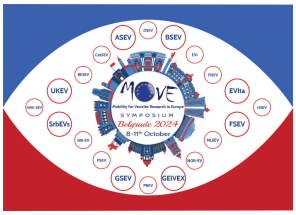
Introduction: Extracellular vesicles (EVs) released from the placenta are increasingly being explored as a new approach to cancer treatment. They are being examined for their role in regulating proliferation and invasion, although the exact mechanism of modulating cancer cell function remains largely unknown. The study aimed to evaluate if trophoblast cells' EVs (TC-EVs) could influence viability and metabolic function of choriocarcinoma cells (JAR) and whether they could sensitize cells *in vitro* to the effects of cisplatin (CisP) as a known anti-cancer therapeutic.

Methods: TC-EVs were isolated from conditioned media of healthy human extravillous trophoblast cells HTR-8/SVneo, by ultracentrifugation. JAR choriocarcinoma cells were treated with 1 µM CisP and/or TC-EVs (50 µg/mL protein) for 24h. Cell viability and levels of reactive oxygen species (ROS) were determined by MTT and H2DCFDA assays, respectively. DNA damage was evaluated by detecting γ-H2AX foci using immunofluorescent staining.

Results: Significant inhibition of JAR cells' viability was observed in monotreatment with TC-EVs as well as with CisP, while the most pronounced cytotoxicity was achieved in co-treatment. ROS production was significantly increased in cells after 24h co-treatment with TC-EVs and CisP compared to control cells, while there were no significant changes in the monotreated cells. In terms of DNA damage, TC-EVs, CisP and combination of TC-EVs with CisP produced 40.56%, 70.15% and 72.30% cells positive for γ-H2AX foci in nuclei, respectively.

Conclusion: We have demonstrated that TC-EVs used as concomitant treatment with CisP were able to influence the metabolic activity of choriocarcinoma cells. The results showed a greater response to cytotoxic drug treatment and increased production of ROS, while DNA damage levels were similar. Although TC-EVs may represent a mean to sensitize tumor cells to chemotherapy, further work is required to establish whether these effects could be translated in the *in vivo* setting.

Funding information: Study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Agreement no. 451-03-66/2024-03/200019)



P-T-16

Collagen Hydrogel Embedded SHED-EVs for Enhanced Osteogenesis

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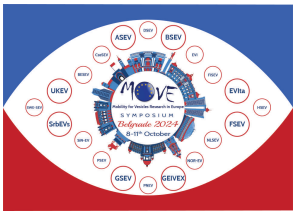
Introduction: Mesenchymal stem/stromal cells (MSCs) from the dental pulp of human exfoliated deciduous teeth (SHED cells) have shown promising results in bone tissue regeneration. The application of SHED-derived extracellular vesicles (EVs) to bone defects can be achieved using biomaterial scaffolds. Collagen scaffolds, which are natural polymers with slow degradation times, are particularly suitable for EVs entrapment. This study aimed to examine the release rate of embedded SHED-EVs from collagen scaffolds and to evaluate their osteogenic capacity when combined with collagen and gradually released.

Methods: SHED-EVs were isolated using differential ultracentrifugation and characterized using nanotracking particle analysis (NTA), Western Blot (WB), and scanning electron microscopy (SEM). The SHED-EVs were labeled with PKH67 fluorescent lipophilic dye and embedded in a collagen hydrogel matrix (0.3% collagen in PBS). The cumulative release of the fluorescently labeled EVs was monitored for 35 days using NTA. The osteogenic potential of the collagen scaffolds with EVs was assessed by analyzing the relative expression of key osteogenic genes in treated SHED cells using RT-PCR.

Results: The isolated SHED-EVs exhibited a uniform size distribution, as confirmed by NTA and SEM analyses, and were positive for CD63 as shown by WB analysis. The release of EVs from the collagen matrix was gradual, with half of the entrapped EVs being released within the first 10 days and an additional 10% released over the subsequent 10 days, followed by a plateau phase. The SHED-EVs embedded within the collagen hydrogel influenced the expression of osteogenic genes.

Conclusion: The use of a collagen matrix for embedding and gradually releasing SHED-EVs provides a promising strategy for enhancing the osteogenic potential of these vesicles in regenerative therapies.

Funding information: This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract No. 451-03-65/2024-03/200135, 451-03-66/2024-03/200287, and 415-03-66/2024-03/200019)



P-T-17

Extracellular Vesicles as Therapeutic Agents for Inflammation Resolution and Tissue Regeneration

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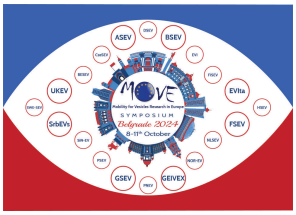
Introduction: Apoptosis plays central role in the resolution of inflammation. Recent evidence suggests that extracellular vesicles (EVs), released from dying immune cells, play a critical role in communicating with other immune cells to promote tissue regeneration, but the exact molecular mechanisms governing this phase remain unclear. This study aims to explore the biomolecular composition of EVs to identify key biomolecular components capable of immunomodulation and translate those findings towards informing new therapeutic strategies to support tissue regeneration.

Methods: EVs were isolated from apoptotic primary human T cells and characterized using various analytical techniques, including TRPS, flow cytometry, proteomics, and lipid-metabolomics to define their biophysical and biomolecular profile. Enzyme activity, lipid mediator profiles, and macrophage polarization were assessed using enzymatic assays and flow cytometry. Functional assays included detailed macrophage phenotyping, both in vitro and in in vivo mouse models of inflammation.

