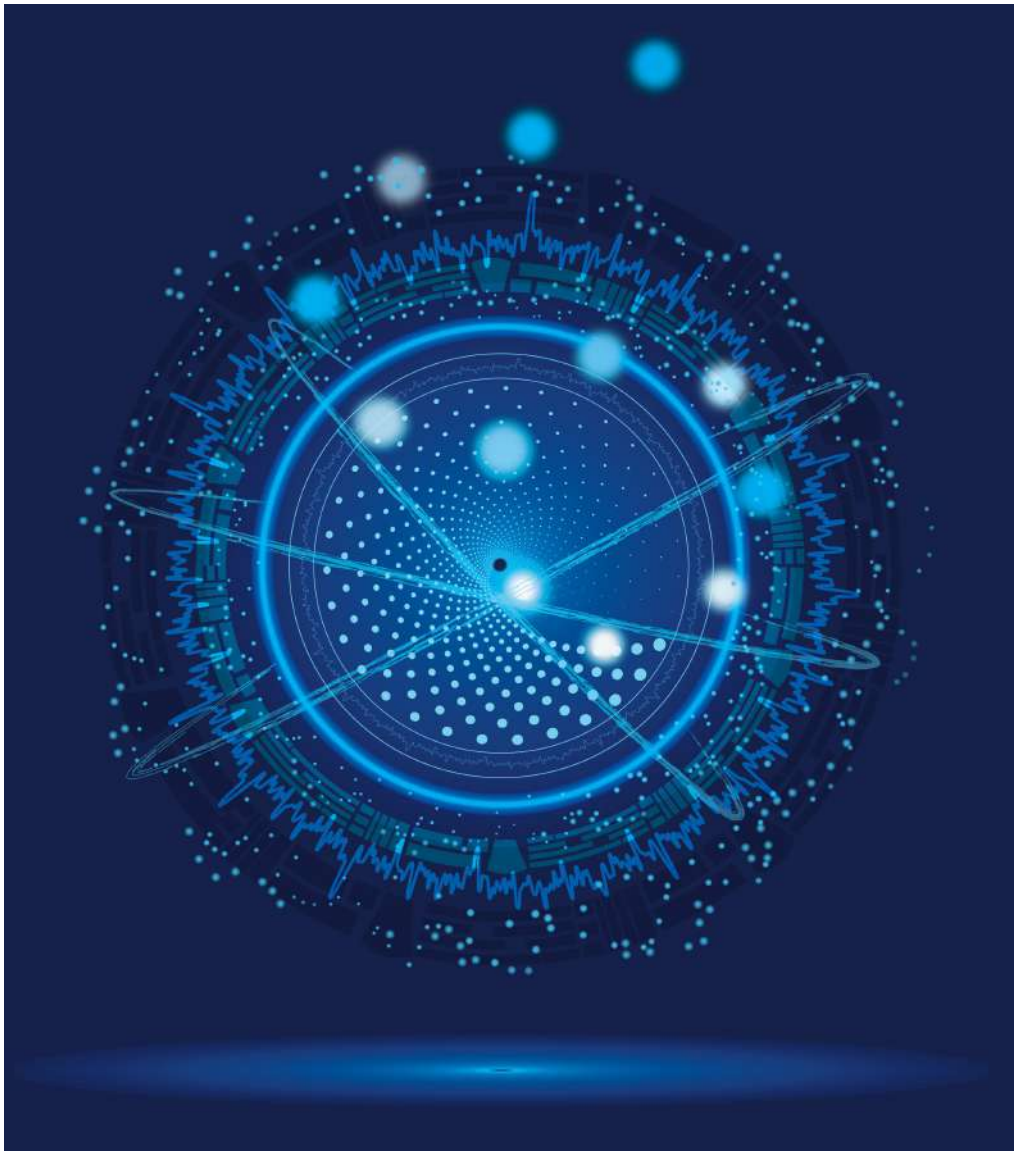
Small New World 2.0

4-5 September 2023

Abstract Book



Medical University Graz, Austria

Small New World 2.0

4-5 September 2023., Graz, Austria

Joint Meeting of

Austrian Society for Extracellular Vesicles - ASEV
Hungarian Section for Extracellular Vesicles - HSEV
Slovenian Network for Extracellular Vesicles - SiN-EV
Serbian Society Extracellular Vesicles - SrbEVs

Organizing committee:
Beate Rinner, ASEV
Wolf Holnthoner, ASEV
Edit Buzas, HSEV
Metka Lenassi, SiN-EV
Maja Kosanović, SrbEVs

Scientific committee:
Beate Rinner, Medical University Graz, Austria;
Wolf Holnthoner, Ludwig Boltzmann Institute for Traumatology, Austria;
Edit Buzas, Semmelweis University, Hungary;
Metka Lenassi, Faculty of Medicine, University of Ljubljana, Slovenia;
Maja Kosanović, Institute for the Application of Nuclear Energy, INEP, Serbia;
Zoltan Giricz, Semmelweis University, Hungary;
Bernd Giebel, Institute for Transfusion Medicine, University Hospital Essen, Germany

Small New World 2.0

4-5 September 2023., Graz, Austria

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PROGRAM
for Monday, 4th September 2023

8:30 - 10:00	Registration and poster placement	
10:00-10:15	Welcome note from the Presidents of ASEV, HSEV, SiN-EV, SrbEV Welcome note from the local organizers & organizational introduction	
10:15-12:00	**EV therapeutics - regenerative medicine and beyond** Chairs: Wolf Holnthoner (Austria) + Zala Jan (Slovenia)	
	Keynote: **Bernd Giebel** (Germany)	Clinical potential of MSC-EVs and translational challenges
	Beáta Szebeni	Origin of extracellular vesicles from peritoneal dialysate and their immunomodulatory effect
	Mateja Manček Keber	Therapy for cancer-specific MyD88L265P signaling based on exon skipping using LNP-mediated ASO delivery
	Katharina Schallmoser	A protein corona around human platelet-derived EVs promotes regenerative functions
	Maximilian Haertinger	Small extracellular vesicles derived from multipotent adipose stromal cells in peripheral nerve regeneration: jack of all trades, master of none?
	Alexander Otahal	Hoffa-derived MSCs primed with IL1β in bioreactor culture yields extracellular vesicles hindering chondrocyte recovery
12:00-13:30	Lunch break / General assembly of ASEV	
13:30-15:00	**Methodology advances in EV analysis** Chairs: Beate Rinner (Austria) + Sofija Glamočllija (Serbia)	
	Keynote: **Pia Siljander** (Finland)	Methods to study EVs - what do we (need to) look for?
	Jaroslaw Jacak	Tracking individual extracellular vesicles: from purification analysis to intracellular co-localization using atomic force- and single-molecule fluorescence microscopy
	Pietro Parisse	Substrate stiffness modulates extracellular vesicles' release in triple-negative breast cancer models
	Maria Jaritsch	Quantitative serum pharmacokinetics of EVs from diverse sources using high content single vesicle imaging
	Anna Lischnig	Quantitative proteomics and nFCM analysis of subpopulations of immune cell-derived EVs
15:00-15:30	Coffee break	

PROGRAM
for Monday, 4th September 2023

15:30-16:40	**News from industry and development - "Rising projects"** Chairs: Dirk Strunk (Austria) + Pia Siljander (Finland)	
	Clemens Helmbrecht ParticleMetrix	NTA goes colocalization: Characterization of Multi-labelled bionanoparticles
	Mehdi Madi and Quentin Lubart Abbelight	Quantitative analysis of single EV and their subpopulations with super-resolution solutions
	Core Facilities MedUni Graz	EV technologies at the MedUni Graz
	BioTechMed consortium "iNterAcD+"	Extracellular vesicle in exercise: sporty messengers in interorgan communication
	Christian Wadsack and Michaela Klaczynski	Fetal immune priming by placenta-derived small extracellular vesicles
	Beate Rinner and Mariangela Garofalo	Patient-derived tumor models, EVs and oncolytic viruses
16:40-16:45	Short break	
16:45-17:30	Special guest lecture: Translation of EV into the clinics - **Eva Rohde** (Austria)	
17:30-23:00	Poster party and Social evening	

PROGRAM
for Tuesday, 5th September 2023

09:00-10:45	**EV numbers and cargo** Chairs: Maja Kosanović (Serbia) and Nicole Maeding (Austria)	
	Keynote: **Paolo Bergese** (Italy)	Extracellular vesicles by the numbers
	Hargita Hegyesi	Cardioprotective role of extracellular vesicle-mediated mir-sponge transfer
	Christa Noehammer	Small RNA biomarker profiling from extracellular vesicles in immune-mediated inflammatory diseases
	Tasvilla Sonallya	Systematic investigation and classification of membrane active peptides based on their affinity for interaction with extracellular vesicles
	Ilona Barbara Csordás	Extracellular Vesicles (EVs) miRNA-cargo loading and alterations after ionizing radiation induced cellular stress
	Marija Holcar	Characterization and Interindividual Variability of Plasma Extracellular Vesicles in Healthy Adults
10:45-11:30	Coffee break	
11:30-12:30	**NETWORK SESSION + MOVE** Chairs: Beate Rinner and Wolf Holnthoner	
	Wolf Holnthoner	ASEV - Austrian Society for Extracellular Vesicles
	Edit Buzas/Zoltan Giricz	HSEV - Hungarian Society for Extracellular Vesicles
	Metka Lenassi	SiN-EV - Slovenian Network for Extracellular Vesicles
	Maja Kosanović	SrbEVs - Serbian Society for Extracellular Vesicles
	Johannes Oesterreicher	MOVE news from Finland
	Martin Wolf	MOVE news from Sweden
12:30-13:30	Lunch break	

PROGRAM
for Tuesday, 5th September 2023

13:30-15:00	**Diversity of EV sources** Chairs: Edit Buzas (Hungary) + Djenana Vejzovic (Austria)	
	Keynote: **Pieter Vader** (The Netherlands)	Extracellular vesicle-mediated RNA delivery: from mechanistic insights towards therapeutic applications
	Astrid Laimer-Digruber	Unraveling the pathogenic and pro-inflammatory potential of extracellular vesicles secreted by Bacillus cereus
	Vendula Pospíchalová	Proteomic analysis of ascitic extracellular vesicles describes tumor microenvironment and predicts patient survival in ovarian cancer
	Kaja Ujčič	Effects of placental extracellular vesicles on maternal hematopoiesis
	Veronika Kralj-Iglič	Mechanisms of formation of extracellular particles in diverse samples from human, animal, plant and microalgae

15:00-15:30	Coffee break

15:30-17:00	**Purity meets function** Chairs: Metka Lenassi (Slovenia) + Krisztina Nemeth (Hungary)	
	Keynote: **Saara Laitinen** (Finland)	To EV, or not to EV: that is the question
	Martin Wolf	Functional implications of protein EV corona
	Johannes Grillari	EV therapeutics - regenerative medicine and beyond
	Maria Cavinato	Alternative mechanisms of mitochondria quality control elicited by EVs in skin aging and disease
	Irma Schabussova	Outer membrane vesicles of the probiotic E. coli O83 activate innate immunity and prevent allergic airway inflammation in mice

17:00-17:15	Awards: Best poster & Best oral presentation Farewell notes

18:00	City tour Graz

Dear friends and colleagues, working on these tiny bubbles which we call „EVs“,

Last autumn I was – as usually on Sundays - cycling along the Danube in Vienna. While riding my bike many thoughts came into my mind: I recently visited Edit Buzas in Budapest, I was invited by Metka Lenassi for a talk at the annual meeting of the Slovenian Network for Extracellular Vesicles, and I got acquainted and befriended with Maja Kosanović. Consequently I thought it would be a really nice idea to organize a joint annual meeting, bringing our communities from Austria, Hungary, Slovenia and Serbia together. So I asked the board members of the Austrian Society for Extracellular Vesicles, and of course Edit, Metka and Maja, and I was absolutely thrilled that everybody agreed enthusiastically.

So, here we are!

Extracellular Vesicles gained tremendous scientific interest in the last decade. From the basic understanding of the biology, the recent technological advances in the purification and characterization of EVs, straight to the application in diagnostic and therapeutic areas: Here at the Medical University in Graz we come together in the SmallNewWorld2.0 to exchange (and ignite) our thoughts on all these aspects of EVs.

This congress is the continuation of SmallNewWorld 2022, when our colleagues from Salzburg/Austria organized the recent annual meeting together with our sister society from Germany (GSEV). I am really thankful for their great experience, which helped us organizers in all the necessary steps to prepare our joint meeting in 2023 in Graz.

The modern facilities at the Medical University of Graz will for sure be the perfect surrounding and sparkle exchange of the EV research of our communities in Austria, Hungary, Slovenia, Serbia and participants from over 15 countries. Especially young scientists are encouraged to get in contact with experienced researchers and of course with our international keynote speakers. I cordially invite you all to learn from eachother.

Together we will not only have fun and learn to know all of us better on a personal level, but also extend our knowledge of these fascinating tiny bubbles.

On behalf of the organizing committee I heartily welcome you here in Graz, and I wish us all an inspiring great time!

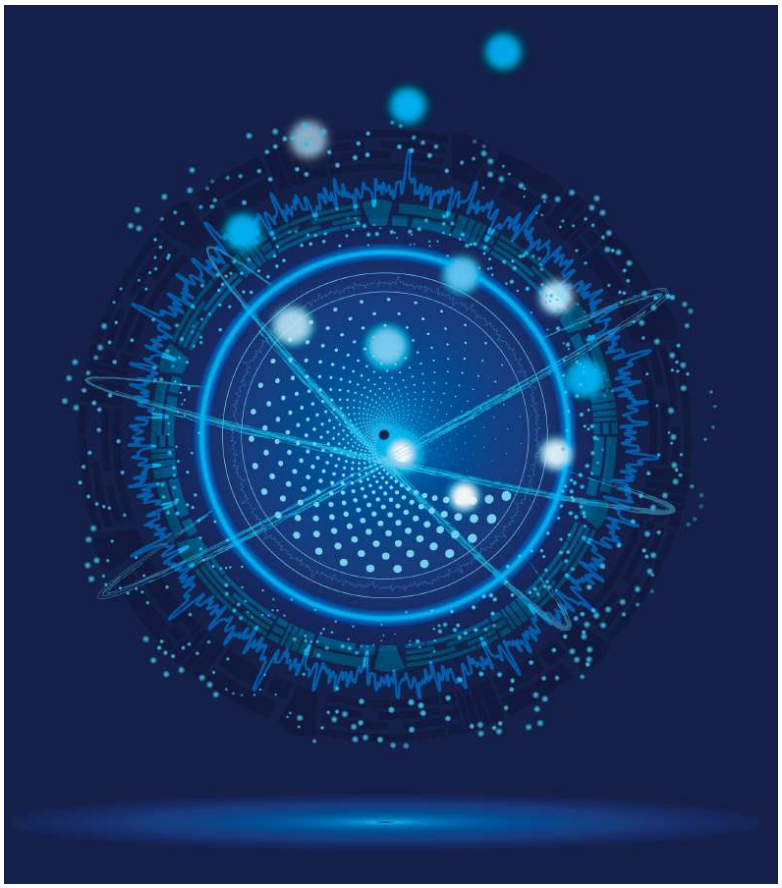
Wolf Holnthoner
President of the Austrian Society for Extracellular Vesicles (ASEV)

EV therapeutics
- regenerative medicine and beyond

Monday, 4th September 2023

10:15-12:00

Chairs: Wolf Holnthoner (Austria) + Zala Jan (Slovenia)



Keynote presentation

Clinical potential of MSC-EVs and translational challenges

Bernd Giebel

Institute for Transfusion Medicine, University Hospital Essen, Germany

• Bernd Giebel studied biology in Cologne and received his PhD in 1996. 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. It is his goal to efficiently translate MSC-EVs into the clinics and to set up appropriate quality control platforms. BG is the president of the German Society of Extracellular Vesicles (GSEV), co-chairing the exosome working group of the International Society of Gene and Cell Therapy (ISCT) and part of the scientific advisory board of four SME companies, Innovex Therapeutics, Mursla LTD, PL BioSciene and ReNeuron. Furthermore, he is a consultant of FUJIFILM Wako Chemicals Europe GmbH and founding director of Exosla LTD. •

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.

Oral presentation

Origin of extracellular vesicles from peritoneal dialysate and their immunomodulatory effect

Beáta Szebeni[1,2]; Péter Bokrossy[1]; Apor Veres-Székely[1,2]; Domonkos Pap[1,2]; Csenge Szász[1]; Mária Bernáth[1]; György Reusz[1]; Attila J. Szabó[1,2]; Ádám Vannay[1,2]

[1]Dept. of Paediatrics Bókay street Unit, Hungary; [2]ELKH SE Pediatrics and Nephrology Research Group, Hungary

Introduction: Due to their low immunogenicity and unique immunosuppressive properties, mesenchymal stem cells (MSCs) are considered one of the most promising cell types in human therapy. Furthermore numerous studies confirm the regenerative and anti-inflammatory properties of MSC derived extracellular vesicles (EVs). Our present aim was to investigate the possible stem cell origin and role of EVs isolated form the peritoneal dialysate (PDE) of patients with peritoneal dialysis (PD).

Methods: EVs were isolated from PDE using ultrafiltration and size exclusion chromatography. Following a quality check, Western blot was carried out to investigate the presence of stem cell -(CD75, CD90, CD105), endothel- (CD31), mesothel- (CK-18, E-Cadherin) and mesenchymal (fibronectin) markers as well as the absence of CD14, CD34 and CD11b immune cell markers on the isolated EVs. The effect of PDE-EV treatment on cytokine production of peripherial mononuclear cells (PBMCs) under physiological and pathological (inflammatory) conditions was investigated using RT-PCR and ELISA.

Results: Presence of stem cell marker CD105, mesothelial marker CK18 and E-cadherin and mesenchymal marker fibronectin was confirmed on PDE-EVs. At the same time, the absence of CD11b (monocyte, granulocyte marker) and CD34 (hematopoietic progenitor cells, marker of endothelial cells) was confirmed. Our data suggest that the sources of PDE-EVs may be mesothelial cells, had previously undergone mesenchymal dedifferentiation/transition and detached from the mesothelial layer into PDE. EV treatment increased the mRNA expression of interleukin (IL)-10 and monocyte chemoattractant protein-(MCP)-1 of PBMCs. Moreover, the expression of these factors became even more pronounced after their induction with lipopolysaccharide. Phytohemagglutinin induced production of tumor necrosis factor (TNF)-α or IL-6 of PBMCs was not affected by EV treatment.

Discussion: Based on our results PDE derived EVs have mesothelial origin and may affect the cytokines balance, they can moderate the "excessive" activation of the immune system and promote tissue regeneration.

Funding: K-142728, ELKH-POC-2022-024,TKP2021-EGA-24, ÚNKP-22-4-II-SE-12, ÚNKP-22-5-SE-17, János Bólyai Research Scholarship.

Oral presentation

Therapy for cancer-specific MyD88L265P signaling based on exon skipping using LNP-mediated ASO delivery

Peter Pečan, Duško Lainšček, Roman Jerala and **Mateja Manček Keber**

National Institute of Chemistry, Slovenia

Introduction: MyD88 is a central signaling adaptor in innate immunity, downstream of TLRs. Somatic MYD88L265P mutation enables its spontaneous oligomerization, resulting in constitutive activation and inflammation contributing to cancer development such as Waldenström`s macroglobulinaemia (WM). MyD88 signaling is tightly regulated. MyD88S transcript lacks exon 2 and is inhibitory as it prevents binding of IRAK4 to myddosomal complex. Antisense oligonucleotides (ASO) designed to skip exon 2 can increase translation of MyD88S, which inhibits NF- B signaling, but no one has shown whether MyD88SL265P can act inhibitory.

Methods: We designed ASOs targeting different sites in introns and exons. ASOs were transfected/electroporated to HEK293 or WM cells and mRNA transcripts, cytokines, apoptosis were analyzed. Lipid nanoparticles (LNPs) with defined compositions to increase B cell uptake and organ tropism for ASO delivery were prepared and tested in mice.

Results: We showed that MyD88SL265P efficiently inhibits MyD88 signaling. Different ASOs and their combinations improved exon skipping shown by decrease of exon 2 containing mRNA transcripts and inhibited cytokine release from cells. A combination with ibrutinib or venetoclax further increased apoptosis. WM is B cell lymphoma with the main pathology in the bone marrow and spleen. LNPs were uptaken by the cells in the BM and labelling of LNPs with anti-CD38 increased tropism for the spleen in vivo.

Discussion: MyD88L265P presents a survival signal for cancer cells, therefore inhibition of its signaling could be used for treatment. By improving alternative splicing and in vivo targeted delivery of ASOs we deliver novel therapy for cancers that involve MyD88-dependnt signaling.

Oral presentation

A protein corona around human platelet-derived EVs promotes regenerative functions

Katharina Schallmoser[1]; Fausto Gueths Gomes[1,2]; André Cronemberger Andrade[2]; Martin Wolf[2]; Sarah Hochmann[2]; Linda Krisch[1,2]; Christof Regl[3]; Rodolphe Poupardin[2]; Patricia Ebner-Peking[2]; Christian Huber[2]; Nicole Meisner-Kober[3]; Dirk Strunk[2]

[1]Dpt. of Transfusion Medicine, Paracelsus Medical University Salzburg, Austria; [2]Cell Therapy Institute, Paracelsus Medical University Salzburg, Austria; [3]Dpt. of Bioscience, Paris Lodron University Salzburg, Austria

Introduction: Clinical efficiency of platelet derivatives still lacks evidence, but human platelet lysate (hPL) has proven as potent substitute for fetal bovine serum during clinical cell manufacturing. We have observed accelerated skin organoid formation and in vivo wound healing by hPL. As previously shown, extracellular vesicles (EVs) bear a biologically active protein corona, depending on preparation mode and protein milieu. We asked whether hPL-derived EVs or platelet-derived soluble factors mediate these trophic effects of hPL.

Methods: EVs were separated from soluble factors to understand the mode of action during skin organoid formation and immunomodulation as model systems for tissue regeneration. EVs were concentrated by tangential-flow filtration (TFF-EVs) and further purified by size-exclusion chromatography (TSEC-EVs) separating EVs from (lipo-) protein-rich soluble factors (TSEC-sol.F). Samples were characterized by tunable resistive pulse sensing, western blot, tandem mass-tag proteomics and super-resolution microscopy and functionally tested.

Results: We identified three major protein clusters by proteomic principle component analysis separating TSEC-EVs from hPL clustering with TFF-sol.F and TFF-EVs clustering with TSEC-sol.F. TFF-EVs induced significantly improved skin-organoid formation and inhibition of T-cell proliferation, compared to TSEC-EVs or to TSEC-sol.F. Reconstituting the corona on TSEC-EVs with TSEC-sol.F re-established functionality, super-resolution imaging confirmed corona formation.

Discussion: TFF enables scalable enrichment and separation of functional corona-bearing EVs and soluble factors. Depletion of the TFF-EV corona by SEC abrogated functionality indicating a novel mode of action. The corona could be artificially reconstituted on TSEC-EVs by add-back of sol.F, showing similar effects compared to TFF-EVs. This enables EV engineering with selected corona proteins for therapeutic applications.

Oral presentation

Small extracellular vesicles derived from multipotent adipose stromal cells in peripheral nerve regeneration: jack of all trades, master of none?

Maximilian Haertinger[1]; Julian Krause[1]; Flavia Millesi[1]; Paul Supper[1]; Tamara Weiss[1]; Andreas Spittler[2]; Christine Radtke[1]

[1]Department of Plastic, Reconstructive and Aesthetic Surgery, Medical University of Vienna; [2]Core Facility Flow Cytometry, Medical University of Vienna

Introduction: The peripheral nervous system has only limited intrinsic repair capabilities following axonal disruption, hence severe axonal injuries remain one of the biggest challenges in reconstructive surgery. Schwann cells (SCs) emerged as key players of the inert injury response and are in the focus of novel therapeutic approaches. In this study, we investigate how small extracellular vesicles derived from multipotent adipose stromal cells (ASC-EVs) interact with, and modulate the SCs response to injury.

Methods: We employed high-resolution live confocal microscopy to visualize the initial interaction between ASC-EVs and SCs. Further imaging flow cytometry was used to dissect internalization dynamics. Although the effects of ASC-EV signaling on SCs are manyfold, we have focused on key stages of the SC response to peripheral nerve injuries. We investigated the initial immunomodulation via bead-based multiplex immunoassay and assessed effects on myelin clearance efficiency. Further, we looked into the proliferation of repair phenotype SCs upon ASC-EV stimulation, and lastly investigated the remyelination process. Based on the knowledge obtained from these experiments, we then applied ASC-EVs in a rodent nerve injury model, and discuss the outcome.

Results and Discussion: Considering the results obtained in this study as well as the current literature, small extracellular vesicles, especially derived from multipotent stromal cells, offer a promising therapeutic approach to peripheral nerve regeneration. However, the clinical translatability of these findings is questionable, not only owing to the heterogeneity of small extracellular vesicles. For successful and targeted therapeutic application, the underlying mechanisms of EV signaling need to be investigated in more detail.

Oral presentation

Hoffa-derived MSCs primed with IL1β in bioreactor culture yields extracellular vesicles hindering chondrocyte recovery

Alexander Otahal[1], Karina Kramer[1], Markus Neubauer[1,2], Zsombor Lacza[3,4], Stefan Nehrer[1,2], Andrea De Luna[1]

[1]Center for Regenerative Medicine, Department for Health Sciences, Medicine and Research, Danube University Krems; [2]Universitätsklinikum Krems, Krems, Austria; [3]University of Physical Education, Department of Sport Physiology, Budapest, Hungary; [4]Semmelweis University, Inst. Clinical Experimental Research, Budapest, Hungary

Introduction: Extracellular vesicles (EVs) from mesenchymal stromal cells (MSCs) have anti-inflammatory and immunomodulatory properties. Adipose-tissue derived MSCs are investigated to find the ideal source of MSC EVs as therapeutic agents, for example in osteoarthritis (OA) therapy. The infrapatellar Hoffa's fat pad might harbour MSCs that are involved in tissue homeostasis of a knee joint. Hoffa MSCs might respond to OA as well as intra-articular treatments such as injection of blood products. They could release EVs that could promote regeneration of OA chondrocytes.

Methods and Materials: Hoffa MSCs were obtained from surgical waste of five patients undergoing total joint replacement surgery. Primary cells were isolated from tissue via enzymatic digestion, before MSCs were seeded onto polystyrene microcarriers in vertical wheel bioreactors for culture in suspension. Cells were treated with IL1β, followed by blood product supplementation (citrate anti-coagulated platelet-rich plasma or hypACT serum). EVs were isolated from conditioned supernatant via ultrafiltration. Primary OA chondrocytes were treated with isolated EVs at different cell:EV ratios to observe dose-response effects. Gene expression changes were assessed via qPCR and cytokine release was monitored via ELISA.

Results: Hoffa MSC 3D culture in vertical bioreactors was successfully established and yielded up to 100-fold higher amount of EVs compared to 2D culture. Treatment of chondrocytes with EVs derived from unstimulated MSCs at ratios of 500 to 50.000 EVs per cell resulted in dose-dependent upregulation of aggrecan and collagen type I and II expression in blood product-dependent manifestation. EVs from IL1β-primed MSCs did not change expression of extracellular matrix proteins, while strongly increasing MMP3 and MMP13 expression. Cytokine release was modulated favouring IL6 release in response to stimulated MSC EVs.

Conclusion: Hoffa MSCs in OA patients with active inflammation could release EV-mediated signals that exacerbate the disease, while EVs from unstimulated MSCs can have beneficial effects on chondrocytes and cartilage homeostasis.

EV therapeutics
– regenerative medicine and beyond

– poster presentations –

Poster presentation P01

Enhancing Targeted Peripheral Nerve Regeneration through Laminin-Binding Extracellular Vesicles Derived from Adipose Derived Stem Cells

Mai Quyen Nguyen[1,2]; Johannes Oesterreicher[1,3]; Madhusudhan Reddy Bobbili[1,3], Regina Grillari[4]; Sébastien Couillard-Després[2]; Johannes Grillari[4,1,3]; David Hercher[1]

[1]Ludwig Boltzmann Institute for Traumatology, Austrian Cluster for Tissue Regeneration; [2]Paracelsus Medical University; [3]University of Natural Resources and Life Sciences Vienna; [4]Evercyte GmbH

Peripheral nerve injuries result in impaired sensory and motor function, with current regeneration methods often providing suboptimal recovery. Schwann cells (SCs) play a crucial role in facilitating the regeneration process, by providing guidance cues for regrowing axons via their basement membrane -primarily consisting of laminin. Extracellular Vesicles (EVs) are vital for intercellular communication and the transfer of biological information. Recently, adipose derived stem cell- EVs (ASC-EVs) have emerged as a promising drug delivery system. However, the precise targeting of EVs to the desired location upon intravenous administration remains a significant challenge. To address this hurdle, the tetraspanin protein CD81, an EV surface marker protein, has been modified to enhance its affinity for laminin, thereby enabling preferential homing to the target site. This study focuses on producing laminin-binding EVs in ASCs to enable specific targeting to injured peripheral nerve sites.

To allow evaluation of uptake and biodistribution, we produced stable ASC cell lines which express fusion proteins of CD81-LEL sequences with eGFP or Akaluciferase using a lentiviral system. Subsequent characterization of ASC-EVs revealed a dominant peak in 100-120nm size and a substantial positivity for GFP signals of up to 10%. Performed fluorescence imaging showed an enhanced binding affinity of the produced engineered ASC-EVs on laminin.

Moving forward, we will focus on the uptake, biodistribution and proregenerative capacity of the laminin-binding EVs in vitro on primary Schwann cells as well as in vivo in a median nerve defect model.

Poster presentation P02

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

Djenana Vejzovic[1,2]; Christina Karner[3]; Iva Brcic[4]; Bernadette Liegl-Atzwanger[4]; Dirk Strunk[5]; Martin Wolf[5]; Beate Rinner[3]

[1]Ludwig Boltzmann Institute forTraumatology, Austrian Cluster for Tissue Regeneration; [2]Paracelsus Medical University; [3]Division of Biomedical Research, Medical University of Graz, Graz, Austria; [4]Diagnostic and Research Institute of Pathology, Medical University of Graz, Graz, Austria; [5]Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI-TReCS), Cell Therapy Institute, Paracelsus Medical University (PMU) Salzburg, Austria

Introduction: Extracellular vesicles (EVs) contribute to tumor growth, drug resistance, metastasis and remodeling of the tumor immunological microenvironment. Using patient-derived autologous tumor models mimicking this microenvironment enables investigation of EV impact on drug resistance. In the present project, we focus on Ewing-like sarcomas (ELS). Chemotherapy is usually only used for metastatic ELS, as surgery is still the golden standard for primary tumors. However, metastatic ELS often exhibit a drug-resistant phenotype that differs from the primary tumor. Although experimental studies have shown that chemotherapy can have metastasis-promoting effects, the role of EVs and tetraspanins in ELS resistance remains unclear.

Material & methods: In this study we generated a patient-derived in vitro model of the Ewing-like clear cell sarcoma (CCS) harbouring the EWSR1::ATF1 oncogenic fusion consisting of cell lines derived from the primary tumor and the kinase inhibitor crizotinib resistant metastatic lesion. Cell lines and EVs were comprehensively characterized based on their morphology, transcriptomic signatures and tetraspanin co-localization before and after treatment with crizotinib.

Results: Interestingly, a triple tetraspanin co-localization (CD63+/CD81+/CD9+) could only significantly be detected in tumor-associated EVs, whereas EVs from normal fibroblasts did not exhibit this feature. Treatment altered co-localization patterns of primary tumor EVs but showed no effect on metastatic EVs. Triple co-localization decreased after treatment in a dose-dependent matter in primary tumor EVs but resulted in an increase in single CD9+ EV populations of cancerous and normal entities.

Discussion: The results give insight into a potential resistance mechanism involving tetraspanins and allow monitoring and improving of CCS treatment.

Poster presentation P03

Immortalization of mesenchymal stromal cells by hTERT does not affect the functional properties of secreted extracellular vesicles

Alessia Brancolini[1]; Zahra Mazidi[1]; Matthias Wieser[1]; Johanna Gamauf[1]; Marieke Roefs[1]; Giulia Corso[1]; Harini Nivarthi[1]; Marianne Pultar[2]; Bobbili Madhusudhan[3,4,5]; Matthias Hackl[2]; Grillari Johannes[3,4,5]; Regina Grillari-Voglauer[1]

[1]Evercyte GmbH, Vienna, Austria; [2]TAmiRNA GmbH, Vienna, Austria; [3]Institute of Molecular Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria; [4]Ludwig Boltzmann Institute for Traumatology, The Research Center in Cooperation with AUVA, Vienna, Austria; [5]Austrian Cluster for Tissue Regeneration, Vienna, Austria

Background: Mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) promote therapeutic activities comparable to the ones attributed to their cell sources, while avoiding most of the safety concerns associated with cell-based therapies. However, the donor heterogeneities, the limited replicative life span and changes in the cellular phenotype throughout in vitro cultivation, are major hurdles for scalable EV production for clinical applications.

Methods: The cells were characterized for expression of cell-type specific markers CD73, CD90 and CD105, tri-lineage differentiation capacity and tumorigenic potential using soft agar assay. EVs secreted by the cells were enriched using tangential flow filtration followed by characterization using NTA, BCA, multiplex bead array, western blotting, and electron microscopy. Moreover, the miRNA cargo was characterized by small RNA sequencing. Finally, anti-fibrotic, wound healing and anti-inflammatory activity was tested using relevant in vitro bioassays.

Results: hTERT expression did not affect the cellular morphology and the canonical expression of surface markers while ensuring unlimited, stable in vitro growth. Moreover, typical EV characteristics and the biological activity were not affected by immortalization of the cells. Finally, the lack of full-length mRNA and hTERT protein in EVs from telomerized cells in addition to the lack of a tumorigenic potential suggests that telomerized WJ-MSC-EVs have an adequate safety profile.

Conclusions: Immortalization of MSCs by hTERT does not affect the inherent molecular, biological, and functional properties of either donor cells or MSC-derived EVs. Thus, the use of telomerized MSCs as cell factories for EV production is a suitable strategy to foster scalable and standardized EV production.

Poster presentation P04

Therapeutic potential of fetal MSC derived EVs on inflamed chondrocytes and synoviocytes in vitro

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Osteoarthritis is a highly prevalent degenerative joint disease for which no disease-modifying treatment is available. Mesenchymal stem cells (MSCs) show promising therapeutic effects, mainly mediated by paracrine signals, with fetal cells demonstrating greater efficacy than adult cells. Therefore, this study aims to compare the therapeutic efficacy of ovine fetal MSC-derived conditioned medium (CM) and EVs on inflamed (IL-1β and TNF-α) chondrocytes and synoviocytes in vitro. Following dose titration studies, the inflammatory stimulus that elicited the maximum inflammatory response while still being reversible with the gold standard treatment (Dexamethasone, 100ng/mL) was selected (1ng/ml IL-1β+TNF-α) for subsequent EV therapeutic efficacy studies. Ovine fetal umbilical cord blood derived MSCs (43x106 cells) were cultured in a hollow fiber bioreactor (6ml volume) in xeno-free medium for 6 weeks. A total volume of 186mL CM was obtained with a protein concentration of 0.11 mg/mL and particle count of 5.81x108 particles <200nm/mL (total particle count: 7.09x108particles/mL). CM was harvested and either directly concentrated by Crossflow filtration (4x, protein: 0.23mg/mL, 3.31x109particles <200nm/mL) or subjected to tangential flow filtration to isolate EVs (10x, protein: 0.827mg/mL, 9.03x109particles <200nm/mL). The effect of different concentrations of EVs (1x108 and 1x109particles/ml) alone or in combination with CM on inflamed chondrocytes and synoviocytes, was tested using a scratch assay and qPCR (IL6, MMP1, MMP3, MMP13, Col2, Col10, and Sox9, PRG4 and Vimentin) as readouts. A dose of 109 EVs and CM supplemented with 109 EVs showed superior immunomodulatory and pro-regenerative effects compared to CM or EV concentrate with lower particle numbers or Dexamethasone.

Poster presentation P05

EV corona-associated factors differentially contribute to immuno-modulatory function of AML-EVs on innate and adaptive immunity

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Introduction: Extracellular vesicles (EV) enriched in acute myeloid leukemia (AML) patient's blood were found to contribute to migration, invasion and chemoresistance of AML cells. The mechanisms, however, by which AML-derived EVs promote the immunosuppressive microenvironment remain poorly understood. We and others have recently published that EV functions are partially mediated by factors within a protein corona around EVs. Here we asked if immune functions of AML-EVs depend on corona-associated factors.

Material & Methods: AML-derived EVs from conditioned medium were enriched >100x using sequential rounds of tangential flow filtration (TFF), separating AML-derived soluble factors from EVs. AML patient plasma-derived EVs were isolated by size exclusion chromatography. Purified AML-derived EVs were used as active agent in assays interrogating T cell proliferation and NK cell-dependent cytotoxicity. To determine the contribution of corona-associated factors, EVs were further purified by ultracentrifugation resulting in corona-depleted core EVs (TUCF) and corona-associated factors (solF).

Results: Purified EVs showed a significant dose-dependent inhibition of PHA-stimulated T cell proliferation. Furthermore, cell-line and primary patient-derived AML-EVs were able to reduce NK cell-mediated lysis of target cells. EV corona-associated factors (solF) were responsible for inhibition of cytotoxicity as corona-depleted EVs did not show an inhibitory effect. In contrast, modulation of PHA-stimulated T cell proliferation was independent of corona factors.

Discussion: We show that AML-EVs inhibit T cell proliferation and NK cell functionality with differential contributions of EV corona- and core-associated factors. These findings highlight target-dependent modes of action of AML-EVs and could aid in implementing novel strategies for specific AML-EVs-based therapeutic interventions.

Poster presentation P06

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

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

Poster presentation P07

Comparative analysis of extracellular vesicles derived from SHED and osteogenically differentiated SHED

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Introduction: Restoring large bone defects in orthopedic surgery remains a significant challenge due to limited bone tissue regeneration. While mesenchymal stromal/stem cell (MSC) therapy shows promise, it has certain limitations. An alternative approach involves utilizing extracellular vesicles (EVs) derived from MSCs of diverse origins, which have demonstrated pro-osteogenic properties. Stem cells from human exfoliated deciduous teeth (SHED) represent highly proliferative MSCs capable of differentiating into multiple lineages. They can be conveniently collected and stored during natural teeth replacement. Although SHED-derived EVs have shown promise for bone regeneration, investigations on EVs derived from differentiated SHED cells have been limited. This study aims to compare the characteristics of EVs derived from undifferentiated and osteogenically differentiated SHED cells.

Methods: SHED were subjected to osteogenic differentiation and EVs were separated by UC from both differentiated or not cells. EVs were analyzed by Western blot, NTA, electron microscopy, and their internalization in SHED was confirmed, after PKH67 labeling, by confocal microscopy and FACS analysis.

Results: SHED cells were successfully differentiated into osteoblasts as revealed by FACS analysis, morphology, alkaline phosphatase, and gene expression. Differentiated SHED cells were found to produce smaller-sized EVs. Both confocal microscopy and FACS revealed internalization of both EVs types after 4h incubation.

Conclusion: Obtained results represent the first step in the investigation of the differential impact of EVs from undifferentiated and differentiated cells on osteogenic differentiation and pave the way for the development of targeted strategies utilizing SHED-derived EVs for enhanced bone defect restoration in orthopedic surgery.

Poster presentation P08

Preservation of extracellular vesicles with polyvinylpyrrolidone-based nanofibers

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The use of extracellular vesicles (EVs) for therapeutic purposes is of growing interest. However, their storage conditions are not yet optimized. They are most often stored at -80°C, however the freezing and thawing may damage their structure. Moreover, the frozen storage makes the transport difficult. A solution could be to encapsulate EVs in nanofibers, which provide long-term stability at higher temperatures and easy access to EVs once they are dissolved.

Our aim was to incorporate HEK293T-palmGFP-derived medium sized EVs (mEVs) into water soluble, electrospun polyvinylpyrrolidone-based nanofibers. The presence of mEVs associated with nanofibers was determined by transmission electron microscopy, confocal microscopy and flow cytometry. The stability of EVs encapsulated in nanofibers was investigated for 12 weeks either at room temperature or at 4°C by using a flow cytometer and confocal microscope.