Results: Apoptotic human T cells actively release high levels of EVs which carry a specialized pro-resolving mediators of inflammation, including active lipoxygenase enzymes responsible for their synthesis, with an overall balance strongly supporting pro-resolving and anti-inflammatory phenotypes of secreted EVs. 'Active' EVs can transfer active enzymes to host cells where enzymes retain their metabolic activity. Similarly, apoptotic EVs transfer enzymatic activity to macrophages, polarizing them towards a pro-repair M2 phenotype, as supported by surface phenotyping and metabolome profiling. In vivo, mice treated with EVs exhibited increased recruitment of homeostatic macrophages and reduced inflammatory macrophages and eosinophils in inflammatory models.

Conclusion: This study highlights the role of EVs as an active metabolic compartment, capable of modulating immune responses to support tissue repair. Future research now focuses on engineering lipid nanoparticles (LNPs) loaded with mRNA to specifically promote repair of non-healing wounds. These findings underscore the potential of EVs and mRNA-loaded LNPs as therapeutic tools in regenerative medicine.

Funding information: BBSRC responsive mode (BB/M006298/1, BB/S00324X/1); BBSRC ALERT23 (APP25332); BBSRC Super Follow-on (APP3887); BBSRC ICURe and ICURe Follow-on; Innovate UK (10088667); Dunhill Medical Trust (AIS2110\33).



The Mechanisms and Effects of Autologous Extracellular Vesicles on Joints and the Nervous System

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Introduction: Autologous Conditioned Serum (ACS) is a blood preparation from extended coagulation (EC) of whole blood at physiologic temperature in suitable containers. ACS is known for its content of cytokines (e.g. IL-1Ra), growth factors (e.g. IGF1), lipid mediators (SPM) and as a source of heterogeneous Extracellular Vesicles (EV). Here, the term "Extracellular Vesicles" is used synonymously to "Exosomes".

ACS and ACS-EV are readily obtainable, safe injections solutions. ACS is successfully in clinical routine use for chronic musculoskeletal and neuroskeletal pain conditions since 1998. Ultracentrifuge generated ACS-EV concentrates are in a successful clinical use since 2010 for autoimmune-/chronic pain conditions.

We recently learned that ACS-EVs are crucial in resolution of a Paclitaxel chemotherapy-induced peripheral neuropathy model.

We provide a preliminary mode of action for the clinical efficacy and safety of ACS in chronic nerve pain, suggesting a substantial role for ACS contained EVs. This approach is able to de-chronify patients' pain after long suffering since clinical studies with periradicular/epidural ACS injections in patients with chronic radicular pain were clinically significant.

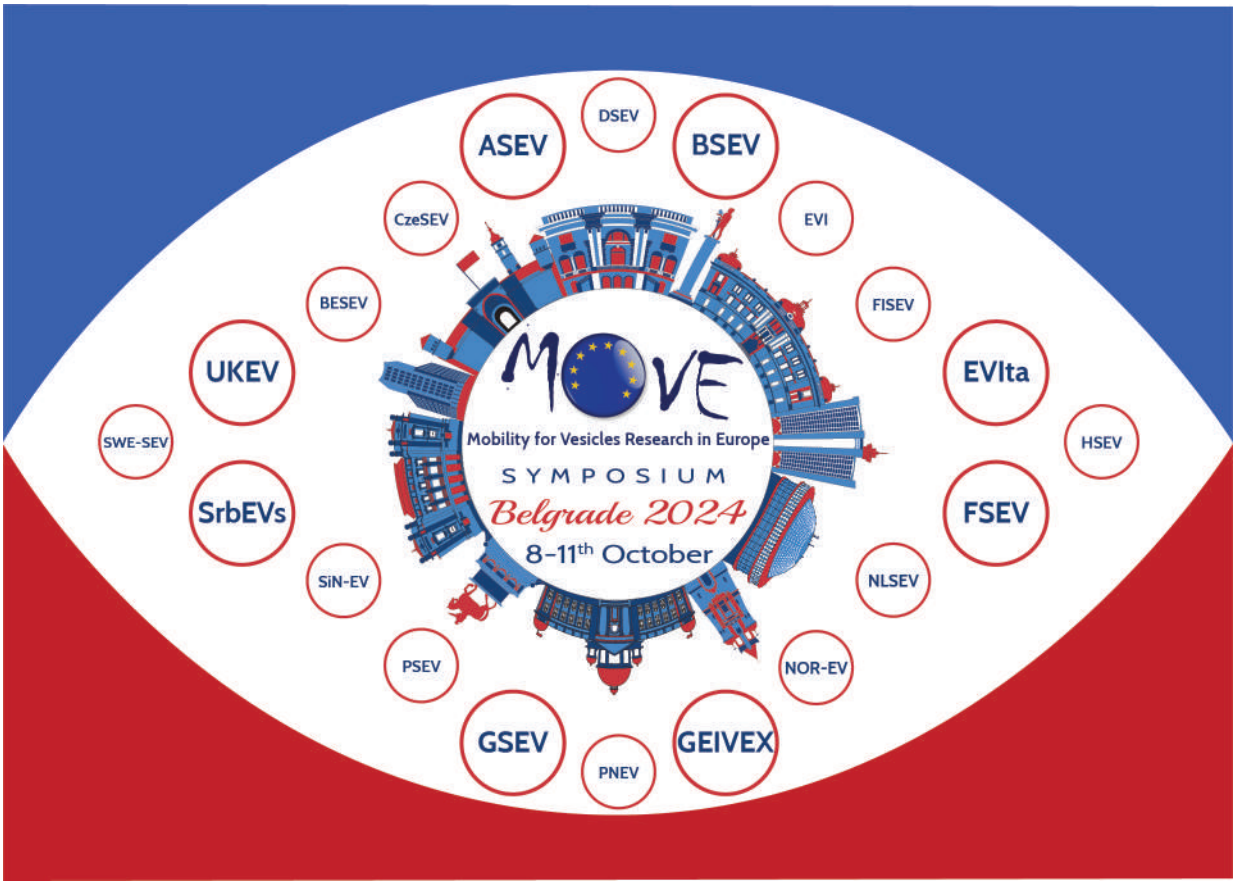
Methods: Main focus will be the characterization of the action of ACS contained EVs. In this project the characteristics of ACS in terms of components and mechanism will be advanced in that well defined standard commercial cell culture systems will be employed to analyze biochemical and culture technical models to define possible modes of action. It is planned to establish in vitro models with nerve cells, muscle cells, cartilage and skin cells. In an open clinical setting patients with neurological deficits are treated with ACS and ACS-EVs.