EVs were detected successfully in the nanofibers based on their GFP signal. After sample solubilization, GFP positive particles were successfully identified using a flow cytometer. Compared to free EVs, they were resistant to TritonX-100 lysis, their CD81 accessibility was reduced, while their AnnexinV was not detectable. Regardless of the storage temperature, nanofiber-associated EVs remained detectable throughout the experimental period. While in the case of free EVs, the CD81 signal and the EV number decreased continuously throughout the experiment, in the case of EVs encapsulated in nanofibers, these parameters did not change.

In summary, electrospinning offers a good alternative for fast, cost-effective, reliable storage of EVs. The technique may offer a new opportunity for the development of EV-based therapeutic strategies.

Poster presentation P09

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

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The effectiveness of therapeutic extracellular vesicles (EVs) is limited by their rapid elimination from circulation by the liver. The involvement of different liver cells in this process is not fully known. In addition, the protein corona surrounding EVs may also influence their sequestering to the liver.

HEK293T-palmGFP-derived medium EVs (mEVs) and small EVs (sEVs) were labelled with 99mTc-HYNIC-duramycin. The biodistribution of EVs was analysed by SPECT/CT one hour after intravenous injection in mice. The role of hepatocytes, Kupffer cells and liver sinusoidal endothelial cells (LSECs) in the uptake of EVs was investigated in vitro. To examine the effect of protein corona on the biodistribution, sEVs were incubated with human serum albumin (HSA) or a mixture of HSA-ApoB. We determined the effect of proteins on the size, particle number, TritonX-100 and phospholipaseA2 sensitivity of sEVs.

EVs accumulated mainly in the liver. All tested cell types were able to take up EVs. Kupffer cells were the most effective ones in the uptake of mEVs, while LSECs have taken up sEVs most efficiently. Although no difference was observed in the liver, the ApoB corona significantly reduced EV accumulation in the spleen. The lysis of sEVs by TritonX-100 or phospholipase A2 was reduced by the artificially built HSA-ApoB EV corona.

In conclusion, we provide evidence that different cell types in the liver differentially take up EVs in a size-dependent manner. According to our data, although the ApoB-rich protein corona alters the physicochemical properties of EVs, it does not affect hepatic EV uptake.

Poster presentation P10

Investigation of large-scale production-optimized exosomes in a hepatocellular carcinoma model

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Membrane vesicles released from prokaryotic and eukaryotic cells, such as exosomes, microvesicles, and apoptotic bodies, represent a dynamic extracellular vesicular domain that is increasingly recognized in basic research and translational clinical development. Exosomes are nanovesicles 20-100 nm in size, called natural liposomes. Research into the use of exosomes in therapeutic therapy as delivery systems has increased rapidly since the discovery of exosomes in the 1970s. There is a lot of research focusing on its use not only in oncology, but also in the treatment of cardiovascular diseases, autoimmune syndromes, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, and infections caused by viruses such as tuberculosis, diphtheria, toxoplasma and HIV, and the delivery of drugs and vaccines. In this study, it is aimed to obtain a large amount of exosomes for therapeutic purposes in a short time by producing exosomes in a controlled bioreactor. For this purpose, the human monocyte cell line (THP-1) was adapted to a chemically defined serum-free medium so that the exosomes, which are primarily intended to be used for therapeutic purposes, do not cause cross-reaction. Then, the optimization of cell production in a stirred tank bioreactor, the optimization of the ultrafiltration method, which is more suitable for large scale during exosome isolation from cells, and the characterization of the exosomes are provided. Finally, the therapeutic effect of exosomes was investigated in 3D in vitro hepatocellular carcinoma cell spheroid model.

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Poster presentation P11

Therapeutic Effect of Exosomes Isolated from Bone Marrow-derived Stem Cells on Breast Cancer Spheroids

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Secretion and intercellular exchange of cellular contents by extracellular vesicles (EVs) are common aspects of all cell types. Exosomes, defined as extracellular vesicles, have become important beyond their function of waste transport in terms of contributing to the biological processes of various diseases, including cancer, due to their outstanding biological roles in cell-cell communication. Exosomes secreted from stem cells have recently been stated to have therapeutic effects and functionality similar to their source such as cell differentiation, immunoregulation, angiogenesis, and tumor suppression. Since cell-cell communication plays an essential role in tumor progression and metastasis, the role of exosomes in cancer progression has drawn considerable attention, as well as their therapeutic potential. Here, the isolation of exosomes from bone marrow-derived stem cells (BMSCs) was optimized by using a statistical design to evaluate the maximum isolation of exosomes where, passage number, initial cell concentration, and depletion time in an FBS-free medium were elicited as independent variables. Then, morphological STEM analysis, size and Zeta potential, Western blotting, and FACS analysis for isolated CD9+ exosomes under optimized conditions were performed. Furthermore, CD9+ exosomes were administered at concentrations of 70,000/spheroid to non-invasive (MCF-7/HUVEC) and invasive (MDA-MB-231/HUVEC) breast cancer spheroids, which were developed under microgravity conditions and compared with that of 2µM doxorubicin. Cell viabilities of 80% and 88% both in non-invasive and invasive breast cancer spheroids showed the potential therapeutic application of CD9+ exosomes.

Poster presentation P12

Genetic engineering of the CD81 large extracellular loop for targeted delivery of mesenchymal stem cell derived vesicles for spinal cord injury repair

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Spinal cord injury (SCI) is a devastating condition. Its consequences are related with the acute and chronic inflammatory response, as well as the formation of an inhibitory environment for axonal outgrowth that limits the onset of regenerative processes, resulting in lifelong impairments. Our labs have previously shown that intravenous injections (IV) of mesenchymal stem cell (MSC) secretome lead to improvements in motor phenotype in SCI rodent models and that the large extracellular loop (LEL) of CD81, present in extracellular vesicles, can be engineered towards specific antigen recognition units. Since most of the IV injected extracellular vesicles are captured by organs of the reticuloendothelial system, the aim of this project is to genetic engineer the CD81 LEL for targeting of MSC derived extracellular vesicles towards the post-injury spinal cord environment (myelin and glial scar proteins), maximizing the benefits of systemic administrations of MSC secretome for SCI.

Poster presentation P13

Extracellular vesicles of the probiotic bacteria E. coli O83 activate the immune system and prevent allergy in mice

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Introduction: Escherichia coli A0 34/86 (E. coli O83; serotype O83:K24:H31) is commercially available as a live oral vaccine due to its probiotic properties. When administered orally, it reduces allergic sensitisation, but not allergic asthma. We have shown that intranasal administration of E. coli O83 reduces allergic airway inflammation induced by ovalbumin (OVA) in mice by targeting the airways directly.

Results: Here, we isolated outer membrane vesicles from E. coli O83 (EcO83-OMVs), characterized them and investigated their immunomodulatory properties. EcO83-OMVs are spherical structures about 110 nm in size that contain a parent bacterium-derived cargo, such as LPS and proteins. We identified 136 proteins enriched in EcO83-OMVs compared to the whole bacterium, including several flagellar proteins. Stimulation of human embryonic kidney cells transfected with TLR2, TLR4, TLR5, NOD1 or NOD2 resulted in the production of interleukin-8 indicating that EcO83-OMVs can interact with these receptors. When administered intranasally to OVA-sensitized and challenged mice, EcO83-OMVs reduced airway hyperresponsiveness, airway eosinophilia, and allergen-specific Th2 cytokines in re-stimulated lung and spleen cells.

Discussion: In ongoing experiments, we further investigate the mechanism of the beneficial effect of EcO83-OMVs on allergy and the effects of different purification methods on the immunomodulatory potential of OMVs. We demonstrate for the first time that intranasally administered OMVs from probiotic Gram-negative bacteria have an anti-allergic effect and can serve as a promising tool to modulate immune responses in the host, in a safer manner than live bacteria.

Poster presentation P14

Small Extracellular Vesicles from Human Amniotic Membrane as a potential source in tissue regeneration

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The human amniotic membrane (hAM) has been used in tissue regeneration. A promising alternative is the usage of cell-derived bioactive factors released from the viable native hAM. Regarding the EVs derived from hAM, only little information is available. The aim of this study is to characterize EVs derived from preconditioned human amniotic membrane.

To establish EV enrichment, supernatants from hAM punch biopsies of the placental amnion (P) and reflected amnion (RA) were collected at different time points and subjected to size exclusion chromatography (SEC), characterized for particle concentration and size distribution by nanoparticle tracking analysis (NTA) and fluorescence-triggered flow cytometry (FT-FC) for EV content, size, and composition.

For both P and RA, the number of extracellular vesicles and similar size particles increased after 48 h and 72 h, compared to 24 h of incubation, (for P samples, 24 h = 5.95 x 108 particles/mL, 48 h = 9.2 x 108 particles/mL, 72 h = 1.1 x 109 particles/mL), with particles smaller than 200 nm (mean = 140 nm, SD = 14; n = 3 biological donors). FT-FC confirmed the presence of particles smaller than 200 nm (88 % of EVs). Of the characterized EV surface markers, CD81 was prominent in all samples (38 %, n = 2).

Our results showed information about conditioning time, size, and number of EVs secreted by hAM using SEC to isolate EVs. In our next step, we want to investigate the cargo of hAM-derived EVs pursuing a potential therapeutic approach to support tissue regeneration.

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Poster presentation P15

Characterisation of extracellular vesicles directly in diluted human and canine blood and plasma for therapeutic plasma

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Introduction: Plasma is considered a fluid with potential diagnostic and therapeutic value as regards extracellular vesicles. However, isolation of extracellular vesicles presents a challenge as the samples are sensitive to processing procedures. To minimally disturb the samples, the measurements of the concentration and size of extracellular vesicles were performed directly in blood and in plasma. Also we characterized particles in samples by scanning electron microscopy and cryogenic transmission electron microscopy.

Material and Methods: After blood sampling, human and canine plasma were prepared by centrifugation of respective blood at low centripetal acceleration of the centrifuge rotor. The concentration of extracellular vesicles and their average hydrodynamic diameter were determined by using the interferometric light microscope (Myriade, Paris, France). Samples were also imaged by cryogenic transmission electron microscopy and by scanning electron microscopy.

Results: The average concentration of extracellular particles in plasma was of the order of 10^7/uL and in blood it was of the order of 10^8/uL, and it varied between subjects. The average hydrodynamic diameter in blood and in plasma was between 100 and 200 nm, with a standard deviation of about 80 nm, as determined from several hundred vesicles.

Discussion: Interferometric light microscopy enables a high throughput estimation of concentration and size of extracellular particles directly in human and canine blood and plasma. The morphology of extracellular particles as observed under electron microscopes agrees with the results of the interferometric light microscopy..

Poster presentation P16

Raman spectral signatures of plasma-derived extracellular vesicle-enriched isolates support the diagnosis of different cancerous diseases

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Introduction: Spectroscopic analysis of the molecular composition of small extracellular vesicles (sEVs) is a promising but underexplored method for diagnosing cancerous diseases, particularly central nervous system tumors. Using a sufficient number of clinical samples and Raman spectroscopic analyses, we attempt to elucidate the potential role of plasma-derived sEVs in diagnosing seven distinct patient groups.

Methods: The study is conducted in accordance with the Declaration of Helsinki, informed consent forms are collected and the study was approved by national ethics committee. Up to 490 plasma samples will be obtained from seven patient groups (glioblastoma multiforme, meningioma, melanoma and non-melanoma brain metastasis, colorectal tumors, melanoma and a control group). SEV isolation is performed through differential centrifugation. The isolates are characterized by Western Blot, transmission electron microscopy and nanoparticle tracking analysis. Principal Component Analysis–Support Vector Machine algorithm is performed on the Raman spectra for classifications. Classification accuracy, sensitivity, specificity and the Area Under the Curve (AUC) value are used to evaluate the performance of classification.

Results: According to preliminary results, the patient groups are distinguishable with 80–95% sensitivity and 80–90% specificity. AUC scores of 0.82–0.9 suggest excellent classification performance.

Discussion: Our findings indicate that Raman spectroscopic analysis of sEV-enriched plasma isolates is a promising strategy for the development of noninvasive, cost-effective methods for the clinical diagnosis of various cancers.

Poster presentation P17

Effects of cardiomyocyte-derived extracellular vesicles on gene expression profile of macrophages

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Introduction: The anti-cancer drug doxorubicin (Dox) is known to cause both severe heart damage and local inflammation. Macrophages maintain heart homeostasis and influence the progression and resolution of cardiac injuries. However, the interactions between cardiomyocytes and macrophages remain poorly understood. Extracellular vesicles (EVs) have been identified as important mediators of intercellular communication, carrying specific cargo molecules that influence target cells. As potential biomarkers, EVs have attracted significant scientific interest in the context of cardiovascular diseases (CVDs) and associated inflammatory conditions. Objective: Here we aimed to investigate the effects of cardiomyocyte-derived EVs on macrophage activation. We examined the direct impact 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 the gene expression of macrophages.

Methods: HL-1 mouse cardiomyocytes were treated with Dox or DL-11, and cell viability was assessed with xCELLigence platform. EVs were isolated from the conditioned media by using gravity-driven size filtration and differential centrifugation. The EV concentration and size distribution were determined by using nanoparticle tracking analysis (NTA). Peritoneal macrophages were exposed to isolated cardiomyocyte-derived EVs, and the resulting macrophage inflammatory response was evaluated. RNA was extracted from macrophages, and differences in gene expression were analyzed by using NanoString PanCancer Immune Profiling panel. The differentially expressed genes were further validated by quantitative real-time polymerase chain reaction (Q-RT-PCR).

Results: DL-11 treatment at 500nM concentration was less cytotoxic to cardiomyocytes and macrophages than Dox. EVs derived from cardiomyocytes showed similar size distribution regardless of Dox or DL-11 treatment, but the number of EV particles increased significantly in the case of treated cells. EVs from Dox-treated cardiomyocytes exhibited gene expression patterns associated with early aging and inflammation, while DL-11-treated EVs showed more moderate changes. Notably, the expression of Cdkn1a (Cyclin Dependent Kinase Inhibitor 1) was prominently altered in both groups, with a 7.8-fold increase for Dox-treated EVs and a 4.5-fold increase for DL-11-treated EVs.

Conclusion: Our study of the impact mediated by cardiomyocyte-derived EVs indicated a tendency towards accelerated cellular aging and the presence of an inflammatory phenotype of macrophages. However, this effect was less pronounced when HL-1 cells were treated with DL-11, resulting in fewer significantly altered genes. Additionally, it was noted that macrophages expressed senescent-related genes when exposed to both treatments, although the rate of change was lower and the response to stress signals was less severe in the case of DL-11. These findings suggest that DL-11 may possess a more favorable side effect profile and could potentially be incorporated into a novel anti-tumor therapeutic strategy in the future.

Poster presentation P18

Response of blood cells to Listeriolysin O and its mutant LLO Y406A

Apolonija Bedina Zavec[1]; Rebeka Podgrajšek[1]; Ana Špilak[1]; Maja Jamnik[1]; Matic Kisovec[1]; Gašper Šolinc[1]; Nejc Petrišič[1]; Veronika Kralj-Iglič[2]; Gregor Anderluh[1]; Marjetka Podobnik[1]

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Listeriolysin O (LLO) is a toxin from the intracellular pathogen Listeria monocytogenes and its major virulence factor. LLO is a pore-forming protein, which forms pores in cholesterol-rich lipid membranes of host cells. A distinctive property of LLO is its pH-dependent stability, with the optimum LLO activity at pH 5.5. Mutant LLO Y406A has even more pronounced pH-dependent activation than the wild-type protein, and this property holds its promise as a new potential agent in the development of new anticancer agents. The purpose of our research was to determine the effect of LLO wt and LLO Y406A on blood cells. We compared the response of various cancerous blood cells (K562, Jurkat, Raji) with the response of healthy cells isolated from blood (PBMC). The condition of the cells after treatment with LLO wt and LLO Y406A was determined by means of cell death and tracking of secreted extracellular vesicles (EVs). The results showed that in comparison to LLO wt the toxicity of LLO Y406A to blood cells was very low at physiological pH (7.4). Treatment with LLO Y406A resulted in reduced cell viability by 20% at concentrations 300 to 7000-fold higher than treatment with LLO wt. In addition, LLO wt and LLO Y406A were more toxic to cancer blood cells than healthy blood cells under physiological conditions. Treatment of healthy blood cells with LLO wt resulted in reduced cell viability by 20% at concentrations 1.7 to 300-fold higher than cancer blood cells, and LLO Y406A at concentrations 3 to 30-fold higher than cancer blood cells. As pH decreased, the activity of the proteins continued to increase. Treatment of healthy blood cells with LLO wt at pH 6.5 resulted in reduced cell viability by 20% at concentrations 4-fold higher than cancer blood cells, and LLO Y406A at concentrations 4 to 40-fold higher than cancer blood cells. Increased extracellular vesiculation in response to LLO wt and LLO Y406A was detected at protein concentrations 10 to 100-fold lower than initial cell death. The differences in the toxicity of LLO wt and LLO Y406A to cancer and healthy cells, as well as the increased activity of both proteins at pH 6.5, confirm that both, LLO wt and its mutant LLO Y406A, are of potential interest for medical applications.

Poster presentation P19

Hybridosomes from spruce needle homogenate

Spasovski Vesna[1,2]; Romolo Anna[1,3]; Kisovec Matic[4]; Zagorc Urška[5]; Arrigler Vesna[5]; Arko Matevž[1]; Bedina Zavec Apolonija[4]; Iglič Aleš[3,6]; Kogej Ksenija[5]; Kralj-Iglič Veronika[1]

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Introduction: Being of compatible structure with biomembranes, lipid–based nanoparticles are considered as convenient platforms for drug delivery systems. In the proposed work we considered formation of lipid nanovesicles associated with bioactive phytochemicals from spruce needle homogenate (here called hybridosomes). We formed hybridosomes by mixing appropriate amounts of lecithin, supernatant of isolation of extracellular particles from spruce needle homogenate and glycerol.

Methods: We visualized hybridosomes by light microscopy and cryogenic transmission electron microscopy and assessed them by flow cytometry, dynamic light scattering, ultraviolet–visual spectroscopy and interferometric microscopy.

Results: We found that the particles consisted of a bilayer membrane and a fluid-like interior. Flow cytometry and interferometric light microscopy measurements showed that the majority of the particles were nano-sized. Dynamic light scattering and interferometric light microscopy measurements agreed well with the determined average hydrodynamic radius of the particles Rh (between 140 and 180 nm) while their number densities were in the range between 10^13 and 10^14/mL indicating that hybridosomes present about 2/3 of the mixture, excluding solvent and other small molecules.

Discussion: Simple and low-cost preparation method, non-demanding saving process and efficient formation procedure suggest that large scale production of hybridosomes from lipids and spruce needle homogenate is feasible.

Poster presentation P20

Isolation and analysis of extracellular vesicles from recombinant Lactococcus lactis

Tina Vida Plavec[1,2]; Kristina Žagar Soderžnik[1]; Giulia Della Pelle[1]; Nina Kostevšek[1]; Robert Vidmar[1]; Aleš Berlec[1,2]

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The Gram-positive bacterium Lactococcus lactis represents a well-known system for recombinant protein expression and a promising vector for in vivo delivery of bioactive proteins. Gram-positive bacterial extracellular vesicles (EVs) have recently been described and hypothesized to have a possible physiological role. The bacteria producing recombinant proteins could be used as cell factories for incorporation of functional and bioactive proteins in the EVs with potential health benefits. In this study, we analysed the release of EVs from recombinant L. lactis, which was genetically engineered for different recombinant protein expression.

We followed a standard EVs isolation protocol using an ultracentrifuge at 130.000 × g for 2 hours (Beckman Coulter Optima, XPN-90), discarded the supernatant and harvested the EVs. EVs were characterised with transmission electron microscopy (TEM), flow cytometry and proteomic analysis.

We successfully isolated EVs from recombinant L. lactis expressing different proteins, and characterized them by flow cytometry, transmission electron microscopy and proteomic analysis. Flow cytometry and TEM revealed a difference in the amount of secreted EVs, depending on the recombinant protein that was expressed in L. lactis. The size of the isolated EVs was within the expected nano-scale range, mostly 50 - 200 nm. The presence and quantity of individual recombinant protein inside the vesicle was confirmed by proteomic analysis. We checked the stability of the EVs at -80 °C, and confirmed their preservation after 6 months of storage.