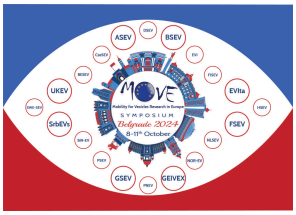
Results: The clinical progress are routinely monitored. Quantity and quality of ACS-EVs are routinely correlated to clinical progress.

Conclusion: We expect that this model will help optimize dosage and ACS production procedure with regards to maximum clinical effect.

Manufacturing of native and engineered EV products

- oral presentations -





O-M-1

Targeting the tumor microenvironment: a click chemistry-based surface-functionalization method and a therapeutic-loading strategy for artificially produced erythrocyte-derived extracellular vesicles

Maria Chiara Ciferri[1], Ulla Impola[2], Petra Ilvonen [2,3], Maria Elisabetta Palamà[4], Silvia Scaglione[4], Ambra Costa[5], Enrico Millo[1], Katia Cortese[1],Saara Laitinen[2], Roberta Tasso[1,5]

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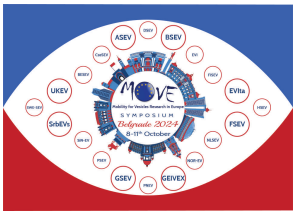
Introduction: Because of their ability to transport functional cargoes and the possibility to modify their surface to incorporate ligands, EVs have been recently considered valuable delivery vehicles, especially for targeted therapy. Particularly, erythrocytes-derived nanoparticles, which are beneficial in yield and bioavailability have shown promising potential as efficient targeted drug delivery systems.

Methods: In this study, we propose a two-step strategy for Nanoerythrosome (NanoEs, artificial erythrocyte-derived vesicles) engineering: (i) surface functionalization by a copper-free click chemistry approach with a fluorescent peptide able to target an oncofetal variant of the fibronectin (Extra Domani B (EDB)-fibronectin), normally absent in healthy adult tissues but upregulated in various pathological conditions, including cancer; (ii) loading with Paclitaxel (PTX) by sonication. Imaging flow cytometry was employed to evaluate the achievement of membrane functionalization. Then the internalization of anti-EDB-NanoEs vs naïve NanoEs by EDB-positive and negative cells was evaluated through an organ-on-chip technology in a millifluidic environment.

Results: Click chemistry turned out to be a successful approach for NanoE surface engineering producing about 50% of Click-NanoEs and we observed a higher internalization of functionalized NanoEs compared to the naïve ones by EDB-expressing cells. The fluorescent anti-EDB peptide was shown to significantly recognize EDB-fibronectin both in its free and NanoE-conjugated form thus demonstrating that the conjugation by click chemistry doesn't affect the targeting ability. Moreover, the loading efficiency of PTX was analyzed by HPLC-MS revealing a 3% drug encapsulation, and at the same time, the cytotoxic effect of PTX-NanoEs, in comparison to naïve (empty) NanoEs, was evaluated by MTT assay. PTX-loaded NanoEs showed a significant reduction of viability indicating the achievement of the therapeutic effect.

Conclusion: These data suggest that click chemistry for NanoE surface functionalization, and sonication for PTX encapsulation, represent two valid and powerful strategies to produce engineered NanoEs directed toward the tumor microenvironment.

Funding information: "Programmi Operativi Nazionali" (PON), European Community,grant number: ARS01_00693; PNRR, National Center for Gene Therapy and Drugs based on RNA technology, CN3_Spoke 8 to B.B.



Gla-Rich Protein-loaded EVs for cardiovascular research: Production via baculovirus-insect and human cell systems

Joana Carreira[1], Carla Viegas[1,2], Arnaud Poterszman[3], Simon Pichard[3], Charlotte Schnitzler [3], Teresa Maia[5], Tiago Faria[6,7], Sara Nascimento[6,7], Cristina Peixoto[6,7], Anjos L. Macedo[8], António Matos[9], Dina Simes[1,2]

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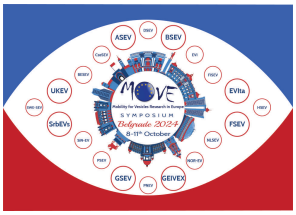
Introduction: Gla-rich protein (GRP) is a vitamin K-dependent protein with anti-inflammatory and anti-calcification activity, dependent on its gamma-carboxylation for maximum functionality. Reduced GRP levels are associated with renal dysfunction, vascular calcification, and systemic inflammation, with high therapeutic potential for pathological calcification and chronic inflammatory diseases such as cardiovascular disease (CVD). This study aims to establish a cell system able to produce gamma-carboxylated GRP (cGRP) loaded into extracellular vesicles (EVs) as new nanoformulations to increase GRP efficiency/bioavailability with potential application as new treatments for CVD.

Methods: To produce cGRP-EVs we engineered a baculovirus-insect cell system (BEVS) co-expressing GRP and essential enzymes for gamma-carboxylation (gamma-glutamyl carboxylase (GGCX); vitamin K epoxide reductase (VKOR), and protein processing (Furin), followed by the translation to a more therapeutic relevant system, using human Expi293F cells transiently overexpressing GRP. All cells were supplemented with vitamin K. EVs were isolated from the culture media of overexpressing cells by ultracentrifugation at 30,000g (30K) and 100,000g (100K), and characterized by nanoparticle tracking analysis, transmission electron microscopy, and proteomics. GRP content and gamma-carboxylation status in EVs were assessed using ELISA and Western blot.

Results: EVs isolated from the BEVS co-expression system are loaded with cGRP, GGCX, VKOR and Furin, although differentially associated with specific EVs populations. Expi293F derived cGRP-EVs were obtained only with GRP overexpression, although at lower levels than in BEVS. In the BEVS GRP was predominantly found in 30K EVs, while in the human cell system GRP was present in both 30K and 100K EVs. In both systems, vitamin K supplementation was essential for GRP gamma-carboxylation and resulted in increased GRP loading into EVs.

Conclusion: These results show that cGRP can be loaded into EVs from bioengineered cells and highlight the potential use of Expi293F cells for a scalable and efficient platform to produce cGRP-EVs to develop novel EV-based therapies.

Funding information: This research was funded by Portuguese National Funds from FCT—Foundation for Science and Technology, through PhD grant 2022.12777.BD and transitional provision DL57/2016/CP1361/CT0006, and projects EXPL/BTM-TEC/0990/2021, UIDB/04326/2020 (DOI:10.54499/UIDB/04326/2020), UIDP/04326/2020 (DOI:10.54499/UIDP/04326/2020) and LA/P/0101/2020 (DOI:10.54499/LA/P/0101/2020); by transnational access within Instruct ERIC/EU/project PID7060 and the European Proteomics Infrastructure Consortium – Providing Access (EPIC-XS2019 project #7); by AAC nº 41/ALG/2020—Project nº 072583—NUTRISAFE; and by the Portuguese Society of Nephrology (SPN) through projects funding 2019.



O-M-3

Comprehensive Classification of Antimicrobial and Cell-Penetrating Peptides in Vesicle Interactions

Tasvilla Sonallya[1], Imola Cs. Szigyártó[1], Tünde Juhász[1], Kinga Ilyes[1], Priyanka Singh[1], Delaram Khamari[2], Edit I. Buzas[2], Zoltán Varga[1], Tamás Beke-Somfai[1]

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Host defense peptides (HDPs) are promising biomaterials with potential applications in antimicrobial and anticancer therapies due to their ability to disrupt or lyse cell membranes. These peptides interact with membranes via mechanisms like the carpet, toroidal pore, and barrel stave models. Additionally, cell-penetrating peptides are key in delivering cargo and enhancing the uptake of small molecules and nanoparticles. While extensive research has been conducted on the interactions of these peptides with model membranes, their interactions with extracellular vesicles (EVs) remain underexplored. Understanding the interplay between EVs and HDPs could be significant, from their cooperative presence at infection sites to their potential roles in EV cargo loading.

The study selected a variety of HDPs, including Indolicidin, Aurein 1.2., Dermcidin (DCD-1), DHVAR 4, Bactenecin, Protegrin-1, Transportan, Buforin IIb, KLA, Temporin-La, LL37, FK16, Mellitin, Polybia MPI, Histatin 5, PNC-28, CM15, Buforin II, Gramicidin, Arg-1, Macropin I, Lasioglossin LL-III, R8, and Penetratin. Techniques such as polarised light spectroscopy (linear dichroism), flow cytometry, nanoparticle tracking analysis, zeta potential measurements, and freeze-fracture TEM were employed to study these interactions.

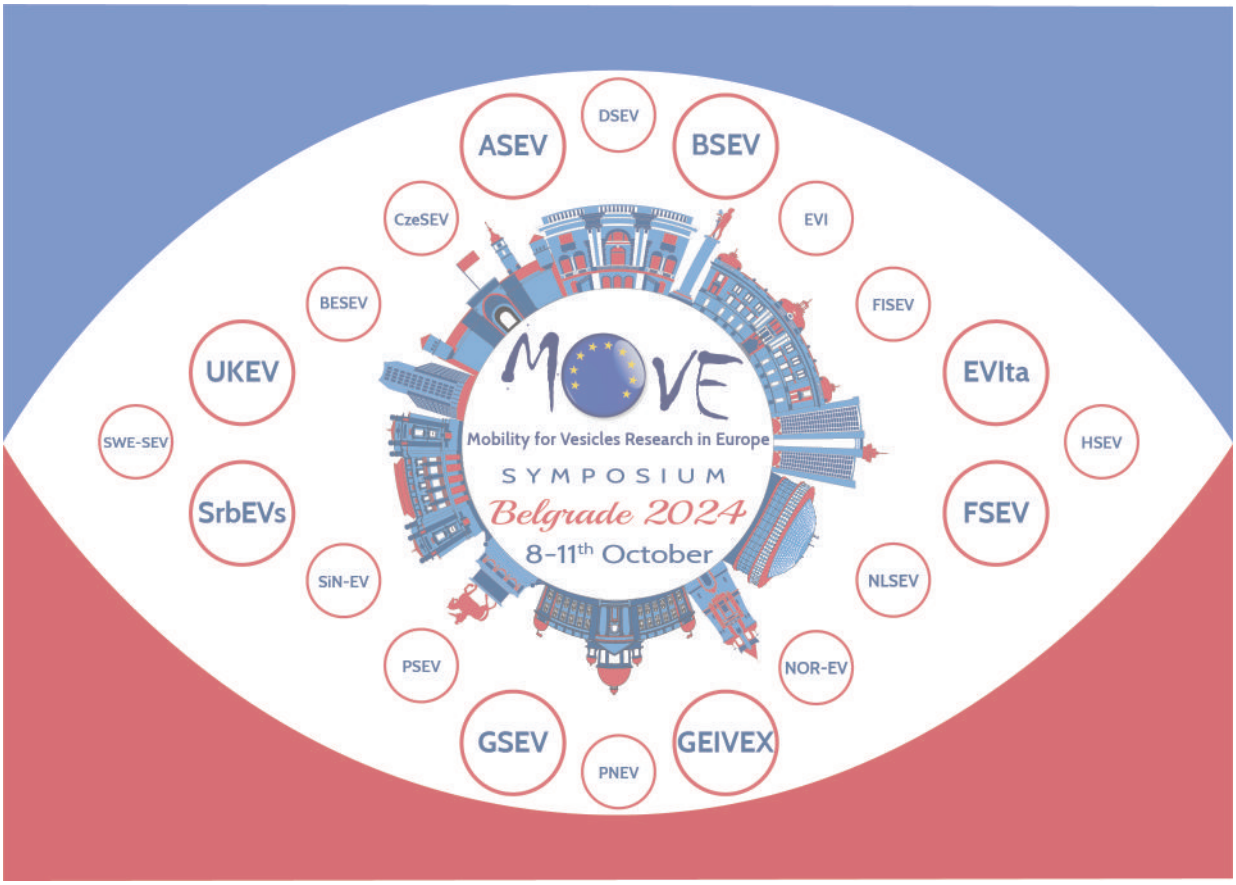
Biophysical analyses revealed that different HDPs employ distinct mechanisms, including vesicle penetration, lytic actions, and protein corona removal. The study categorizes HDPs into eight groups based on these mechanisms. For instance, LL37 and Lassioglossin are efficient at removing surface proteins, Melittin strongly disrupts membranes, and Octaarginine and Penetratin exhibit less disruptive effects on the original vesicle composition.