We have shown that recombinant L. lactis can be used to successfully produce EVs. We expressed different recombinant proteins, and shown that they were packaged inside the isolated vesicles. Our study highlights the potential of L. lactis for the production of EVs loaded with recombinant protein with potential therapeutic benefit.

Poster presentation P21

Immunomodulatory Potential of Trophoblast-Released Extracellular Vesicles on Activated PBMCs

Andrea Pirković, Aleksandra Vilotić, Milica Jovanović-Krivokuća, **Maja Kosanović,** Žanka Bojić-Trbojević, Mirjana Nacka-Aleksić

University of Belgrade, Institute for application of nuclear energy, INEP

During pregnancy, trophoblasts express and secrete a battery of immunomodulatory molecules to sustain immune tolerance at the feto-maternal interface. The study aimed to investigate the effects of extracellular vesicles (EVs) released by first trimester extravillous trophoblast HTR-8/SVneo cell line on activated peripheral blood mononuclear cells (PBMCs), and their ability to modulate cellular proliferation and the expression of inflammatory molecules.

EVs were isolated from HTR-8/SVneo cell-conditioned medium and characterized for size, quantity, and exosomal markers using nanoparticle tracking analysis (NTA) and Western blot, respectively. Subsequently, PBMCs were isolated from healthy human donors and activated by PHA.

Following 24h treatment with trophoblast EVs, a significant decrease in PBMC proliferation was observed, providing robust evidence for the inhibitory effects of trophoblast EVs on PBMC activation-induced proliferation. Quantitative real-time polymerase chain reaction (qPCR) analysis revealed a substantial downregulation of proinflammatory cytokines IL-6 and TNF-α, in trophoblast EVs-treated PBMCs compared to control groups. Additionally, our results suggest a protective role for trophoblast EVs in H2O2-induced oxidative stress in PBMCs.

The previous findings shed light on the potential role of trophoblast EVs in maintaining immune homeostasis at the feto-maternal interface and suggest their therapeutic potential for managing inflammatory disorders and pregnancy-related complications. Further investigations are warranted to unravel the underlying mechanisms and to explore the full range of immunomodulatory capabilities of trophoblast EVs.

Poster presentation P22

Engineered bacterial outer membrane vesicles for antibiotic delivery

Leila Pourtalebijahromi[1,2]; Ioulia Exapicheidou[2]; Markus Rothammer[1]; Philip Maier[1]; Eleonora Diamanti[2]; Anna K. H. Hirsch[2,3]; Gregor Fuhrmann[1,2]

[1]Department of Biology, Faculty of Science, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany; [2]Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) – Helmholtz Centre for Infection Research (HZI), Saarbrücken, Germany; [3]Department of Pharmacy, Saarland University, Saarbrücken, Germany

Introduction: Antimicrobial resistance of pathogenic bacteria is one of the growing concerns globally. Nanoparticles (NPs) are potential drug carriers to overcome some of the resistance mechanisms. Outer membrane vesicles (OMVs) are NPs released spontaneously by gram-negative bacteria for various purposes. Our group has previously purified and studied the intrinsic antibiotic effects and biocompatibility of the OMVs from several strains of Myxobacteria. Taking a step forward, we have attempted to engineer these OMVs to enable targeting, tracking, and drug loading.

Methods: Ciprofloxacin was loaded into SBSr073 OMVs via passive drug incubation or hybridization with ciprofloxacin-loaded liposomes. The drug and particle concentration were measured using liquid chromatography-mass spectrometry and nanoparticle tracking analysis, respectively. In addition, the potential of Cbfe23 OMVs' surface for being modified with bioorthogonal reactions was studied. First, azide groups were installed on the OMVs, which further reacted with either of the two designed linkers carrying a dye and biotin. In each step, the efficiency of the reactions was measured by measuring the fluorescence intensity of the modified particles.

Results: With the incubation method, the ciprofloxacin concentration was 2.1±1.1 ng/1.0E10 particles, while it was 20.0±5.1 ng/1.0E10 particles for the hybrid vesicles. In the first step of surface modification, the installation of 201±25 azide groups on the Cbfe23 OMVs was confirmed. Further, it was revealed that surface modification with the trans-cyclooctene-containing linker is more successful because the reaction of methyltetrazine and trans-cyclooctene was more efficient compared to that of methyltetrazine and norbornene in the experimental settings.

Conclusion: OMVs of myxobacteria show great flexibility for antimicrobial drug delivery. We successfully loaded a model antibiotic into SBSr073 OMVs via different strategies. Moreover, in a proof-of-concept study, surface modification of the Cbfe23 OMVs was achieved based on bioorthogonal reactions. Yet, the efficiency of the engineered OMVs needs to be investigated in further steps.

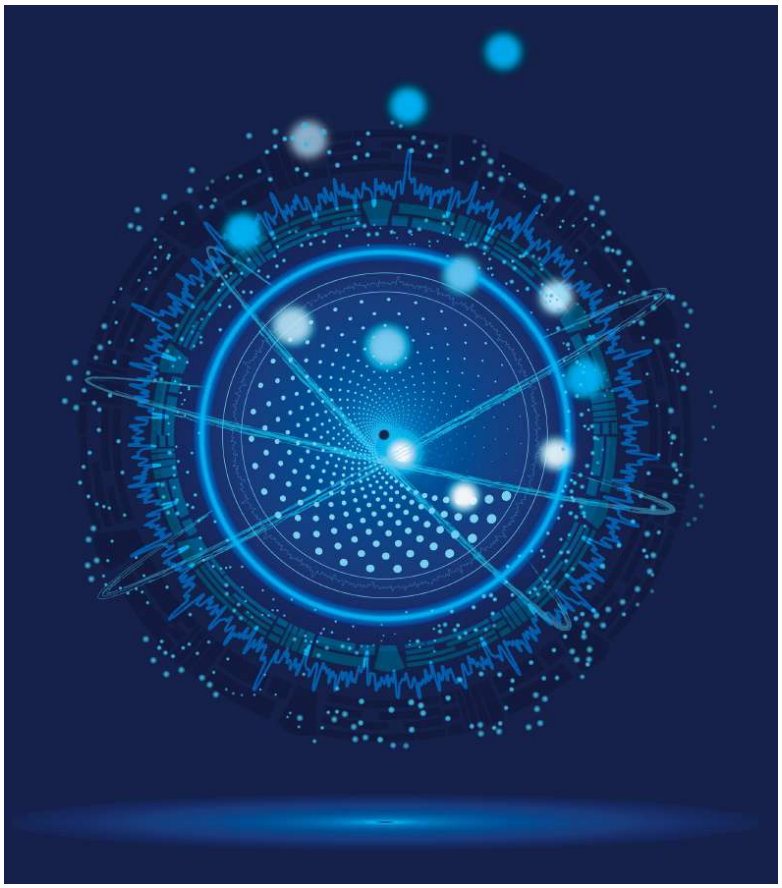
Acknowledgments: This work was supported by a grant from the Federal Ministry of Research and Education of Germany (NanoMatFutur grant, 13XP5029A) and a European Research Council starting grant (Gels4Bac; 945602).

Methodology Advances in EV analysis

Monday, 4th September 2023

13:30 - 15:00

Chairs: Beate Rinner (Austria) + Sofija Glamocllija (Serbia)



Keynote presentation

Methods to study EVs – what do we (need to) look for?

Pia Siljander

University of Helsinki, Helsinki, Finland

• Pia R-M Siljander is a principal investigator of the EV group and an adjunct professor at the University of Helsinki. Pia did her biochemistry Master's at the University of Helsinki. During her PhD at the Wihuri Research Institute, she focused on collagen receptor interplay in platelet activation, which started her ongoing work on platelet-derived extracellular vesicles (EVs) with her first EV paper in 1996 on microparticles. After her Postdoc at the University of Cambridge U.K., she returned to the University of Helsinki as a senior university lecturer at the Faculty of Biological and Environmental Sciences, Molecular and Integrative Biosciences research program. As a principal investigator from 2011, her continuing interest is to explore the biophysicochemical and functional versatility of platelet and cancer EVs and to develop methodologies for the EV field. The latter led to the founding of the world's first EV core facility in 2016. Her research is driven both academically and through industry-collaboration. She has published over 50 EV-related publications, 22 of which have been cited > 100 times, has an H-index of 38 according to Google scholar, and was cited over 15 000 times during the past 5 years. Pia is the vice president of the Finnish Society of Extracellular Vesicles. •

Extracellular vesicles (EVs) are lipid bilayer-enclosed nanoparticles which ferry biological cargo such as metabolites, proteins, RNAs and DNAs relevant for local and distant cellular communication or waste management. EVs have since been found to play a role in development, homeostatic regulation as well as in major diseases including cardiovascular and neuronal diseases and cancers. Thus, there is a huge interest in their use in theranostic applications. However, basic EV biology also still requires intensive research, due to the vast heterogeneity of the EV populations (from 30 nm to several micrometers) impacted by spatiotemporal regulation of subpopulations and cargo dynamics. Due to the small size and yield of EVs, we are still heavily analysing EV batches vs being able to analyse single EVs in a time-dependent and cell location/tissue -specific manner.

The phases of EV methodologies can be divided into preanalytical, isolation and post-isolation analytics. The EV field has now recognized that all these stages can heavily impact on the results, and importantly, their reliability and comparability. Hence there is an ongoing major endevour to increase standardization in the EV field and to develop materials (standards and test samples) to improve data quality and increase transparent reporting.

Our research focus, the heterogeneity of platelet-derived extracellular vesicles provides an excellent example to illustrate the methodological challenges and (some) solutions. Using our platelet EV research and experiences from the world's first EV core facility (Est 2016), I will discuss the analytical methods from different perspectives. The aim of my talk is to spark discussion on what kind of analytical data/information is needed in the EV field and are we able to obtain it with our current methodologies. Do we have trustworthy work horses, or do we have to go after glittering unicorns? What should we look (out) for?

Oral presentation

Tracking individual extracellular vesicles: from purification analysis to intracellular co-localization using atomic force- and single-molecule fluorescence microscopy

Jaroslaw Jacak[1]; D. Sivun[1]; S. Puthukodan[1]; M. Hofmann[1]; M. Mairhofer[1]; H. J anout[2]; J. Schurr[2]; F. Hauser[1]; C. Naderer[1]; J. Preiner[1]; **Stephan Winkler[2]**

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Extracellular Vesicles (EVs) are nanometer-sized particles enclosed in lipid bilayers that are released by cells and contain lipids, metabolites, proteins and nucleic acids. They are linked to numerous cellular processes, including physiological and pathophysiological intercellular communication, immune modulation and inflammation.

We present a toolbox for comprehensive localization of EVs, their cellular uptake dynamics and nanoscopic 3D co-localization to cell organelles at the single molecule level. Small EVs isolated from transfected HEK293T cells. There, the N-terminus of the tetraspanin protein CD63 is labelled with a green fluorescent protein (eGFP). The combination of Atomic Force Microscopy and 2D Single Molecule Fluorescence Microscopy (SMFM) and 3D Single Molecule Localization Microscopy (SMLM) was used to characterize fluorescently labelled endosome-derived EV population in terms of size, elastic modulus and number of eGFPs per EV. We performed two-color 3D SMFM to track EVs and simultaneously visualize the cell membrane. The trajectories obtained allowed us to decipher a variety of EV diffusion inside and outside the cell. In a post-uptake showcase study using two-colour 3D SMLM and a new analysis software, we measured the nanoscopic 3D co-localisation of EVs with transferrin, which is involved in early endosome recycling. Stepwise photobleaching analysis was performed and provided information on EV clustering inside the cells.

Quantification of aggregation, uptake dynamics and co-localization of uptaken EVs with cellular compartments and proteins at the single-molecule level is essential for deciphering biological processes associated with EVs at the individual cell level.

Oral presentation

Substrate stiffness modulates extracellular vesicles' release in triple-negative breast cancer models

Beatrice Senigagliesi[1,2]; **Pietro Parisse**[2,3]; **Loredana Casalis**[2]

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Introduction: Small extracellular vesicles (sEVs, 30 to 200 nm) are increasingly being recognized as potent messengers in intercellular communication. Indeed, sEVs can mediate both cell-cell and cell-matrix communication by transferring oncogenic molecules that promote proliferation, migration, invasion, and metastatic spreading. Here we focus on the inter-relation between extracellular matrix (ECM) and sEVs, highlighting the quite unexplored aspect of the influence of the ECM stiffness on sEVs release.

Methods: Here, we use Triple Negative Breast Cancer (TNBC) cells to study the sEV release from cells plated on substrates at different stiffness. We plated MDA-MB-231 cells on two collagen coated Polydimethylsiloxane (PDMS) substrates at different stiffness (0.2 and 3.6 MPa), comparing them with the glass substrate, and then we isolated sEVs by differential ultracentrifugation. After a careful control of the cell growth conditions (vitality, morphology by immunofluorescence microscopy, stiffness by Atomic Force Microscopy (AFM)) we performed a multi-parametric analysis based on complementary techniques (AFM, Nanoparticle Tracking Analysis, and Asymmetric Flow Field Flow Fractionation with a Multi-Angle Light Scattering detector) for the TNBC-derived sEV characterization.

Results: We observe that soft substrates induce TNBC cell softening and rounding. This effect promotes the release of a high number of sEVs, differing also in average dimensions (larger from cells grown of soft substrates), as observed by the multiparametric analysis.

Discussion: Here we showed how ECM physical properties have an important role in the regulation of sEV-mediated signaling in a TNBC model. The effect needs further investigation to unravel the molecular mechanisms regulating it, but already represents a step towards a better understanding of ECM-cell-sEVs crosstalk.

Oral presentation

Quantitative serum pharmacokinetics of EVs from diverse sources using high content single vesicle imaging

Maria Jaritsch[1]; Tanja Plank[1]; Melanie Schürz[1]; Joachim Danmayr[2]; Cristian Tudor-Matea[1]; Anna Müller[1]; Jana Kiefer[1]; Laurens Kober[1]; Eva Klinglmayr[1]; Vesna Stanojlovic[1]; Petra Reinthaler[3]; Angelika Sales[3]; Martin Wolf[4]; Dirk Strunk[4]; Mario Gimona[4]; Martin Hintersteiner[5]; Nicole Meisner-Kober[1]

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Introduction: Research on extracellular vesicles (EVs) as therapeutics or for drug delivery exploded in recent years, but knowledge of quantitative in vivo pharmacokinetics/dynamics (PK/PD), bioavailability, and biodistribution is still limited. Here, we present a simple, robust, and miniaturized method for quantifying the pharmacokinetics of administered EVs directly in serum.

Method: Different EV-labelling strategies with fluorescent dyes were characterized for their selectivity, efficiency, stoichiometry, and serum stability in-vitro, and a miniaturized assay was developed using high-content single vesicle imaging (HC-SVI) to quantify labeled EVs directly in serum. To investigate the serum pharmacokinetics of EVs from different sources (HEK, bovine milk, goat milk, umbilical cord), labeled vesicles were administered intravenously to mice. Serum from various timepoints was analyzed by HC-SVI and quantified by EVAnalyzer (Schuerz M., 2022).

Results: Using covalent fluorescent labeling, the miniaturized HC-SVI assay was validated in mouse, rat, non-human primate, and human serum. NIRF dyes provided the best signal-to-noise, resulting in a detection limit of 1-5x10e7 EVs/mL serum. The possibility to quantify EVs directly in serum avoids any bias from additional EV extraction methods and allowed to reduce sample volumes to below 20µL, allowing multiple bleedings per mouse and significantly reducing animal numbers. Applying this assay to EVs from various sources revealed a uniformly short serum half-life (T1/2 5min) tested in mice, which was also confirmed in non-human primates for the most relevant sample. This suggests rapid extravasation into tissues rather than clearance, which we confirmed by histological liver sections (abstract submitted; Müller A.).

Conclusion: This new HC-SVI serum assay allows quantification of the circulation half-life of EVs from different sources and in various species by using small amounts of serum without further purification and only standard laboratory equipment, fluorescence microscopes and an open source software.

Oral presentation

Quantitative proteomics and nFCM analysis of subpopulations of immune cell-derived EVs

Anna Lischnig[1], Nasibeh Karimi[1], Rossella Crescitelli[2], Karin Ekström[2], Cecilia Lässer[1]

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Introduction: Small EVs (S-EVs) are the most studied EV subpopulation, but less is known about other subpopulations e.g. large EVs (L-EVs). Consequently, subpopulation markers are missing. Therefore the aim of our study was to compare the differential protein expression between EVs of different sizes and densities.

Methods: HMC-1 and THP-1 were cultured and L-EVs (16,500xg, 20min) and S-EVs (118,000xg 2.5h) were isolated by differential ultracentrifugation and purified by a density cushion. Low density EVs (LD; 1.11 g/mL) and high density EVs (HD; 1.17 g/mL) were collected from both L-EV and S-EV. Purity, morphology, and yield of EVs were determined by nanoparticle tracking analysis (NTA), protein measurement, and transmission electron microscopy (TEM). The proteome of the different samples was analysed with quantitative mass spectrometry and nFCM.

Results: TEM and NTA showed different size and concentration between EV subpopulations. In total 3972 and 4735 proteins were quantified in HMC-1 and THP-1, respectively. PCA showed that the EV subpopulations were well separated. Several protein groups were enriched, among these groups we found that mitochondrial proteins were enriched in L-EV LD. KIF proteins were enriched in the L-EV HD. Tetraspanins ESCRT and ADAM proteins were enriched in the S-EV. Interestingly, nFCM showed that ADAM10 was exclusively detected on vesicles that were also positive for any of the three tetraspanins CD63, CD81 or CD9.

Conclusion: This study identified several protein groups differently enriched in L-EV and S-EV, which validate previous findings. In addition the proteome of EVs isolated at different densities had significant differences.

Methodology Advances in EV analysis

– poster presentations –

Poster presentation P23

Analysis of EV research over the past decade: Cargo and function correlate with EV source and isolation method

Rodolphe Poupardin[1], Martin Wolf[1], Nicole Maeding[1], Liliia Paniushkina[1,2], Sven Geissler[3], Paolo Bergese[5], Kenneth W. Witwer[2], Katharina Schallmoser[5], Gregor Fuhrmann[6], Dirk Strunk[1]

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Introduction: The evolution of extracellular vesicle (EV) research enabled fascinating discoveries by introducing nanotechnology into biomedical cell communication science.

Material & Methods: Through a text mining-oriented comprehensive analysis of 20,364 PubMed-recorded EV-related entries from 2013-2022, we undertook an impartial investigation to map out the development of EV isolation techniques, phenotype, cargo, and functional analysis over the past decade.

Results: We discovered a significant connection between EV isolation, cargo and function, suggesting an inherent link between EV production methods and their practical use. Blood/plasma/serum, urine, and cell culture media emerged as the most investigated sources of EVs. We observed a notable increase in studies that integrated 2-4 methods for EV isolation, characterization, or functionality. However, out of 2,386 papers providing particle counts, only 156 (5.9%) reported a normalized particle-protein ratio.

Discussion: To promote transparency within the field, we compiled an online database of more than 20,000 open access EV publications, allowing users to carry out detailed targeted searches within specific publication sections such as methods or results (www.myevcheck.org). We also introduced an online checklist to standardize method and result reporting, thereby fostering further research and development. Completion of the checklist generates a downloadable PDF with a summary, intended for the benefit of scientists, journal editors, and reviewers in this rapidly evolving field.

Poster presentation P24

Removal and identification of external protein corona members from red blood cell derived extracellular vesicles by surface manipulating host defense peptides

Imola Csilla Szigyártó[1]; Priyanka Singh[1]; Maria Ricci[1]; Anikó Gaál[1]; Livia Fülöp[2]; Judith Mihály[1]; Diána Kitka[1]; Lilla Túriák[1]; László Drahos[1]; Zoltán Varga[1]; Tamás Beke-Somfai[1]

[1]Research Centre for Natural Sciences; [2]University of Szeged, Hungary

Extracellular vesicles (EVs), in the last years have shown great interest and extreme potential in biomedical applications. Increasing number of studies suggest that a prote in corona could adhere to the surface which can have a fundamental effect on function, on their targeting and thus on their therapeutical efficacy. However, removing and routinely identifying these corona members is currently a challenging task to achieve.

The interactions of EVs with host defense peptides (HDPs) were followed by flow-linear dichroism spectroscopy, size exclusion chromatography, microfluidic resistive pulse sensing and proteomics analysis.

Based on these results, HDPs can be categorized based on their action mechanism: some remove the surface proteins, others can penetrate into and some can disrupt the vesicle. TEM images demonstrate a smoother EV surface lacking protein aggregates for those who display "carpet model" mechanism type activity. When comparing the identified proteins for the control and treated EVs, we have assigned seventeen proteins as protein corona members, which were removed from EV surface by at least two HDPs in separate experiments. These proteins include plasma membrane components or have cytosol, cytoplasm origin, which are likely to associate with the surface of EVs during biogenesis.

Our results envisage the applicability of HDPs as a useful tool in specific surface engineering of nanoparticles from both synthetic and natural origin.

This work was funded by the National Research, Development and Innovation Fund: 2020-1-1-2-PIACI-KFI_2020-00021, TKP2021-EGA-31, KKP_22 -144180 and by Eötvös Loránd Research Network: SA-87/2021, KEP-5/2021.

Poster presentation P25

Development of synthetic reference materials for the optical detection of extracellular vesicles

Zoltán Varga; Anikó Gaál; Mohammed Al-Khafaji
Research Centre for Natural Sciences, Budapest, Hungary

Introduction: Extracellular vesicles (EVs), key entities in intercellular communication and potential diagnostic biomarkers, necessitate accurate size and concentration determination. In comparison to traditional polystyrene beads, this study explores hollow organosilica beads (HOBs) as improved reference materials for flow cytometry analysis of EVs.

Methods: HOBs were synthesized utilizing a hard template sol-gel method. To further refine our approach, we also developed mesoporous HOBs by modifying the original synthesis method and incorporating a surfactant, facilitating the production of a mesoporous structure. To enhance their functionality and detection capabilities, the surface of these mesoporous HOBs was modified using 3-aminopropyl(diethoxy)methylsilane (APDEMS). This surface modification was then followed by a labeling procedure using fluorescein isothiocyanate (FITC), which led to the creation of fluorescent HOBs, increasing their potential for use in sophisticated optical detection systems.

Results: The HOBs exhibit monodisperse size distribution and homogeneous shell thickness, scattering significantly less light than similarly sized solid beads. The scattering intensity of HOBs overlaps with EVs, leading to more accurate EV concentration measurements within size gates set by HOBs.

Discussion: HOBs resemble EVs in structure and light scattering properties, and can set size gates independent of a flow cytometer's optical configuration. They offer a practical reference material to standardize EV measurements across instruments and laboratories. Furthermore, our work also highlights the optimized synthesis of porous hollow organosilica particles with amino surface modification and fluorescent labeling, offering an advanced tool for more precise optical detection of EVs.

Poster presentation P26

Optimization of EV Upstream Process Using PATFixTM Analytical Tools

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Extracellular vesicles (EV) are secreted by any kind of cells. To study their potential production in a lab scale is sufficient. The choice for the most suitable plain medium supplement is important to provide sufficient nutrients for cell expansion and EV secretion. Often an animal derived product like FBS is initially chosen which can be purchased at reasonable costs and with acceptable growth performance of cells. Unfortunately, FBS contains EV that cross contaminate the EV preparation. To study produced EVs, cross contamination of FBS derived EVs is unwanted. Multiple companies offer exosome-depleted FBS (EV (-) FBS) generated in a variety of approaches to prevent EV cross contamination.

In this work we evaluated commercially available EV (-) FBS supplements for residual EV content and tested their upstream performance in respect to secreted EV content. The analysis was performed with a PATfix™ Biochrom System capable to detect fluorochrome coupled EV marker after inline separation of EVs by SEC as an analytical method (IF-SEC).

Poster presentation P27

Identification of extracellular vesicle interactors at the blood-brain barrier in vitro by proximity-dependent biotinylation

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Introduction: Small extracellular vesicles (sEVs) are released by cells and present in body fluids. Having reached the blood-brain barrier (BBB), sEVs can trigger interactions with brain capillary endothelial cells (BECs) [1]. At the BBB, molecular components involved in sEV internalisation and transport are as-yet-unidentified. We aim to investigate these molecular interactors.

Material & Methods: Systemic cancer and brain cell sEVs were labelled with CellTracker™Orange and their effects on barrier tightness and uptake were assessed. Selected cell lines were transfected with plasmid containing GPI-anchored GFP-biotin ligase (BioID-GPI and TurboID-GPI). Western blotting detected biotinylation in sEVs. Biotinylated sEVs were applied to immortalised BECs – hCMEC/D3. Lysates were harvested and biotinylated proteins were captured by magnetic streptavidin beads. Interactome will be revealed by proteomics - mass spectrometry and bioinformatic analysis.

Results: The transwell setup was optimised for sEV/BEC studies using 1.0 µm pore size inserts and 1% BSA in the receiver compartment. Interactions of sEVs from seven different cell lines showed different effects on the BBB model dependent on sEV cell origin and donor compartment. Western blots detected biotinylated proteins along with recombinant protein in transfected cells and sEVs. From sEV-BBB cell lysates, proteomics will reveal interacting cellular surface candidates whose role will be validated by pathway-blockers.

Discussion: We established and characterised stable cell lines releasing sEVs expressing BioID-GPI and TurboID-GPI. Tracking sEV pathways in BECs will enable us to determine sEV interactors and elucidate their fate inside cells, which could pave the way for cancer treatment or drug delivery application into the brain.

This work was founded by the Austrian Science Fund FWF (project P 34137-B).

Poster presentation P28

Development of a robust protocol for EVs isolation from non-invasive biomaterials for diagnostic application

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Introduction: Extracellular vesicles (EVs) possess key roles in cell-to-cell communication in health and disease. Their presence in most tissues and body fluids makes them an excellent target for biomarker discovery. Here we are developing a theranostic Parkinson´s disease (PD) biomarker to select candidate patients for vaccination and monitor their response. We selected, PD driving oligomeric α-synuclein (α-syn) as primary diagnostic target. Not knowing the location of α-syn inside EVs or as surface cargo, we apply methods preserving natural EV corona enabling extended downstream biomarker analysis.

Materials and methods: EVs were enriched from healthy donor urine (uEV) via tangential flow filtration (TFF). Influence of storage at -80°C on EV quantity and size was analysed by tunable resistive pulse sensing (TRPS), and quality by tetraspanin+ biomarker expression in dot plots and super-resolution microscopy.

Results: Small uEVs with a mode size 89.75±0.59 nm (mean±SEM) were isolated by TFF. EV CD9/63/81 tetraspanin expression did not show significant differences before/after cryo-storage despite 63-86% loss of particle number from 3.0±0.5x1010 to 1.6±1.0x1010 particles/mL (mean±SEM) after thawing. Spiking -syn-EVs as a representative biomarker into uEVs enabled comparing both sources for setting up a sensitive detection assay. Titration of a-Syn-EVs will determine optimum pre-analytic conditions for subsequent biomarker detection based on ISEV uEV task force recommendations.

Discussion: Developing new theranostic biomarker analysis methods from easy accessible non-invasive sources is important for patient acceptance and study performance. We develop a standardized protocol regarding sample retrieval, pre-processing, storage and EV isolation for biomarker studies.

Poster presentation P29

Specific Extracellular Vesicle Detection and Isolation in Complex Samples using FO-SPR

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FOx Biosystems NV

Introduction: We introduce an automated method for the specific detection, quantification and isolation of extracellular vesicles in complex samples, such as culture medium or blood plasma, using fiber-optic surface plasmon resonance (FO-SPR).

Material & Methods: FO-SPR was performed on WHITE FOx analytical instrument. MCF7 cell-derived EVs were analyzed using anti-EpCAM as capture antibody and anti-CD9, anti-CD63, anti-CD81 as detection antibody on 100-fold diluted plasma. Recombinant EVs were detected in DMEM cell medium supplemented with 10% exosome depleted fetal bovine serum using anti-CD63 as capture antibody and anti-CD9 as detection antibody. For calibration curves and positive controls, EVs were spiked in respectively the 10% FBS supplemented cell culture medium, or in 100 x diluted pooled plasma from healthy donors.

Results: The potential of the bioassay to detect MCF7 cell–derived EVs in 100-fold diluted blood plasma was investigated using an anti-EpCAM as capture antibody and an anti-CD9/anti-CD63/anti-CD81 combination antibody mix as detection, obtaining an LOD of 1.1 × 10^8 particles/mL. Furthermore, the specificity of the bioassay was shown by the absence of signal when analyzing plasma samples from 10 healthy donors compared to a spiked sample.

Discussion: With the FO-SPR technology it is feasible to detect and quantify EVs directly in complex matrices, with minimal processing, thanks to its dip-in configuration. FO-SPR has a great potential for sensitive EV analysis, particularly for analyzing EV subpopulations with specific membrane proteins (biomarker), with LODs much lower than the normal EV concentration in human plasma of patient samples.

Poster presentation P30

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

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

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

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

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

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

Poster presentation P31

Is it possible to detect HER2-positive extracellular vesicles in human plasma?

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Introduction: Extracellular vesicles (EVs) are produced in both physiological and pathological conditions, where tumor cells produce significantly more vesicles. However, tumor-derived EVs represent only a small fraction of the total amount of EVs in peripheral blood. For this reason, we aimed to find a method to detect small amounts of HER2-positive EVs in plasma from patients with breast cancer.

Methods: EV isolation from BT-474 cell line supernatant and plasma from both healthy donors (n=1) and patients with HER2-positive (n=3) and triple-negative breast cancer (TNBC) (n=3) were performed using IZON qEV1 70 nm columns. To determine detection limit, EVs from BT-474 were spiked into 0.1ml of plasma from healthy donor. Particle and protein concentrations were measured by NTA, nFCM and Qubit protein assay. HER2+ EVs very analysed by nFCM using CD340-APC antibody.

Results: EVs isolated from BT-474 cells showed 20% HER2 positivity, and EVs from healthy donor plasma were 0.6% positive. HER EVs were detected in the spike-in samples in a dose-dependent way, and the positive events were close to the expected values. There was no significant differences in total protein or number of particles between samples from healthy donors/TNBC or HER2. In samples from HER2+ breast cancer, EVs with HER2 positive events were observed (0,7-1,7%), but not statistically significant from patients with TNBC (0,5-1,5%) or healthy donors (0,6%).

Conclusion: As HER2-positive EVs can also be found in healthy individuals, a detection method should be sensitive enough to measure small changes in a certain protein expression on EVs. nFCM is a promising technique, however further optimizations are needed to enhance sensitivity and more experiments are required to test the detectability of HER2 EVs in larger patient cohorts.

Poster presentation P32

Microfluidics as tool for the analysis of the size distribution of plasma-derived microsomal fraction

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Introduction: The amount and composition of secreted vesicles in human blood can vary depending on the patient's health status. Microfluidic methods enable the analysis of the size distribution of floating particles based on their diffusion characteristics (influenced by shape, size and density etc.). Thus, microfluidic channels might be a potential and inexpensive method for analyzing EVs from different sources.

Methods: During our investigations, microsomal fraction was isolated from human plasma with SEC. The samples were fluorescently labeled with a protein marker (Atto 488 NHS ester), then the free dye was removed using another SEC step. The labeled EV samples were examined using two types of microfluidic channels. In the first type, the sample is focused to the center of a straight channel by loading buffer from both sides. The second type of channel is based on dean flow dynamics. In a spiral-shaped channel, the sample is pressed against the outer curve, by loading buffer from the inner curve side. This device has three outlets enabling the collection ofthree fractions with different size ranges. The intensity of the fluorescent signal was examined in cross-sections with increasing distance from the inlet.

Results and Conclusion: Various patterns in fluorescent intensity were detected upon the analysis of the composition of the microsomal fractions isolated from samples of patients with different oncological statuses rising the hope, that the method can be further improved for diagnostic and prognostic applications.

Poster presentation P33

Benefits of lactadherin based membrane labelling in EV fluorescent microscopy

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Extracellular vesicles (EVs) are phospholipid bilayer enclosed particles in the extracellular space without the ability of self-replication. As the phospholipid membrane is the key defining component of EVs, its visualisation during microscopic observations is essential. During studies on EV biogenesis, it is also important to define the location of plasma membrane of the EV releasing cells. The latter makes it possible to decide whether an EV-specific marker is present inside or outside of the cell during immunocytochemistry. Since an ideal membrane labelling does not exists, here we tried to find an optimal method (fit for purpose), which may help to follow the release of medium and large size EVs during confocal microscopy.

Lactadherin is a phosphatidylserine binding protein. Its advantage over annexin V is its Ca2+ independent binding. The lactadherin-based labelling is therefore, more stable during immunocytochemistry than any annexin V based method.

We conjugated the lactadherin protein with fluorophores and tested the membrane- and EV labelling properties on different fixed cell lines (such as HEK293, HEK293T-palmGFP, HepG2, HT29, H9c2, HeLa). The results were analysed by Leica SP8 Lightening confocal microscope.

Based on our data, lactadherin specifically labelled the external plasma membrane leaflet of fixed but not permeabilized cells. As phosphatidylserine is enriched in the EV membrane, the detected fluorescence signal of EVs was even stronger than the one of the plasma membrane. Thus, according to our data, application of lactadherin-based labelling is highly recommended during fluorescence microscopy of EV studies.

Poster presentation P34

High resolution scanning electron microscopy (SEM) of extracellular vesicles in breast tumor matrix

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Introduction: Extracellular vesicles are released by cells that are implicated in various biological processes, including cancer. In the study presented here, scanning electron microscopy (SEM) has been used to reveal presence, distribution and density of extracellular vesicles (EVs) in breast tissue tumor. Aim: The aim of our study was to visualize the native arrangements of extracellular vesicles association with the extracellular matrix in breast tumor by SEM.

Material and Method: The tumor tissue was surgically removed and followed by a core biopsy to obtain the tumor and surrounding tissue. The tissue was immediately chemically fixed (4% paraformaldehyde, 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated and died) and prepared for inspection by SEM (SEM, JEOL JSM-6500F, Tokyo, Japan). Permission for this study was received from the Commission for Medical Ethics of University Medical Center of Maribor.

Results: We provide evidence that SEM could be used for detailed inspection of vesicles in solid tumor. Our results confirm that conventional SEM could be valuable supplementary tool to advanc our understanding of extracellular vesicles in the local environment of tumor tissue.

Discussion: We suggest that the morphological markers such presence and abundance of matrix nanovesicles in different parts of tumor could be used as a complementary tool to monitor the state and progress of tumor. The application of reliable intuitive methods like 3D imaging could significantly contribute to translation of scientific findings to clinical practice.

Acknowledgement to COST Action "Focused Ion Technology for Nanomaterials" (FIT4NANO) CA19140.

Poster presentation P35

Microalgae communities – assay for in-vivo effects of extracellular particles and hybridosomes

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Introduction: Unicellular planktonic algae are well suited for bioassays because they are easily cultured and sensitive to organic and inorganic substances. We examined the effect of hybridosomes composed from soyabean lecithin, glycerol and supernatant from isolation of extracellular particles (EPs) from spruce needle homogenate on the conditioned media of marine microalgae strain Phaeodactylum tricornutum.

Methods: To prepare hybridosomes, needles from Picea abies tree were homogenized in ultraclean water and EPs were isolated by centrifugation whereas filtered supernatant was mixed with soyabean lecithin and glycerol. Number density of microalgae was assessed by flow cytometry. Number density and hydrodynamic diameter of hybridosomes and extracellular particles were assessed by interferometric light microscopy. We performed microbiological analysis of the samples. Microalgae were imaged by the scanning electron microscope. Hybridosomes were imaged by cryogenic transmission electron microscope.

Results: At microbiological testing we confirmed monoculture with presence of coliform bacteria and fungi. The number density of microalgae was of the order of 10^3/micro L and the number density of extracellular particles was of the order of 10^6/micro L. In the treated samples we observed considerable increase of the number density of microalgae and of extracellular particles with respect to control (untreated) samples in the interval of 13 days. The proportion of microalgae to other microorganisms in the samples remained largely unchanged during the follow up.

Discussion: Microalgae communities proved a convenient system for in vivo effect. Hybridosomes from spruce needle homogenate accelerated growth of microalgae Phaeodactylum tricornutum communities.

Poster presentation P36

Isolation and characterization of extracellular vesicles from pleural effusion samples of patients with advanced non-small cell lung cancer

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Introduction: The incidence of lung cancer (LC) in Serbia has increased over the last three decades. Up to 40% of patients with advanced non-small cell lung cancer (NSCLC) develop pleural effusion (PE). The aim of this study was to evaluate the usability of existing methods for isolation of extracellular vesicles from PE samples of patients with advanced non-small cell lung cancer and to characterize them for further use in the clinical/diagnostic/research setting.

Material & Methods: PE samples diluted in PBS (1:1) from patients with advanced NSCLC were used. In-house spherical porous methacrylate-based copolymer coupled with VHH antibodies was used for the isolation of extracellular vesicles (EV) from PE samples. Flow-cytometry was performed for detection of exosomal markers.

Results: A pool of PE was prepared from 5 patients with advanced NSCLC. Flow cytometry confirmed that the isolation of EVs was successful using the in-house affinity chromatography method. The presence of CD9 antigen was detected, as well as a decrease in the signal after the addition of Triton X-100. Further plans include the analysis of CD63 and CD81 antigens using flow cytometry, NTA analysis to determine the number and diameter of obtained vesicles, as well as TEM and SEM to determine their morphology.

Discussion: We aim to investigate for the first time whether this method is applicable to pleural effusion samples as a new cancer liquid biopsy sample type. We also plan to evaluate the method in comparison with a commercial isolation kit.

Poster presentation P37

Surface-Associated Glycans as a Possible Distinct Factor for Establishing the Molecular Properties of Prostasomes

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Glycans, complex carbohydrates bound to lipids and proteins, play important roles in biological processes. Prostasomes, extracellular vesicles from prostate epithelial cells, have a glycan composition influenced by their cellular origin and by the composition of the seminal plasma into which they are secreted. We hypothesized that the membrane-associated glycans could be used as selective targets for separation of prostasomes and as relevant parameter for distinction of their populations.

Prostasomes from seminal plasma of normozoospermic and oligozoospermic men were separated by ion-exchange- and lectin-affinity chromatography using concanavalin A (ConA) lectin, specific for mannosylated structures, and wheat germ agglutinin (WGA), specific for sialic acid. The presence of tetraspanins (CD63, CD9 and CD81), galectin-3 (gal-3) and gamma-glutamyl transferase (GGT) as known vesicle markers were monitored in association with distinct glycans. Their distribution was also analysed upon treatment of prostasomes with non-ionic detergent.

Membrane-associated GGT in the context of Con A- and WGA-reactive glycans mark prostasome populations from normozoospermic and oligozoospermic men. The assembly of tetraspanins, gal-3, and distinct N-glycans defines the solubilisation signature of prostasomes. WGA-reactive glycoproteins co-localize with CD9 and gal-3 in detergent-resistant domains, whereas ConA-reactive glycoproteins were distributed in detergent-sensitive domains along with CD63 and GGT. Subtle differences in the composition/presentation of examined molecules made difference among vesicles sharing the same physical properties in each group as well as between them.

The results obtained suggest the potential of glyco-parameters as reference markers for EVs populations.

Poster presentation P38

EVAnalyzer, an Open- Source ImageJ Plugin for automated, quantitative, high content single vesicle imaging: further developments in standardization

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Introduction: With the rapid growth of the EV field, rigorous characterization, and quantitation at the single vesicle level has become of increasing importance. Robust methods are essential to improve comparability and reliability of published data. Guidelines and data on calibration strategies have been published for flow cytometry-based quantification of EV sizes and fluorescence intensities at different instruments, using commercially available calibration beads or reference EVs. Emerging single vesicle imaging technologies provide a complementary opportunity for in-depth and quantitative EV characterisation.

Methods: We recently developed 'EVAnalyzer', an ImageJ plugIn for automated, quantitative single vesicle analysis from diverse and large sets of imaging data. Additionally, we established a robust protocol for capture, (immuno-) labelling and fluorescent imaging of EVs using exclusively standard reagents and laboratory equipment. The process has been optimised and validated for a number of routine EV applications (Schürz et al, JEV 2022) and is continuously being extended for new functions and applications. Here we describe the EVAnalyzer tool with a focus on new applications and features, including the standardization using flow cytometry calibration beads.