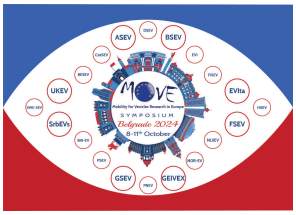
These findings offer an extensive view of the surface interactions between HDPs and EVs, providing insights that could be instrumental in customizing the surfaces of EVs with short HDPs for bioengineering purposes.

Funding: This research was funded by the Ministry of Innovation and Technology of Hungary through the NKFI Fund under the TKP2021-EGA-31 and 2020-1-1-2-PIACI-KFI_2020-00021 funding schemes, with additional support from ELKH, grant no. SA-87/2021.

Manufacturing of native and engineered EV products

- poster presentations -





P-M-1

Comparison of the effectiveness of anion exchange chromatography, ultracentrifugation, tangential flow filtration and size exclusion chromatography in EV isolation

Reetta Pusa[1,2], Heikki Saari[1], Ulla Impola[1], Milla Lampinen[1], Saara Laitinen[1]

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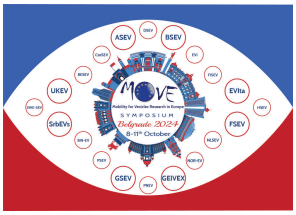
Introduction: Finding an effective method for extracellular vesicle (EV) isolation from complex biological fluids, such as blood, has been a long running challenge in the EV field. The current gold standards for EV isolation – size exclusion chromatography (SEC) and ultracentrifugation (UC) – both come with their own challenges. As many current isolation protocols are based on size we have been focusing on finding new isolation methods that separate EVs based on other characteristics – such as surface charge. In this study we were comparing the new player on the field – ion exchange chromatography (IEC) to these widely used methods.

Methods: We used clinical-grade platelet concentrates of four blood-group matched donors. Cells were removed by centrifugation and the supernatant was split into four different isolation methods: IEC, UC, tangential flow filtration (TFF) and TFF+SEC. The final EV product was analysed using nanoparticle tracking analysis, Amnis ImageStream flow cytometer, ELISA, western blot and electron microscopy.

Results: In all four isolation methods EVs were highly enriched from cell-free filtrate. UC and TFF+SEC giving the highest purity (less ApoB in final product). Different methods had specific limitations and advantages. SEC diluted the sample and no large volumes were able to be used. From all four methods IEC was easiest to up-scale to large volumes producing concentrated sample volumes.

Conclusion: Isolation and purification of EVs is always a competition between conflicting interest of yield and purity. A more pure sample equals less yield. Another challenge is the up-scaling to manufacturing. Our results show that compared to purity of UC isolated EVs and easiness of TFF technique, IEC gave compromise of these two: easy to use technique with results showing large yields with satisfying purity. IEC was also gentle and made no selection based on size that is important for research use as it does not lead to too early selection of EV populations produced by cells.

Funding information: Finnish Cultural Foundation, 00240980



P-M-2

Membrane vesicles of Lactiplantibacillus plantarum as nanocarriers for synergistic antimicrobial combinations to treat skin wound infections

Liubov Shishaeva, Katharina Maniura, Qun Ren

Laboratory for Biointerfaces, Empa, Swiss Federal Laboratories for Materials Science and Technology, St. Gallen, Switzerland

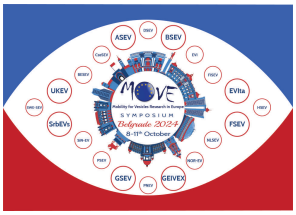
Introduction: Skin wounds represent a health condition that affects an increasing number of patients. Often contaminated with pathogenic bacteria, wounds are challenging to treat due to the increasing prevalence of antimicrobial resistance (AMR). One of the approaches to address this problem is the encapsulation of antimicrobials in nanoscale drug delivery systems. Membrane vesicles (MVs) from probiotics represent a natural alternative to artificially synthesized liposomes. In this project, we aim to investigate the potential of MVs from probiotic bacterium Lactiplantibacillus plantarum as a nanocarrier for synergistic combinations of a conventional antibiotic erythromycin with antimicrobial peptides (AMPs). This system is intended to improve erythromycin and AMPs stability and sustained release as well as to reduce their cytotoxicity and AMR development.

Methods: *L. plantarum* MVs were isolated via ultracentrifugation and characterized with transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), SDS-PAGE and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). *L. plantarum* MVs cytocompatibility and influence on cell migration were studied with normal human dermal fibroblasts (nHDFs). Erythromycin was encapsulated via passive loading, while AMPs were added via passive loading or co-culture with *L. plantarum*. The antimicrobial activity of the system was studied on *Staphylococcus aureus* and *Escherichia coli*.