Results: Diverse applications of EVAnalyzer will be described, including the quantification of EV subpopulations based on immunostaining, validation of EV labelling reagents or optimisation of genetic EV engineering by determining population fractions and loading densities at the single vesicle level. Further we show new developments for EV quantification in histological sections or serum, as well as size and intensity calibrations.

Summary/Conclusion: We propose that EVAnalyzer can be used in virtually every lab for routine high-content single vesicle quantification and characterisation supporting both in vitro and in vivo applications in EV and nanoparticle research. Additional calibration using reference beads will enable to standardise EV quantifications across different labs.

Poster presentation P39

Quantitative histology of EVs from diverse sources on frozen tissue sections with high-content imaging

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Introduction: Pharmaceutical applications of EVs have gathered enormous attention due to their breakthrough potential as both therapeutics and drug delivery vehicles. Quantitative information on bioavailability and biodistribution, however, remains limited, with scarce side-by-side comparisons of diverse EV sources. We developed a simple and robust histology and automated high-content imaging workflow for quantification of EVs in tissue down to the single-vesicle level. The method is generically applicable to EVs from diverse sources and enables quantitative investigation of EV spatial biology and biodistribution.

Material & Methods: EVs from HEK293 cells, cow milk, goat milk and umbilical cord MSCs were covalently labelled with two orthogonal NIRF dyes and administered i.v. to mice. Livers were PFA-fixated, OCT-embedded and whole-tissue cryosections were subjected to automated high-resolution widefield imaging. EV signals were quantified by EVAnalyzer (Schürz et al. 2022).

Results: EVs from all sources processed with our workflow were well detected and quantifiable. The use of NIRF dyes was essential for robust discrimination between EV signals and tissue autofluorescence. In line with their short half-life in serum, the highest number of EVs was generally observed in liver sections after 5 min; differences between sources were observed in peak liver concentrations, clearing rates and spot morphology.

Discussion: We successfully established a workflow allowing quantification of EVs from diverse sources in cryosections using NIRF dyes and automated high-resolution widefield imaging. This workflow requires only standard laboratory equipment and open-source/open-access software (EVAnalyzer), enabling generic quantification of EVs in tissue to link pharmacokinetics, biodistribution and spatial biology of EVs in-vivo.

Poster presentation P40

Towards clinical-grade stem cell-derived extracellular vesicles (EV)

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Phoenestra GmbH, Linz, Austria

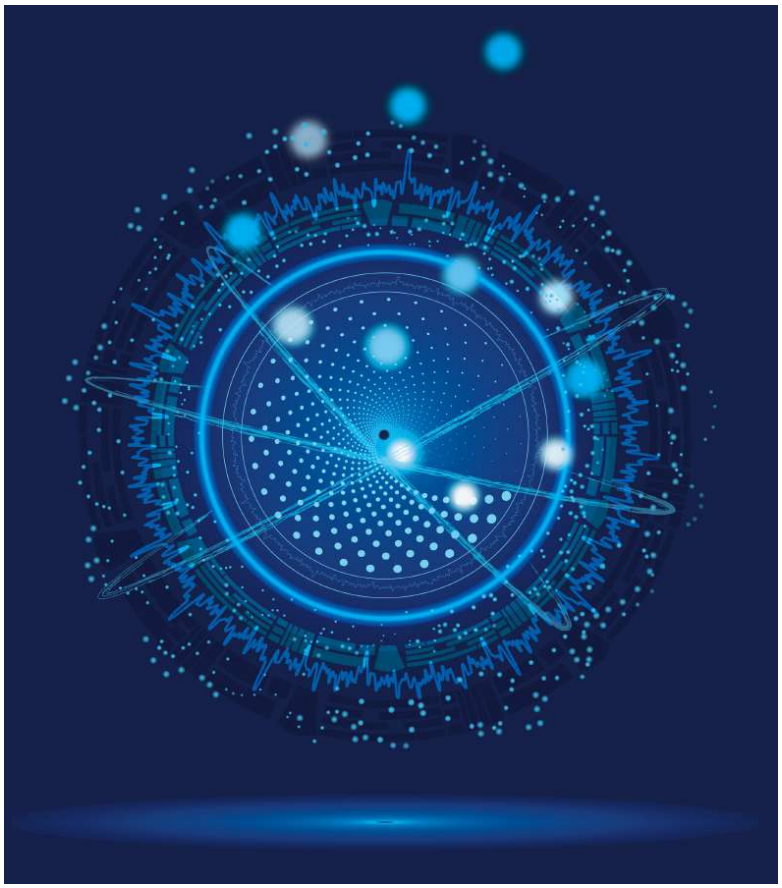
Stem cells and stem cell-derived products, such as extracellular vesicles (EV), provide an outstanding therapeutic perspective for multiple diseases. To ensure translation into viable products, established lab-scale cell proliferation procedures have to be evolved into scalable manufacturing processes. Phoenestra currently develops technology platforms to meet this rising demand of the gene and cell therapy field. We have developed cultivation processes for induced Pluripotent Stem Cells (iPSCs) that apply shake flask cultivation as intermediate scale to seed bioreactors which are then used in perfusion mode to reach cell densities in the million per milliliter range and also respective EV particle productivities. Multipotent Mesenchymal Stromal Cells (MSCs) are promising for therapeutic applications in various diseases and tissue repair (immunomodulatory and regenerative properties), but scalable and consistent cultivation of these adherent and sensitive cells is challenging. We have developed a stable process that uses telomerized MSC lines and cell carriers inside an agitated packed bed bioreactor system. With this system we are able to produce clinically meaningful EV quantities in small bioreactor volumes and a fairly short amount of time. Based on scalable processing and robust handling procedures we employ orthogonal analytical methods in addition to cell-based bioassays to better understand biological functionalities of EV preparations generated from our cell lines and processes. In this presentation, we will share insights from current processing and EV characterization work.

NEWS from INDUSTRY and development – “Rising projects”

Monday, 4th September 2023

15:30 - 16:40

Chairs: Dirk Strunk (Austria) + Pia Siljander (Finland)



Industry presentation

NTA Goes Colocalization: Characterization of Multi-labelled Bionanoparticles

Clemens Helmbrecht

ParticleMetrix

Industry presentation

Quantitative analysis of single EV and their subpopulations with super-resolution solutions

Mehdi Madi[1] and Quentin Lubart[2]

[1]Product Sales Specialist, Abbelight; [2]Project Owner, Senior Scientist, DIAG, Abbelight

Host presentation

EV Technologies at the MedUni Graz

Core Facilities MedUni Graz

Project presentation

Extracellular vesicle in exercise: sporty messengers in interorgan communication

BioTechMed consortium "iNterAcD+"

Project presentation

Fetal immune priming by placenta-derived small extracellular vesicles

Christian Wadsack and Michaela Klaczynski

Project presentation

Patient derived tumormodels, EVs & oncolytic virus

Beate Rinner and Mariangela Garofalo

Special guest presentation

Translation of EV into the clinics

Eva Rohde

Good Manufacturing Practice Laboratory, Spinal Cord Injury and Tissue Regeneration Center Salzburg, Paracelsus Medical University, Salzburg, Austria; Department of Transfusion Medicine, Paracelsus Medical University, Salzburg, Austria.

• Eva Rohde is Head of the Department of Transfusion Medicine at the University Hospital of the Paracelsus Medical University in Salzburg, Austria. She received her M.D. from the Karl-Franzens University of Graz, Austria in 1994, finished training in Transfusion Medicine in 2005 and spent several years in stem cell research as a postdoctoral fellow. Her research focuses on the application of mesenchymal stromal cell-based therapies with special emphasis on their extracellular vesicles (MSC-EVs). Eva Rohde is Founding Member of the Spinal Cord Injury and Tissue Regeneration Center Salzburg (SCI-TRECS), Austria and Director and Qualified Person of the SCI-TReCS GMP Unit. The GMP Unit in Salzburg has achieved the pharmaceutical manufacturing authorization for MSC and MSC-EVs in 2015 and participated a clinical trial testing MSCs in multiple sclerosis. Recent effort is directed towards the preclinical characterization and the regulatory requirements for the clinical assessment and future application of stem-cell based products, including stromal cell-derived extracellular vesicles (EVs). In vitro and in vivo potency assays to define the immunomodulatory, neuroprotective, neuroregenerative and scarless healing support of MSC-EVs are currently developed.

Eva Rohde is involved in the scientific and organizational management of the Transfer Center EV-TT (Extracellular Vesicles Theralytic Technologies; https://evtt.pmu.ac.at/en/) and activities that consolidate nanovesiccular therapeutics in Salzburg (CONSONANT). EVTT is funded by the European Union and the state of Salzburg and treads on new grounds towards the development of so-called Exosome Therapies. It has pioneered the focused activities on theralytic technologies for Nanovesicles. The overarching topic is the development of novel cell-free therapies in conjunction with scientifically solid application-oriented analytical technologies. •

Extracellular vesicles from mesenchymal stromal cells (MSC-EVs) are being investigated as novel promising biologic drug candidates.

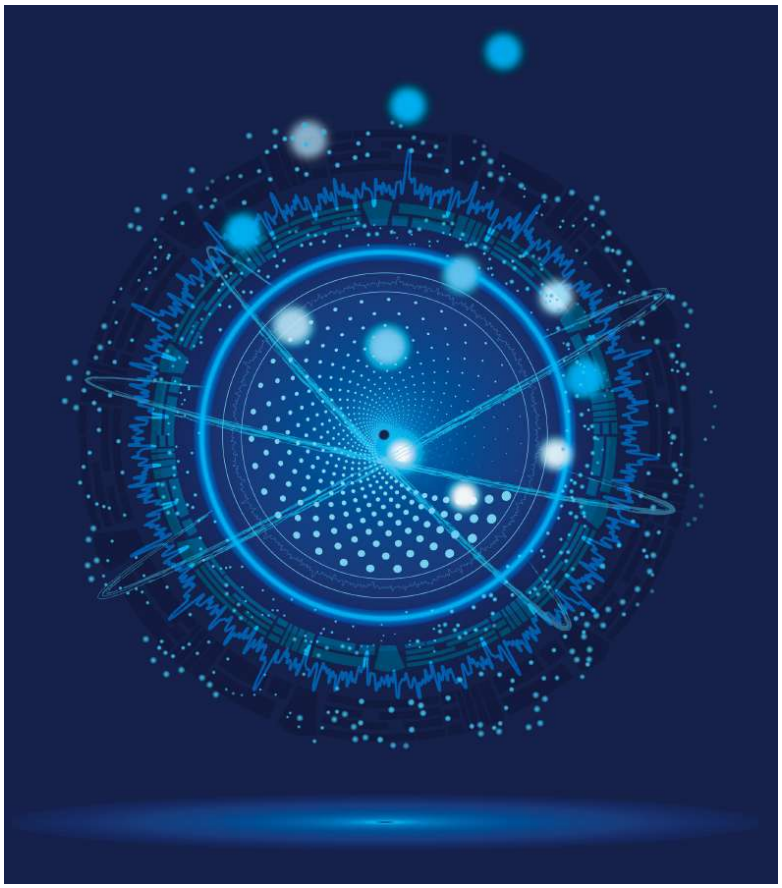
In her talk, Eva Rohde will highlight the pharmaceutical and clinical development of naïve umbilical cord-derived MSC-EVs as candidate therapeutics since about 10 years. Therapeutic concepts for selected target diseases are based on observed anti-inflammatory, anti-fibrotic, and neuropretictive biological activities of MSC-EVs in various in vitro and in vivo models. Non-clinical safety and efficacy data required for clinical evaluation include pharmacodynamic, pharmacokinetic and toxicological results for the selected routes of administration and specific indications. Pilot clincal experiments to confirm the EV-associated prevention of secondary tissue damage following acute traumatic injury or chronic (neuro)degeneration and the developmental road for first-in-human clinical trials are presented.

EV numbers and cargo

Monday, 4th September 2023

09:00–10:45

Chairs: Maja Kosanovic (Serbia) and Nicole Maeding (Austria)



Keynote presentation

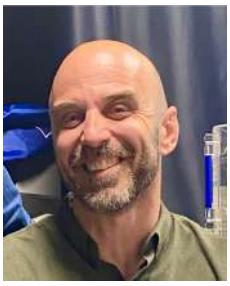
Extracellular vesicles by the numbers

Paolo Bergese

Department of Molecular and Translational Medicine, Università degli Studi di Brescia, Italy

IRIB - Institute for Research and Biomedical Innovation of CNR, Palermo Italy

CSGI - Italian Center for Colloid and Interface Science, Firenze, Italy



- Paolo Bergese Full Professor of Chemistry at the University of Brescia, Associate Researcher at the Institute for Research and Biomedical Innovation of the National Research Council (CNR) and member of the Italian Center for Colloid and Interface Science (CSGI). In 2010 and 2012 he has been visiting Professor at the Massachusetts Institute of Technology (MIT).

Totally fascinated by biogenic (extracellular) nanoparticles, he funded and heads the bioCSI – biogenic colloid surfaces and interfaces – lab at the Dep. of Molecular and Translational Medicine at the University of Brescia; a multidisciplinary team featuring one of the first stories of integration of chemistry, nanotechnology and molecular biology in extracellular vesicle research. He also established within the CSGI, a lab network with the critical know how and facilities for advanced physicochemical characterization of extracellular nanoparticles. Among the projects he coordinated, worth of note are the Horizon 2020 FET projects: evFOUNDRY – The extracellular vesicle foundry (http://www.evfoundry.eu/) and BOW – Biogenic Organotropic Wetsuits (https://www.bowproject.eu/). •

The talk will be about the role of having a feeling for neglected/unnoticed numbers associated with EV biology, including stoichiometry, times and energies.

This will pass through developing a sense of the kinds of physicochemical models that can be built and put in action to explore, with different eyes, the available biochemical and biological (quantitative) data landscape and to spot/extract such numbers.

Overall, I hope to finally demonstrate how physical chemistry is an important aid to advance intuition and insight in EV research, also sharpening the interplay between theory and experiment.

Oral presentation

Cardioprotective role of extracellular vesicle-mediated mir-sponge transfer

Hargita Hegyesi, Balázs Hornyák, Benedek Nagy, Ákos Molnár Csendom, Krisztina Pálóczi, Ágnes Kittel, Edit I Buzás
Department of Genetics, Cell- and Immunobiology, Semmelweis University, Budapest, Hungary

Hypoxia of the myocardium induces changes in the myocardium that can be mediated by extracellular vesicles (EVs). Our previous results suggest that hypoxia induces an increase in the expression of miR-223 and Heart Related circRNA (HRCR) in EVs of neonatal cardiomyocyte (NCMC). HRCR, acting as miR sponge, is able to bind miR-223. The aim of our experiments is to demonstrate the uptake of vesicularly delivered HRCR and miR-223 and to explore the effect of transfered miR-223 on target cells.

Primary NCMC cultures were subjected to 4 h of hypoxia and 16 h of reoxygenation (H/R). EVs were isolated and their concentration was determined by NTA. Expression of HRCR and miR-223 were determined by RT-qPCR, and binding of miR-223 on circular RNA was confirmed by a pull-down assay. NPR3 as a potential target of mir-223 was identified by PCR array. Isolated EVs were incubated with naive NCMCs and the dose-dependence of NPR3 expression was measured by ELISA.

Hypoxia induces a decrease in the expression of NPR3 in NCMCs. Hypoxia increases the release of sEV. H/R sEVs decrease NPR3 expression in acceptor cells, whereas this effect is not significant in the case of H/R mEVs. The miR-223-mimic significantly reduces NPR3 3'UTR sequence-coupled luciferase activity.

Our present model suggests that NCMC-derived EVs are enriched in non-coding RNAs upon hypoxia, and HRCR acts as a miR sponge to promote miR-223 delivery. The miR-223 content of EVs is capable of downregulating the NPR3 expression upon entry into the target cell. Natriuretic Peptide Receptor C, encoded by the NPR3 gene, is responsible for the removal of natriuretic peptides from the circulation, and has been shown to have cardioprotective effects. Thus, we hypothesize that EVs released by the hypoxic myocardium are able to induce cardioprotective effects through inhibition of the NPR3 expression.

Oral presentation

Small RNA biomarker profiling from extracellular vesicles in immune-mediated inflammatory diseases

Christa Noehammer[1]; Lucia Ciglar[1]; Gabriel Vignolle[1]; Samuel Elias Pineda Chavez[2,3]; Silvia Schoenthaler[1]; Maarten Pruijt[4], Iqra Yousaf[1]; Michaela Hendling[1]; Manuela Hofner[1]; Walter Pulverer[1]; Andrea Cafarelli[5,6]; Leonardo Ricotti[5,6]; Robert Haesler[7]; Stephan Weidinger[7]; Geert D'Haens[4]; Stefania Vetrano[2,3]; Klemens Vierlinger[1]; Christa Noehammer[1]

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Introduction: Immune mediated inflammatory diseases (IMIDs) are a heterogeneous group of conditions featured by a dysregulated immune response leading to destructive chronic inflammation. The immune dysregulation can affect various organ systems (e.g. gut, skin) resulting in significant morbidity, reduced quality of life and premature death. As there are no reliable disease progression and therapy response biomarkers currently available, ImmUniverse, a large, EU-funded project, is aiming to develop such biomarkers in two IMIDs, namely atopic dermatitis (AD) and ulcerative colitis (UC) not least by means of small RNA/microRNA profiling in blood-derived extracellular vesicles (EVs).

Materials & Methods: small RNA sequencing was performed on total RNA which has been isolated from plasma- or serum-derived EVs. Analysed samples comprised atopic dermatitis patients, healthy controls as well as blood samples derived from ulcerative colitis patients before or after treatment with biologicals. In addition, mouse plasma samples were investigated for the effect of Low-Intensity Pulsed Ultrasound (LIPUS) as a novel and safe approach to enhance the mucosal release of EVs in experimental models of UC.

Results: We identified a variety of potential EV-derived small RNA biomarkers in AD and UC retrospective patients and could nicely show the impact of LIPUS on EV release into mouse plasma.

Discussion: EV-derived small RNA biomarkers are a novel and promising source of diagnostic biomarkers in IMIDs but by no means are limited to this type of conditions as we also demonstrate by exemplarily showcasing some within AIT identified epigenetic biomarkers for coronary artery disease.

Oral presentation

Systematic investigation and classification of membrane active peptide peptides based on their affinity for interaction with extracellular vesicles

Tasvilla Sonallya[1]; Imola Cs. Szigyártó[1]; Tünde Juhász[1]; Edit I. Buzas[3,4,5]; Delaram Khamari[3]; Kinga Ilyes[3]; Zoltán Varga[2]; and Tamás Beke-Somfai[1]*

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Introduction: Host defence peptides (HDP) are promising biomaterials with antimicrobial and anticancer applications. By disturbing or lysing the cell membrane, they carry out their biological role. These peptides show numerous types of membrane interaction mechanisms i.e., carpet, toroidal pore, and barrel stave. Cell penetrating peptide find application in cargo loading and uptake of small molecules and nanoparticles. The interactive mechanism of these peptides has been studied widely with model membranes however our knowledge with extracellular vesicles (EV) is scarce. There are several aspects where EV – HDP interactions could be relevant, ranging from cooperative presence on infection sites functions to EV cargo loading.

Materials & Methods: 28 well-known peptides were selected, and their interaction with REV was investigated using several techniques from membrane biophysics.

Results: The biophysical studies demonstrated that that some of the MAP mechanisms were vesicle-penetrating, while others lytic, or lead to removal of protein corona. More specific, Melittin has strong membrane disrupting effect whereas, ll37 which has lower disrupting affinity on the original REV composition. Hence, based on their in-depth investigation using biophysical techniques, the binding affinity with extracellular vesicles was studied and categorized as low binding affinity, medium binding affinity and high affinity. This initial categorization gives further insight into its specific interactive mechanism.

Summary/Conclusion: These results give an insight into an overview of the surface interactions of membrane active peptides with the REVs help us to attain a broad perspective on the molecular level interactions which could, in turn, provide vital information on engineering the surface and the interior of Evs with short MAPs.