Results: Isolated *L. plantarum* MVs preparation had a size range from 15 to 350 nm. The most abundant proteins in their composition – ABC transporters, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cell wall hydrolase - were identified with LC-MS/MS. *L. plantarum* MVs demonstrated a high cytocompatibility with nHDFs and a capacity to carry erythromycin and AMPs. *L. plantarum* MVs based drug delivery system inhibited the growth of common skin wound pathogens, *S. aureus* and *E. coli*.

Conclusion: Our study provides an evidence that *L. plantarum* MVs can be potentially used as an efficient nanocarrier for antimicrobial formulations to tackle skin wound infections.

Funding information: Swiss National Science Foundation, 197617



P-M-3

Optimising scalable spheroid-based extracellular vesicle production in stirred-tank bioreactors

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Introduction: Extracellular vesicles (EV)-based therapies have gained widespread interest, but efforts remain to ensure standardisation and high-scale production. Implementing processes in stirred-tank bioreactors (STBr) is mandatory to closely control the cell environment and to scale-up production, but it remains a significant challenge for adherent cells. Using a pancreatic cell line, we investigate the formation of spheroid in spinner flasks (SpF) and STBr at a constant power input per unit volume (P/V) and evaluated the impact of the culture mode on cell fate and EV released.

Methods: 1.4E7 cells were cultured as spheroids in SpF or Ambr®250 STBr. After 24h of culture in serum-free medium, EV were isolated by tangential flow filtration and size exclusion chromatography. EV size and concentration were determined by nanoparticle tracking analysis of tetraspanin-labelled particles. Protein expression was detected by western blot. EV immune properties were assessed through mixed lymphocyte reactions (MLR).

Results: Culturing 1.4E7 cells in suspension reduced their specific growth rate but allowed the formation of homogeneous and viable spheroids. Spheroid formation was dependent on the P/V, and maintaining this parameter constant across scales proved to be the optimal scale-up strategy. However, the process transfer to STBr altered spheroid formation, presumably due to impellers design. Compared to the monolayer process, EV yield decreased (2-fold) in SpF but increased in STBr (2-fold), and EV exhibited distinct EV marker expression and immune responses in MLR.

Conclusion: Anchorage dependency is a key feature of most cell types used in therapeutic EV production. Generating spheroids is a promising approach for scalable EV production in STBr. We demonstrated that maintaining a constant P/V is the best scale-up strategy to standardise the process although we observed a strong impact of impeller design. Finally, culturing cells as spheroids in a stirred system may induce hydrodynamic constraints and affect the EV final attributes.

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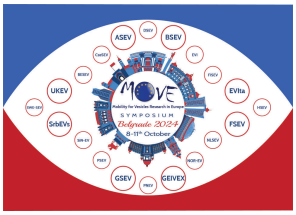

P-M-4

Scalable production of Extracellular Vesicles from Mesenchymal Stem Cells using a Hollow Fiber Bioreactor

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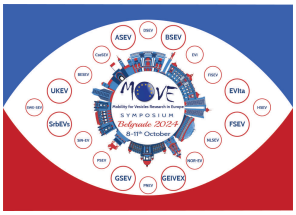
Introduction: Extracellular vesicles (EV) are small vesicles (30-1000 nm) released by cells which contain biomolecules such as microRNAs and proteins that mediate intercellular communication. The full regenerative/therapeutic potential of EV isolated from several types of cells (including mesenchymal stromal/stem cells [MSC]) has been demonstrated in the setting of several diseases, including cardiovascular diseases. Nonetheless, the poor scalability of EVs production hinders its clinical translation. Here, we used a hollow fiber bioreactor to improve the yield of native EVs from MSC whilst maintaining their therapeutic potential.

Methods: We used Wharton's Jelly MSC (WJ-MSC) due to its easy isolation from umbilical cord, retention of primitive stem cells characteristics and well-known therapeutic capacity. A minimum of 100 million WJ-MSC were inoculated and conditioned media was collected every day. EV isolation was done by differential ultracentrifugation with further purification with size exclusion chromatography. EVs were characterized according to their concentration, size, protein amount, surface charge and tetraspanins expression.

Results: Our results showed that EVs produced by the bioreactor are slightly bigger (203.2 vs 161.9 nm), show high expression of CD63 (93.0% vs 97.8%) and similar surface charge (-24.3 vs -20.9 mV) as compared to EVs secreted by cells cultured in 2D systems (T-175 flasks). In addition, we showed a 2.5-fold increase of EV collection per day, as compared to a single isolation using 10-12 T-175 Flasks. We were able to isolate over 1.5×10^{12} EVs in total (after purification) over a period of 15 days. We further validated EVs bioactivity in a scratch assay. Our results showed that the bioactivity of EVs generated in a bioreactor is stronger (73.4% Vs 40.8%) compared to EV from 2D systems.

Conclusion: Overall, hollow fiber bioreactor is a suitable method for the scalable production of EVs, maintaining its therapeutics properties.

Funding information: The authors would like to acknowledge the funding by the FCT PhD Studentship (SFRH/BD/144092/2019), Programa Operacional Competividade e Internacionalização (POCI) na sua componente FEDER e pelo orçamento da Fundação para a Ciência e a Tecnologia na sua componente OE (Project 2022.03308.PTDC; 2022.07615.PTDC; 2022.02803.PTDC); EC projects REBORN (Ref. 101091852); and PRR project HfPT- Health from Portugal (Ref: 02/C05-i01.01/2022.PC644937233-00000047).