Funding: This work was funded by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund, financed under the TKP2021-EGA-31 and the 2020-1-1-2-PIACI-KFI_2020-00021 funding schemes. Support from Eötvös Loránd Research Network, grant n.o. SA-87/2021, is also acknowledged.

Oral presentation

Extracellular Vesicles (EVs) miRNA-cargo loading and alterations after ionizing radiation induced cellular stress

Ilona Barbara Csordás[1,2]; Tünde Szatmári[1]; Kinga Győryné Galgand[1]; Lourdes Cruz-Garcia[3]; Tamás Visnovitz[4]; Christophe Badie[3]; Katalin Lumniczky[1]

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EVs participate in intercellular communication by delivering bioactive molecules, e.g miRNAs. EV-miRNA-cargo is determined by the donor cell status, thus cellular stressors, such as ionizing radiation (IR) exposure modify its composition. We demonstrated in vivo, that IR-modified-EVs induce radiation related damages in the non-irradiated cells, and EV-miRNAs contribute in this process. To better understand the EV-cargo loading mechanisms, the miRNA profile and miRNA-cargo packaging proteins of bone marrow cells (BMC) and BMC-derived EVs were examined and compared.

BMCs and BMC-EVs were isolated 24h after irradiation (0.1, 3Gy) of CBA mice. miRNAs were examined by qPCR. miRNA packaging proteins were analyzed by qPCR and WB, their cellular localization with confocal microscopy. Bioinformatics tools were used to link RNA binding proteins and miRNAs.

EVs miRNA profile alterations did not follow the pattern observed in BMC cells, indicating the existence of the selective miRNA sorting. After IR, the level of certain RNA-binding proteins was modified both in BMCs and EVs, but with a different pattern, which correlated with the detected subcellular redistribution of the proteins. Changes in the level of particular miRNAs carrying recognition motifs for specific RNA-binding proteins followed the quantitative changes of their respective binding partner protein in EVs.

Different miRNA profiles in BMC and BMC-EVs, along with a strong correlation between the level of RNA-binding proteins and the concentration of their binding miRNAs in EV, support the existence of selective miRNA packaging mechanisms. Our results also indicate, that IR affects mechanism of miRNA sorting and incorporation into EVs.

Oral presentation

Characterization and Interindividual Variability of Plasma Extracellular Vesicles in Healthy Adults

Marija Holcar1, Ivica Marić2,3, Tobias Tertel4, Katja Goričar1, Nina Mavec1, Bernd Giebel4 and Metka Lenassi1

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Blood plasma extracellular vesicles (pEVs) are an important source of EV biomarker research, yet their characteristics in healthy individuals remain poorly understood. This study aimed to analyze pEVs of healthy adults and investigate which parameters affect the interindividual variability of pEVs.

Blood and clinical (height, weight, blood pressure, blood type, menopause), demographic (sex, age), lifestyle (exercise, smoking) characteristics were collected from 208 healthy blood donors (ethically approved, informed consent). Complete blood count was performed in blood, CRP and insulin, were analysed from blood sera, free haemoglobin, and plasma factor 4 were analysed in plasma. Imaging flow cytometry was used to phenotype pEVs (25 surface markers) directly in plasma. pEVs were enriched from all plasma samples (ultracentrifugation on a 20% sucrose cushion), then analyzed for concentration (NTA) and 37 surface proteins (MACSPlex). pEV characteristics and correlation with subject parameters were statistically analysed.

Study subjects, evenly distributed by sex and age, exhibited a normal range of blood parameters. The average pEV concentration was 2.3*10^7/mL, but substantial interindividual variability was observed within the same EV type. There was up to a 218-fold difference between medians of different EV types. The concentration of pEVs correlated with subject parameters such as sex, smoking, menopausal status, and blood type. Following enrichment, CD9+/CD63+/CD81+pEVs predominantly displayed blood-cell and endothelial markers, with the highest signal from platelet-derived pEVs. Smoking was associated with increased platelet and endothelial-derived CD9+/CD63+/CD81+pEVs but decreased leukocyte-derived CD9+/CD63+/CD81+pEVs.

These findings highlight significant interindividual variability in pEV concentrations among healthy adults, with correlations to specific patient characteristics.

EV numbers and cargo

– poster presentations –

Poster presentation P41

Characterization of tissue factor-bearing extracellular vesicles in COVID-19 provides evidence for platelet-leukocyte aggregate formation

René Weiss[1], Tanja Eichhorn[1], Silke Huber[2], Marie Ebeyer-Masotta[1], Marwa Mostageer[1], Robert Emprechtinger[3], Reinhard Würzner[2], Viktoria Weber[1]

[1]Center for Biomedical Technology, Department for Biomedical Research, University for Continuing Education Krems, Krems, Austria; [2]Institute of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria; [3]Faculty of Health and Medicine, University for Continuing Education Krems, Krems, Austria

Introduction: Severe COVID-19 is frequently associated with thrombotic complications, which can result in multiple organ dysfunction. There is evidence that increased platelet activation and platelet-leukocyte aggregate formation induces tissue factor (TF) expression on leukocytes in severe COVID-19 patients. In this study, we characterized TF-bearing extracellular vesicles (EVs) and their cellular origin in plasma from COVID-19 patients.

Material & Methods: A total of 12 patients with SARS-CoV-2 infection admitted to the intensive care unit (Hospital Zams) requiring mechanical ventilation (time course, 134 samples in total) and 25 healthy controls were included in this study. Plasma was obtained by centrifugation of whole blood anticoagulated with EDTA at 2000 x g for 15 min. EVs were characterized by flow cytometry using a CytoFLEX LX device (Beckman Coulter) with a combination of PC7-conjugated anti-CD41 as platelet marker, PB-conjugated anti-CD45 as leukocyte marker and FITC-conjugated anti-TF (clone VD8). APC-conjugated Annexin V was used as marker for EVs exposing phosphatidylserine.

Results: EV counts, as well as TF+ EVs, were increased in COVID-19 patients as compared to healthy donors (median [IQR]: 204,000 [122,375-450,000] vs. 53,500 [43,750-70,500] EVs/µl; 18,925 [10,053-33,028] vs. 1880 [1335-3945] TF+ EVs/µl). In COVID-19 plasma, 55.5 [42.9-69.2] % of all EV aggregates (CD41+CD45+) co-expressed TF, whereas a co-expression of only 18.3 [0.0-45.6] % was detected in healthy donors (3974 [1838-8382] vs. 57 [0-140] CD41+CD45+TF+ EVs /µl).

Discussion: To our knowledge, this is the first report on TF+ EVs carrying both, platelet and leukocyte markers, mirroring enhanced platelet-leukocyte aggregate formation in COVID-19 patients.

Poster presentation P42

CDK6 regulates extracellular vesicles in AML

Belinda S. Schmalzbauer[1]; Astrid Digruber[2]; Martin Maw[3]; Silvio Kau[4]; Markus Zojer[1]; Gerwin Heller[5]; Zuzanna Kieron[1]; Jonatan Kendler[1]; Karin Hummel[6]; Ingrid Walter[3]; Waltraud Tschulenk[3]; Elisabeth Gamper[1]; Monika Ehling-Schulz[2]; Veronika Sexl[1]; Thorsten Klampfl[1] and Karoline Kollmann[1]

[1]Institute of Pharmacology and Toxicology, University of Veterinary Medicine, Vienna; [2]Institute of Microbiology, University of Veterinary Medicine, Vienna; [3]Independent researcher; [4]Institute of Morphology, University of Veterinary Medicine, Vienna; [5]Department of Medicine I, Division of Oncology, Medical University of Vienna; [5]Vetcore facility for proteomics, University of Veterinary Medicine, Vienna

Introduction: Extracellular vesicles (EVs) are linked to various diseases with implications in the progression, immune activation, biomarkers and therapy. Acute myeloid leukemia (AML) is a highly aggressive disease with elevated EVs in serum of patients. One crucial regulator and prognostic biomarker for AML is the cell cycle regulator Cyclin-dependent kinase 6 (CDK6). Palbociclib, a CDK4/6 kinase inhibitor, is already FDA-approved for breast cancer and in clinical trials for AML, however the exact mechanisms that are induced and potential combinatorial treatments remain to be elucidated.

Material and Methods: Utilizing a novel murine in vitro model of AML, we analyzed the effects of CDK6 inhibition on cellular states. Transcriptomic changes were measured by RNA-Seq experiments and CDK6 interaction partners were identified by mass spectrometry and westernblots. We measured EVs by nanoparticle tracking analysis, flow cytometry, electron microscopy and mass spectrometry and validated our findings in human AML cell lines.

Results: Human cancer cells are co-dependent on CDK6 and components of intracellular vesicle pathways. CDK4/6 kinase inhibition in AML results in deregulated expression of genes assigned to intracellular vesicles and vesicle trafficking. Inhibition of CDK6 increases EV release of KMT2A-MLLT3+ AML cells and alters the EV size distribution. We identified several proteins of the endosomal-exosomal pathways as CDK6 interactors and detected high levels of CDK6 at endosomes, lysosomes and on EVs.

Discussion: These data identify a novel function of CDK6 at intracellular and extracellular vesicles and propose an important role of CDK6 as a regulator of intercellular communication in AML.

Poster presentation P43

Circulating endothelial extracellular vesicle signatures correspond with ICU requirement: An exploratory study in COVID-19 patients

Johannes Zipperle[1]; Johannes Oesterreicher[1]; Helena Thumfart[1]; Madhusudhan Reddy Bobbili[1]; Johannes Grillari[1]; Marcin F. Osuchowski[1]; Herbert Schöchl[1]; Wolfgang Holnthoner[1]; Christoph J. Schlimp[1]; Matthias Hackl[2]; Teresa Lara Krammer[2]; Marion Wiegele[3]; Judith Schiefer[3]; Marco Valerio Pesce[3]; Stefan Ulbing[3]; Johannes Gratz[3]

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Extracellular vesicles (EVs) represent nanometer-sized, subcellular spheres, that are released from almost any cell type and carry a wide variety of biologically relevant cargo. In severe cases of coronavirus disease 2019 (COVID-19), states of systemic pro-inflammatory activation, EVs, and their cargo can serve as conveyors and indicators for disease severity and progression. This information may help distinguish individuals with a less severe manifestation of the disease from patients who exhibit severe acute respiratory distress syndrome (ARDS) and require intensive care measures. Here, we investigated the potential of EVs and associated miRNAs to distinguish normal ward patients from intensive care unit (ICU) patients (N = 10/group), with 10 healthy donors serving as the control group. Blood samples from which plasma and subsequently EVs were harvested by differential ultracentrifugation (UC) were obtained at several points in time throughout treatment. EV-enriched fractions were characterized by flow cytometry (FC), nanoparticle tracking analysis (NTA), and qPCR to determine the presence of selected miRNAs. Circulating EVs showed specific protein signatures associated with endothelial and platelet origin over the course of the treatment. Additionally, significantly higher overall EV quantities corresponded with increased COVID-19 severity. MiR-223-3p, miR-191-5p, and miR-126-3p exhibited higher relative expression in the ICU group. Furthermore, EVs presenting endothelial-like protein signatures and the associated miR-126-3p showed the highest area under the curve in terms of receiver operating characteristics regarding the requirement for ICU treatment. In this exploratory investigation, we report that specific circulating EVs and miRNAs appear at higher levels in COVID-19 patients, especially when critical care measures are indicated. Our data suggest that endothelial-like EVs and associated miRNAs likely represent targets for future laboratory assays and may aid in clinical decision-making in COVID-19.

Poster presentation P44

Hypercholesterolemia alters the extracellular vesicle secretion of cardiomyocytes

Csenger Kovácsház[1]; Szabolcs Hambalkó[1]; Tamás G. Gergely[1]; Csilla Pelyhe[1]; Dóra Kapui[1]; Alexander L. Hültenschmidt[1]; Éva Pállinger[2]; Edit Buzás[2]; Ádám Zolcsák[3]; Bálint Kiss[3]; Tamás Bozó[3]; Csilla Csányi[3]; Nikolett Kósa[3]; Miklós Kellermayer[3]; David Matallanas[4]; Kieran Wynne[4]; Péter Ferdinandy[1,5]; Zoltán Giricz[1,5]

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Introduction: Hypercholesterolemia (HC) leads to systemic and myocardial inflammation and disrupts cardiac stress adaptation. Extracellular vesicles (EV) play role in both mechanisms. However, how HC affects cardiovascular EV communication is unknown. Aim: To analyse, how HC affects circulating and cardiomyocyte (CM)-secreted EVs.

Materials & Methods: Male Wistar rats were fed with high-cholesterol (HiChol) or control chow for twelve weeks, and platelet-free plasma was collected. EVs were isolated using density gradient ultracentrifugation followed by size exclusion chromatography. Both plasma and EV samples were analysed with MxP® Quant 500 metabolomics kit. AC16 human CMs were treated with Remembrane® HC supplement or with its vehicle or with FBS-free medium and then EVs were isolated using ultracentrifugation. Particle concentration was measured using nanoparticle tracking analysis, biophysical parameters were analysed with atomic force microscopy and proteomics was measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS). THP1-ASC-GFP cells were treated with CM-EVs and GFP expression was measured using flow cytometry.

Results: HiChol diet induced hyperlipidemia in rats and modified the metabolite composition of circulating EVs independently of plasma. HC treatment significantly increased the particle and protein concentration of CM-EVs. Elastic modulus of CM-EVs remained unchanged. LC-MS/MS identified 2135 proteins of which 92 were enriched by HC, meanwhile, the presence of 41 proteins was reduced. CM-EVs, regardless of the treatment, did not induce the activation of THP-1 monocytes.

Conclusions: HC modifies both circulating and CM EVs however, to elucidate the biological roles of these changes, further investigations are needed.

Poster presentation P45

Circulating extracellular vesicle levels are inversely correlated to cholesterol levels in hypercholesterolemia mouse models

Brachyahu Meir Kestecher[1,2,3]; Sayam Ghosal[1,2]; Bernadett Réka Bodnár[1,2]; Adrienn Szabó[1,2]; Nabil Sayour[4]; Tamás Gergely[4]; Krisztina Németh[2,3]; Johannes Oesterreicher[5]; Zoltán Giricz[4]; Zoltán Varga[4]; Péter Ferdinandy[4]; Wolfgang Holnthoner[5]; Edit I Buzás[1,2,3]; Xabier Osteikoetxea[1,2]

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Atherosclerotic Cardiovascular Disease (ACVD) contributes to >40 % of deaths in Western society. Low-density lipoprotein (LDL) is a major protagonist in ACVD. Our laboratory demonstrated the interaction between LDL and extracellular vesicles (EVs) allowing us to hypothesise EV involvement in atherogenesis. Here, 11-week-old male PCSK9-KO (n=6) and LDLR-KO mice (n=6) were used to study the effects these genes play in atherogenesis, cardiac function, body mass (BM), circulating EVs, and cholesterol levels. Mice were fed high fat diet (HFD) for 12-weeks and cardiac function, blood parameters, atherosclerotic plaques, and EVs were analysed. LDL and total cholesterol levels were reduced in PCSK9-KO mice (p=0.0022 and p=0.0006, respectively) and increased in LDLR-KO mice (p=0.0003 and p=0.0004, respectively). Based on annexin-V and CD63, a significant increase in EVs were observed in both PCSK9-KO (p=0.0184 and p=0.0411, respectively) and LDLR-KO groups (p=0.0003 and p=0.0411, respectively). CD81 did not change in PCSK9-KO mice, but a reduction was observed in LDLR-KO mice (p=0.0026). PCSK9-KO mice show increased BM (p=0.0405), but significantly improved cardiac output (p= 0.0005), and ejection fraction (p=0.0022), and fractional shortening was prognostically favourable (p=0.0007). LDLR-KO only show increased (p=0.0087) ejection fraction. PCSK9-KO group show prognostically favourable cardiovascular function after HFD. With increased BM and adipose tissue, however, maintenance of HFD with PCSK9-KO may lead to other health determinants. Despite high cholesterol, LDLR-KO mice do not exhibit signs of decreased cardiovascular function. It was observed within each group, as cholesterol increased, EVs reduced, indicative of an inverse relationship between circulating cholesterol and EVs.

Funding: This work was funded the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No 739593, as well as RRF-2.3.1-21-2022-00003, 2019-2.1.7-ERA-NET-2021-00015 and 2019-2.1.7-ERA-NET-2021-00015.

Poster presentation P46

MiR-200b categorizes patients into pancreas cyst lesion subgroups with different malignant potential

Zoltán Wiener[2]; Márton Benke[1]; Anikó Zeöld[2]; Ágnes Kittel[3]; Delaram Khamari[4]; István Hritz[1]; Miklós Horváth[1]; Bánk Keczer[1]; Katalin Borka[5]; Ákos Szűcs[1]

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Introduction: Extracellular vesicles (EV) carry their cargo in a membrane protected form, however, their value in early diagnostics of diseases is not well known. Although pancreatic cysts are heterogeneous, they can be clustered into the larger groups of pseudocysts (PC), and serous and mucinous pancreatic cystic neoplasms (S-PCN and M-PCN, respectively). In contrast to PCs and S-PCNs, M-PCNs may progress to malignant pancreatic cancers. Since current diagnostic tools do not meet the criteria of high sensitivity and specificity, novel methods are urgently needed to differentiate M-PCNs that may have a malignant potential from other cysts.

Methods: To address this question, we analyzed the EV content and miRNA level of cyst fluid samples.

Results: We show that cyst fluid is a rich source of EVs that are positive and negative for the EV markers CD63 and CD81, respectively. Whereas we found no difference in the EV number when comparing M-PCN with other pancreatic cysts, our EV-based biomarker identification showed that EVs from M-PCNs had a higher level of miR-200b. We also prove that not only EV-derived, but also total cyst fluid miR-200b discriminates patients with M-PCN from other pancreatic cysts with a higher sensitivity and specificity compared to other diagnostic methods, providing the possibility for clinical applications.

Discussion: Since M-PCN may progress to malignant diseases, it should be diagnosed as early as possible to prevent malignant transformation. Our results show that measuring miR-200b in cyst fluid-derived EVs or from cyst fluid may be clinically important in categorizing patients.

Poster presentation P47

Evaluation of EV Biomarkers in Lung Cancer: Insights from IFN-γ Treated A549 Cell Line

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Lung cancer remains a major global health concern, necessitating the identification of novel biomarkers for its early detection and improved therapeutic strategies. Extracellular vesicles (EVs) have emerged as promising candidates for non-invasive biomarker discovery due to their ability to transport and transfer molecular cargo, including proteins and microRNAs (miRNAs). In this study, we investigated EV biomarkers in lung cancer using the A549 cell line treated with interferon gamma (IFN-γ) for 48 hours.

EVs were isolated from the cell culture supernatant using differential centrifugation, and their presence was confirmed by nanoparticle tracking analysis. Furthermore, the expression of EV biomarkers, including CD9, CD63, CD81, TSG101, and Flotillin, was assessed using flow cytometry and Simple Western blotting. Additionally, we investigated the levels of miRNA-21 and let-7, two known miRNAs associated with lung cancer, in both control and IFN-γ-treated cells using reverse transcription-polymerase chain reaction (RT-PCR).

Our findings revealed that the diameter of exosomes ranged from 30 to 150 nm. Interestingly, the number of exosomes decreased following IFN-γ treatment, while the expression of the EV marker CD63 remained unchanged. Conversely, CD9 and CD81 expression exhibited a slight decrease. Notably, several proteins demonstrated significant upregulation (up to 8.9-fold), whereas CD20 expression was reduced by 50%

These results shed light on the impact of IFN-γ treatment on EV characteristics and biomarker expression in lung cancer. The identification of differentially expressed EV biomarkers may contribute to the development of novel diagnostic and therapeutic approaches for lung cancer. Further investigation is warranted to elucidate the functional roles of these altered EV components and their potential as targets for personalized medicine in lung cancer patients.

Poster presentation P48

Characterization and function of extracellular vesicles released by epithelial cells in response to the cysteine protease cathepsin B1 of Schistosoma mansoni

Michael Thaler[1], Lukas Neuninger[1], Magdalena Wysmołek[1], Agnieszka Razim[1], Jan Dvořák[2], Martin Horn[3], Adrian Leontovyč[3], Michael Mareš[3], Anna Schmid[1], Aleksandra Inic-Kanada[1], Ursula Wiedermann[1] and Irma Schabussova[1]

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Background: Schistosoma mansoni migrates through the lungs and releases cysteine proteases such as cathepsin B1 (SmCB1) that can digest host tissue. Host cells produce extracellular vesicles (EVs) that can mediate communication between cells. Here, we tested whether SmCB1 can modulate the release of EVs from alveolar epithelial cells (A549 cell line) and whether these EVs have an immunomodulatory function.