P-M-5

Klotho peptide engineered extracellular vesicles prevent epithelial-mesenchymal transition (EMT) and fibrosis in kidney cells via TGF-β inhibition

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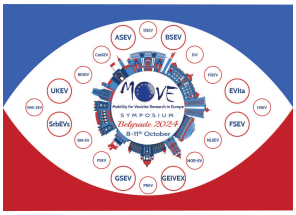
Introduction: Klotho is an anti-aging protein and its expression severely decreases with age and kidney injury. Conversely, studies have demonstrated that augmenting Klotho expression or supplementing it exogenously yields therapeutic benefits in models of chronic kidney disease, notably by mitigating kidney fibrosis. In particular, Klotho was reported to bind to TGF receptor, thus impeding TGF binding and inhibiting TGF-β signaling. We here engineered EVs with Klotho peptide to investigate its effects on epithelial mesenchymal transition (EMT) and kidney fibrosis.

Methods: EVs were isolated from human donors' red blood cells via ultracentrifugation. Klotho peptides were synthesized and linked to the EV membrane using click chemistry. To assess the effects of engineered EVs, human proximal tubular cell lines (HK2 and ciPTEC) were pre-treated with the engineered EVs and subsequently exposed to TGF-β. Changes in fibrosis and mesenchymal markers were evaluated through real-time PCR and flow cytometry analyses.

Results: Targeting of fluorophore-labeled Klotho peptide-engineered EVs was observed in plasma membrane and cytoplasm of target cells. Pre-treatment of kidney cells with engineered EVs prior to TGF-β treatment showed decreased RNA levels of Fibronectin, Collagen 1 and N-cadherin compared to cells treated only with TGF-β. Flow cytometry analysis revealed a reduction in N-cadherin protein expression following EV pre-treatment. Notably, overexpression of Klotho within tubular cells did not alter fibrosis levels after TGF treatment.

Conclusion: This study successfully engineered Klotho peptide-loaded EVs, delivering them to kidney cell models. These engineered EVs effectively prevented fibrosis and epithelial to mesenchymal induction. Further investigations will prioritize elucidating the mechanisms underlying Klotho peptide-driven prevention of EMT and fibrosis, including an exploration of alterations in the TGF-β pathway and the fate of engineered EVs post-uptake by target cells.



P-M-6

The display of an anti-CS1 nanobody by small extracellular vesicles does not improve disease targeting in multiple myeloma

Michiel De Coster[1,2], Lukas Hyka[2], Sofie Meeusen[2], Guido David[2], Eline Menu[1], Kim De Veirman[1], Pascale Zimmermann[2], Elke De Bruyne[1]

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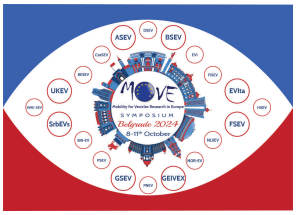
Introduction: Multiple myeloma (MM) is an incurable plasma cell cancer predominantly residing in the bone marrow (BM). Due to inevitable refractory disease, novel therapeutic options are needed. Small extracellular vesicles (sEVs) are promising therapeutic cargo delivery vehicles due to their high biocompatibility and ability to traverse biological barriers. This study aims to engineer HEK293-derived sEVs to display a nanobody (Nb) targeting CS1, a well-established surface marker of MM cells, to increase sEV specificity to MM-associated organs and MM cells.

Methods: HEK293 cells were stably transfected with Nb-SDC1CTF fusion proteins, linked with the juxtamembranal domain of CD4 to prevent membrane cleavage. Western blot and confocal microscopy confirmed sEVs sorting and correct cell membrane topology. Control sEVs without Nb and with an irrelevant Nb were included. Binding of anti-CS1 Nb-displaying sEVs to CS1 was evaluated by incubating sEVs with soluble CS1 and analyzing size-exclusion chromatography (SEC) fractions for co-elution of CS1 with sEVs by western blot. To evaluate the effect of the anti-CS1 Nb on sEV biodistribution, DiR-labeled sEVs were intravenously injected in 5T33MM mice. After 24h, organs were imaged with a Fluobeam800 camera. MM cell-specificity was determined by measuring DiR fluorescence by flow cytometry.

Results: Cleavage-resistant constructs were expressed with the correct topology and were highly enriched in sEVs. Both human and murine anti-CS1 Nb-displaying sEVs showed binding to soluble forms of CS1. Mononuclear cells isolated from spleen, spine and legs showed no enhanced sEV specificity and/or selectivity towards MM cells. Interestingly, in myeloma-bearing mice, anti-CS1 Nb display increased sEV accumulation in liver and lungs.

Conclusion: Anti-CS1 Nbs were successfully displayed on the sEV surface. While these EVs efficiently bind soluble forms of CS1, they do not improve MM targeting in *vivo*. Further work will explore suborgan distribution of sEVs in the lungs and expand the flow cytometric panel to include immune cell populations.

Funding information: This work was supported by Kom Op Tegen Kanker. MDC is a predoctoral fellow of FWO Vlaanderen (1S94421N).



P-M-7

Effects of industrial processing on the functional properties and uptake efficiency of nanovesicles derived from pomegranate juice

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Introduction: Research on extracellular vesicles (EVs) has raised due to their promising applications in disease diagnosis and treatment. Among these, Plant-Derived Nano Vesicles (PDNV) have gained special attention as potential delivery vehicle for bioactive compounds and natural treatments. They are considered a good alternative to traditional drug delivery vehicles (e.g. liposomes) due to their sustainability, safety and biocompatibility. However, the impact ofindustrial scale-up production on PDNVs remains insufficiently explored. This study aimed to examine how freeze-drying and pasteurization, two prevalent techniques in the food industry, influence the functional properties and uptake capacity of pomegranate-derived nanovesicles (PgNVs).

Methods: Juice was obtained manually and the isolation of PgNVs was performed by a combination of differential centrifugation, tangential flow filtration (TFF) and size exclusion chromatography (SEC). PgNVs were then characterized by TEM, NTA and LC-MS/MS, followed by an assessment of their functional properties in Caco-2, HepG2 and THP-1 cell lines. Uptake efficiency was determined in THP-1 cell cultures by flow cytometry and confocal laser microscopy.

Results: Although obtained PgNVs displayed high purity, the yield was reduced by both industrial treatments. Pre-treated PgNVs exhibited lower anti-inflammatory capacity compared to non pretreated PgNVs, although their antioxidant potential remained similar. All PgNVs preparations displayed more efficient uptake rates in THP-1 cells than synthetic liposomes, with NVs derived from non pre-treated juice showing the highest internalization rates at 0.5, 1 and 2 hours. Proteomic analyses suggested that damage of membrane proteins during industrial processing might explain these differences. Tetraspanin-8 was preserved only on non-treated PgNVs and might be a major player in NV-cell interaction.

Conclusion: Industrial processing of pomegranate juice can impact the yield, functional properties and internalization efficiency of PgNVs. Despite this alterations, industrial treated PgNVs maintain bioactivity and demonstrate higher uptake rates than liposomes, indicating their potential as carriers for bioactive compounds.

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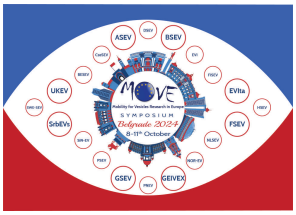

P-M-8

Functionalization of Extracellular Vesicles Surface with Hyaluronic Acid Derivatives: Comparison of Different Approaches

Giulia Duca[1], Stefania Raimondo[1], Nima Rabienezhad Ganji[1], Salvatore Emanuele Drago[2], Fabio Salvatore Palumbo[2], Alice Conigliaro[1], Gennara Cavallaro[2], Riccardo Alessandro[1,3]

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Introduction: Extracellular vesicles (EVs) can be used to deliver therapeutic compounds to specific target cells by modifying the EVs surface. Based on this, focusing on finding the best methods of functionalization, the goal of our project is to develop a targeted EVs-based delivery system for anticancer therapy, functionalizing the EVs surface with hyaluronic acid (HA), whose receptor (CD44) is overexpressed in many cancer cells, by using HA derivatives bearing in the side chain hydrophobic portions able to give interactions with phospholipid bilayer of EVs.

Methods: First, we isolated EVs from HEK293T cells by ultracentrifugation and we decorated them with synthetic Alexa-fluor-labelled HA compounds ($H_{100k}E_{28\%}C_{12\%}$ and $H_{10k}E_{26\%}C_{12\%}$). We tried two different functionalization approaches: the overnight co-incubation between EVs and HA and the EVs electroporation followed by 1h of incubation with HA at 37°C. We tried different electroporation protocols, differing in voltage and pulse number; the most efficient approach was selected evaluating the stability of the EV size through dynamic light scattering (DLS). The decoration efficiency was evaluated by measuring fluorescence emission. Finally, EVs-HA were labelled with PKH26, and the cellular uptake of thyroid carcinoma cells was analyzed by confocal microscopy.

Results: Confocal microscopy images show an extracellular deposition of HA derivatives in cells treated with EVs decorated by co-incubation, suggesting that HA aggregates on EVs surface, also reducing their uptake. Furthermore, fluorescence quantification highlights a low EV decoration with this method. The functionalization was improved by employing electroporation at 1000V for 5 pulses; this approach allows to increase EVs' internalization.

Conclusion: These preliminary data suggest that electroporation can improve the EVs decoration with HA, hence this protocol could be applied to develop a delivery system for anticancer therapy. Further experiments will be performed, loading siRNA into EVs decorated with HA and seeing the functionality of the complex.

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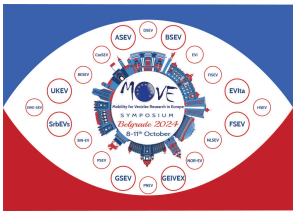

P-M-9

The Importance of Established and Optimized Analytical Capabilities to Develop End to End and Scalable Therapeutic Extracellular Vesicles Processing

Aslan (Mehdi) Dehghani[1], Meng Chai[1], Eric Black[2], Zheng Zhao[1], Camille Trinidad[1], Paul Keselman[2], David Splan[2], Mark Szczypka[2], Sunandan Saha[1], Michael Olszowy[2], David Pollard[1]

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Introduction: Reliable analytical capabilities are critical for the development of Extracellular Vesicle (EV) based therapies. We have established an analytical toolbox consisting of multiple orthogonal techniques to comprehensively analyze EVs samples. This analytical capability was then used to develop an end to end and scalable process for EVs derived from Mesenchymal Stem Cells (MSCs).

Methods: The analytical toolbox consists of nanoparticle tracking analysis (NTA), flow cytometry (FC) and analytical high performance liquid chromatography (HPLC). The assays are established according to MISEV and MIFlowCyt-EV guidelines. MSC-EVs process was established in 2L bioreactor using microcarriers and the collected conditioned media was then processed through clarification, tangential flow filtration, anion exchange chromatography and sterile filtration steps.

Results: Utilizing the optimized analytical capabilities, the EV concentrations, free protein concentrations, free DNA concentrations, EV associated proteins (corona) were monitored throughout the downstream processing. We achieved over 95% free protein and DNA reduction while maintaining over %40 of the EVs. Purified MSC-EVs improved the wound recovery in a dose dependent manner and the EV uptake was shown using live cell imaging. Proteomic analysis showed the common EV markers in agreement with western blot, but also showed that 50% of the identified proteins are linked to diseases, suggesting potential targets for therapeutic applications.

Conclusion: Established and optimized analytical assays are critical to develop therapeutic EVs by systematic evaluation and monitoring of the process. We have successfully established the tools for systematic evaluation of EV biomanufacturing platforms to satisfy the therapeutic needs.

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