Methods: SmCB1 was produced recombinantly by Pichia pastoris and purified by fast protein liquid chromatography and its proteolytic activity was confirmed. A549 cells were treated with SmCB1 and EVs were isolated from the supernatant by ultracentrifugation followed by size exclusion chromatography. The size and number of EVs were determined by Zetasizer. Next, the effect of SmCB1 and SmCB1-induced EVs from A549 cells was evaluated. Mice were treated intranasally with either SmCB1 or EVs for three consecutive days and lung cell populations were analyzed by FACS.

Results: Stimulation of 25 ml culture of A549 cells led to production of 4*1013 EVs. The size of EVs was approximately 100 nm. Intranasal administration of EVs from SmCB1-stimulated A549 cells decreased the number of alveolar macrophages, CD11c+ cells, and NK cells. Administration of SmCB1 only reduced number of NK cells in the lungs

Conclusion: SmCB1 induces the release of vesicles from A549 cells. Intranasal administration of these vesicles reduced the number of immune cells in the lungs. EVs from SmCB1-treated A549 cells reduced the number of cells in the lung to a greater extend than SmCB1. In future experiments, we will characterize the cargo of SmCB1-induced EVs and investigate the mechanisms of their action.

Poster presentation P49

Differential expression of miRNAs in EVs from blood plasma of older compared to younger females

Lukas Kofler[1]; Agnes Mistlberger-Reiner[1]; Johannes Österreicher[2]; Johanna Fleck[2]; Daniela Weber[3]; Barbara Lieder[4,5]; Matthias Hackl[6]; Wolfgang Holnthoner[2]; Tilman Grune[1,3]

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The secretion of EVs and their miRNA cargo are known to be influenced by the senescence associated secretory phenotype. Therefore, circulating EV-associated miRNAs could be used as blood-based biomarkers for processes of ageing and senescence.

EVs from platelet free plasma of 9 younger women (median age 28 (23-34)) and 9 older women (median age 65 years (61-73)) were isolated by size exclusion chromatography. EV concentration and size were determined by nanoparticle tracking analysis. After isolation of small RNAs, NGS was performed to identify differentially expressed RNAs and reverse transcription with consecutive droplet digital PCR was used for validation.

NTA showed that, older female subjects had a 3.3x higher particle concentration ($p = 0.005$), while the mean particle diameter did not differ. Differential expression analysis after NGS identified seven miRNAs as upregulated in the older female group compared to the younger female group and one as downregulated. The identified miRNAs are mainly associated to vascular and blood cell functions. The concentration of these miRNA species measured via ddPCR correlated with the expression levels measured by NGS ($r = 0.800$, $p < 0.0001$) and the level of miRNA hsa-miR-99a-5p ($p = 0.034$) was significantly higher in older females compared to younger females. miRNAs hsa-miR-23b-3p ($p = 0.053$) and hsa-miR-126-5p ($p = 0.062$) showed a trend in this direction, while a trend was identified for hsa-miR-150-5p ($p = 0.055$) with a lower concentration in older females than younger females.

These findings suggest that in older females more particles are present in their bloodstream than in the blood of younger women and their miRNA cargo changes with age.

Poster presentation P50

Extracellular Vesicle Biology of Bacillus cereus: Unraveling the Influence of Growth Media

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Institute of Microbiology, University of Veterinary Medicine Vienna, Austria

The food-pathogen Bacillus cereus is a Gram-positive endospore-forming rod causing emesis and diarrhea. Although there has been an increase in studies on extracellular vesicles (EVs) derived from Gram-positive bacteria, the exact mechanisms of EV secretion remain unknown.

Two widely used bacterial growth media - lysogeny broth and mineral media - were used to investigate the influence of those on vesiculogenesis. Through lipidomic profiling of EVs, the impact of media selection on the composition of EVs derived from emetic B. cereus F4810/72 was examined. Furthermore, EVs were harvested at different time points of the bacterial culture to fully explore the variations in EV secretion dynamics induced by the culturing medium.

EVs were isolated at distinct time points from bacterial cultures using differential centrifugation. To study the influence of the growth media, EV numbers and sizes were measured by NTA. In addition, bacterial growth, protein concentration and lipid composition were determined, by employing untargeted lipidomics, transmission electron microscopy (TEM), and Fourier-Transform-Infrared spectroscopy (FTIR).

Through these approaches, our study reveals that the choice of media exerts a substantial effect on both the secretion dynamics and the compositional profile of EVs. Consequently, we highlight the importance of establishing and employing appropriate culturing conditions to study EV secretion dynamics, ultimately leading to a better understanding of EV vesiculogenesis in Gram-positive bacteria.

Poster presentation P51

Lipidomic profiling of extracellular vesicles from lymphatic endothelial cells under proinflammatory and lymphangiogenic stimulation

Silvio Kau[1,2,3]; Astrid Laimer-Digruber[4]; Johanna Trisko[1,3]; Wolfgang Holnthoner[2,3]; Ingrid Walter[1]; Michal Kyllar[1]; Sebastian Vetter-Lang[5]

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The pathogenesis of lymphedema, a chronic condition that is characterized by impaired lymphatic drainage, tissue swelling, and inflammation, involves an intricate interplay of proinflammatory and lymphangiogenic stimuli. However, the contribution of lymphatic endothelial cell (LEC) extracellular vesicles (EVs) to this complex process remains poorly understood. To unravel their potential involvement, we started with characterizing the lipidome of EVs derived from telomerase reverse transcriptase-transduced human dermal LECs exposed to various stimuli, including proinflammatory factors TNFa and IFNy, as well as the lymphangiogenic factor VEGFC. This investigation aimed to shed light on the specific role of these stimuli observed in inflammatory progression during early and advancing stages of lymphedema, which are hallmarked by skin fibrosis and adipose tissue deposition. EVs were enriched from conditioned media using differential centrifugation and ultracentrifugation and were further subjected to lipidomic profiling using mass spectrometry. Our results revealed distinct lipidomic signatures associated with different stimuli. Specifically, costimulation with TNFa/IFNy/VEGFC showed a significant enrichment of lipid species with known involvement in tissue fibrosis, suggesting a potential role for LEC-EVs in lymphedema fibrotic skin remodeling. This study provides first insights into the lipidome of EVs derived from dermal LECs under proinflammatory and lymphangiogenic stimuli and their potential contribution to lymphedema associated tissue architectural changes. Understanding the role of EVs in lymphatic dysfunction and inflammatory processes could lead to the development of novel therapeutic strategies for managing lymphedema and improving patient outcomes.

Poster presentation P52

Bacteria-derived extracellular vesicles as crucial mediators of intercellular communication in gut

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Introduction: Maintaining the homeostasis of the intestinal tract is a complex process that relies on the crucial intercellular communication between host cells and the intestinal microbiota. In the past, the role of extracellular vesicles derived from intestinal bacteria has been underestimated. Our primary aim is to unravel the significance of bacterial extracellular vesicles (BEVs) derived from representative gram-positive bacterial strains found in the intestines. Specifically, we seek to understand how these BEVs regulate intestinal homeostasis and elucidate the mechanisms underlying their actions.

Material and Methods: We isolate BEVs from Lacticaseibacillus rhamnosus CCM7091 and Enterococcus faecium CCM7167T using ultracentrifugation and sucrose-cushion purification. These BEVs are characterized through various methods, including MADLS, CryoEM, WB, and proteomics. To examine the interaction between BEVs and target cells, we label the BEVs and employ inhibitors of uptake, as well as HEK clones that overexpress TLR and NODs. We visualize the interaction using confocal microscopy.

Results: The diameter of L. rhamnosus BEVs measures approximately 103nm, while that of E. faecium BEVs measures around 86nm. Both strains typically yield approximately 10^13/ml of BEVs. We find that the uptake of both types of BEVs into cells is inhibited by genistein. We currently consider that TLR2 heterodimers are the most likely receptors for these BEVs.

Discussion: It is important to note that BEVs produced by gram-positive bacterial strains have the ability to interact with host cells. To complement our interaction studies, we will conduct experiments to examine the functional effects of BEVs using intestinal cell models (Caco-2) and immune cells (RAW264.7).

Poster presentation P53

Circulating small EV-related alterations in early stage of atherosclerosis

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Introduction: Atherosclerosis is a disease with complex genetic background. Multiple environmental and genetic factors are responsible for its development. Here we focused on circulating extracellular vesicles (EVs) and studied their changes in atherosclerosis.

Methods: Atherosclerosis was assessed by carotid and femoral artery ultrasonography. Blood samples were collected from healthy controls (n=21), from patients with early-stage atherosclerosis (=15) and from individuals with more advanced atherosclerosis (n=18). ACD anticoagulated blood samples were used to prepare platelet free plasma. The PFP samples were subjected to 18,000g centrifugation to remove the medium sized EVs. Then the supernatant was filtered by gravity through a 0.22 micron filter, and was concentrated by ultrafiltration (100kDa cut-off). The filtrate was further purified by SEC (qEV-70), and the EVs were analyzed by Western blotting, NTA, ELISA and by flow cytometry (MACSPlex Exosome Kit). We also measured the blood plasma Lp(a), MMP12 and MCP1 levels, and analyzed the ApoE polymorphism.

Results: We found that MMP12 concentrations were significantly elevated both in the PFP samples and in the small EV preparations in the advanced atherosclerosis group as compared to healthy subjects. Importantly, we also found a significant elevation of MMP12 in small EVs of early-stage atherosclerotic patients, while there was no elevation in the PFP samples of the same patients at this early stage of the disease. We also detected early-stage atherosclerosis-related increase in the number of CD14+ and CD142+ sEVs, while the CD146+ sEVs were significantly elevated only in the advanced atherosclerotic group.

Summary/Conclusion: Our data suggest that by analyzing circulating small EVs, certain atherosclerosis-related alterations can be detected at an earlier stage of the disease than by the analysis of blood plasma samples.

Poster presentation P54

BeWo derived extracellular vesicles regulate T cell differentiation by down-regulation of IL- 6Rα expression on CD4+ T lymphocytes

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Introduction: Maternal immune tolerance requires the action of regulatory T cells at the feto-maternal interface. Their de novo differentiation is influenced by cellular interactions and the local cytokine milieu. Trophoblast-derived medium size extracellular vesicles (TEVs) bind to peripheral T lymphocytes and have an effect on T cell function. We assume that TEVs regulate the local T cell differentiation through the regulation of local cytokine effects.

Materials & Methods: Cytokine secretion and IL6Rα expression of pregnants' lymphocytes were investigated in BeWo choriocarcinoma cell - lymphocyte co-culture model system and measured using multicolor flow cytometry.

Results: There was a significant increase in IL-6 secretion in both T lymphocytes and BeWo choriocarcinoma cells following co-culturing, while simultaneously the CD4+ T cells downregulated the expression of IL-6Rα.

Discussion: Cytokine secretion and IL6Rα expression of pregnants' lymphocytes were investigated in BeWo choriocarcinoma cell - lymphocyte co-culture model system. We demonstrated that BeWo-lymphocyte interaction induced IL-6 production and down-regulation of IL-6Rα on CD4+ T cells. BeWo derived medium size extracellular vesicles (EVs) caused a typical shift in the cytokine production of CD4+ lymphocytes: this EV binding induced IL-10 production but neither IL-17 nor IFNγ could be detected. We concluded that BeWo derived EVs contribute to the development and maintenance of de novo regulatory T lymphocyte differentiation, through the regulation of both local cytokine production and IL-6 sensitivity of the lymphocytes.

Poster presentation P55

Quantitative analysis of the extracellular vesicle-mediated crosstalk in 3D tumor models

Tímea Böröczky[1,2,3]; Mária Harmati[1,2]; Ákos Diósdi[1,4,5]; Ede Migh[1]; Gabriella Dobra[1,2,3]; Mátyás Bukva[1,2,3]; Edina Gyukity-Sebestyén[1,2]; Ferenc Kovács[4]; Péter Horváth[1,4,6]; Krisztina Buzás[1,2]

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Introduction: Tumor evolution relies on the extracellular vesicle (EV)-mediated cross-talk between malignant and stromal cells in the tumor microenvironment (TME). Here, we aimed to establish a multicellular three-dimensional (3D) tumor model system for tracking the EV communication network of different tumors under physiological conditions and cytostatic treatments.

Methods: Human ductal carcinoma, melanoma and osteosarcoma models were established via co-culturing the respective tumor cell line (T-47D/A375/MG-63) with MRC-5 fibroblasts and EA.hy926 endothelial cells on flat- or U-bottom plates after staining with CellTracker dyes (Orange CMTMR, Deep Red, Green CMFDA). To mimic chemotherapeutic stress, low dose doxorubicin was used and the samples were compared using high-content microscopy and machine learning based analysis.

Results: We showed that CellTracker dyes can be used for in-cell labelling of EVs, allowing the quantitative monitoring of EV cross-talk, i.e. EV axes between each cell type and in both directions. The three tumor models differed in their 3D structure, EV cross-talk activity and drug-induced effects as well. We observed distinct temporal kinetics in the development of the EV communication network in 2D and 3D, also priorities of the investigated EV axes varied between the two co-culture systems.

Conclusions: The developed 3D model system is suitable for live tracking of EV cross-talk in the TME, which depends on the microenvironmental conditions. Further data will help to identify potential targets of EV-blocking therapies, and to predict drug-induced changes of the communication in different tumor tissues.

Funding: TKP2021-EGA09, EUGLOH-RIA Research seeding grant, OTKA-K143255.

Poster presentation P56

Cyclodextrins affect extracellular vesicle production in melanoma in vitro

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Introduction: Cyclodextrins (CD) are cyclic oligosaccharides capable to capture lipids and apolar drug molecules due to their toroidal structure. Accordingly, CDs are also used in pharmaceutical applications. Since CDs can interact with membrane lipids, they may effect also extracellular vesicle (EV) trafficking, thereby influence the intercellular communication of tumor cells.

Methods: Nine different cyclodextrins were used to determine their influence on a syngeneic melanoma cell pair and the cell derived EV release, in vitro. The effect of the applied CD-treatment on cell vitality was analyzed using SRB assay. The EVs derived from CD-treated melanoma cells were collected after 72 hours' incubation, then isolated with differential centrifugation. To determine their characteristics dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), protein (Qubit Protein Assay Kit) and lipid assay (Sulfo-Phospho-Vanillin Assay – SPV) were applied.

Results and Conclusion: Although the effect of cyclodextrin treatments on melanoma cells varied widely, some CDs increased the number and decreased the lipid content of isolated EVs without a substantial effect on cell proliferation indicating, that CDs might be used to influence EV secretion selectively.

Poster presentation P57

Characterization of mast cell-derived extracellular vesicles during degranulation

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Semmelweis University department of Genetics, Cell and Immunobiology, Extracellular vesicles research group

Introduction: Mast cells (MCs) are pivotal in allergic reactions and are implicated in various diseases, where degranulating MCs are involved in inflammatory reactions including atherosclerosis. Nowadays, it is recognized that besides 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 characterize and distinguish MC-derived EVs from extracellular granules released during degranulation providing a deeper understanding of their role in disease. This, in turn, may lead to the development of novel diagnostic assays and therapeutic targets and improved methodologies.

Materials & Methods: EVs were isolated from bone marrow derived murine MCs from 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 after stimulation with calcium ionophore or DNP/IgE. Cell activation and recovery was monitored by beta-hexosaminidase, proliferation and viability assays.

Results & Discussion: We established a model system to monitor real time EV production during degranulation by flow cytometry and confocal microscopy. Our findings revealed that during degranulation MCs secrete a heterogeneous particle population. These particles differ in size, concentration and detergent sensitivity suggesting a simultaneous production of EVs, extracellular granules and membrane fragments. The composition of the secreted corpuscles depends also on the stimuli used to induce degranulation. These results emphasize the challenges and importance of distinguishing MC-derived EV secretion and degranulation from one another.

Poster presentation P58

Extracellular vesicles biomarkers for predicting the inhibitory impact of extracellular vesicles on the cytotoxic effect of car t cells

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Introduction: Chimeric antigen receptor (CAR) T cell therapy holds enormous potential for the treatment of hematologic malignancies. Despite its advantages, it is still used as a second line of therapy mainly because of its severe side effects. To improve its therapeutic efficacy, better biomarker models to predict the immune system response to CAR T cell infusion need to be developed. The extracellular vesicles (EVs) mediate immune responses and cancer spread. The aim of our study is to develop EVs biomarker models to predict CAR T therapy progression.

Materials and Methods: EVs were isolated from blood of healthy donors and from supernatant of macrophage THP-1 cancer cell line with ultracentrifugation. They were stained with various combinations of fluorescently labeled antibodies and preliminary measurements were obtained with an Aurora flow cytometer (Cytek biosciences). Their size was compared to the silicon beads' size standards.

Results: The origin of vesicles and their subsets were determined by measuring parent cell markers (CD19, CD3, CD4, CD8). Exhaustion markers (CD279) and activation markers (CD69) were expressed on EVs. CD123 was investigated as a potential biomarker for leukemia cells, but it was expressed on 34 % of vesicles from healthy donors and therefore considered unsuitable for differentiating leukemia vesicles. We observed low expression of CD63, which was tested as a marker for EVs. Other potential EVs biomarkers are being tested.

Discussion: The extracellular vesicles hold great potential in predicting and monitoring the inhibitory effect and immune system response to CAR T cell therapy. However, the identification of more accurate vesicle biomarkers is needed.

Poster presentation P59

Hybridosomes from spruce needle homogenate

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Introduction: Being of compatible structure with biomembranes, lipid–based nanoparticles are considered as convenient platforms for drug delivery systems. In the proposed work we considered formation of lipid nanovesicles associated with bioactive phytochemicals from spruce needle homogenate (here called hybridosomes). We formed hybridosomes by mixing appropriate amounts of lecithin, supernatant of isolation of extracellular particles from spruce needle homogenate and glycerol.

Methods: We visualized hybridosomes by light microscopy and cryogenic transmission electron microscopy and assessed them by flow cytometry, dynamic light scattering, ultraviolet–visual spectroscopy and interferometric microscopy.

Results: We found that the particles consisted of a bilayer membrane and a fluid-like interior. Flow cytometry and interferometric light microscopy measurements showed that the majority of the particles were nano-sized. Dynamic light scattering and interferometric light microscopy measurements agreed well with the determined average hydrodynamic radius of the particles Rh (between 140 and 180 nm) while their number densities were in the range between 10^13 and 10^14/mL indicating that hybridosomes present about 2/3 of the mixture, excluding solvent and other small molecules.

Discussion: Simple and low-cost preparation method, non-demanding saving process and efficient formation procedure suggest that large scale production of hybridosomes from lipids and spruce needle homogenate is feasible.

Poster presentation P60

Enrichment and characterization of lympathic endothelial extracellular vesicles from lymphatic fluid and blood plasma

Tatiana Tyshchuk [1,2,3]; James Ferguson[1,3]; Miikka Vikkula[4]; Johannes Grillari[1,2,3]; Wolfgang Holnthoner[1,2,3]

[1]Ludwig Boltzmann Institute for Experimental and Clinical Traumatology in the AUVA Trauma; Research Centre, Vienna, Austria; [2]Department of Biotechnology, University of Natural Resources and Life Sciences Vienna, Vienna, Austria; [3]Austrian Cluster for Tissue Regeneration, Vienna, Austria; [4]Human Molecular Genetics, de Duve Institute, University of Louvain, Brussels, Belgium

Extracellular vesicles (EVs) are cell-derived, lipid bilayer-enclosed particles which mediate intercellular communication and are therefore a vital component in the transmission of various biological signals. The vascular endothelium is a substantial contributor to the circulating particular secretome with a wide plasticity displayed by endothelial cells, as seen in different molecular signatures and functional properties of the endothelium. These differences are thought to be reflected in the EVs secreted by the endothelium. As endothelial EVs have been linked to a variety of vascular diseases, they garnered interest in their possible use as biomarkers and therapeutic targets. While EVs derived from cell culture have been subject to investigation, much is yet to be done regarding EVs within the vascular network.

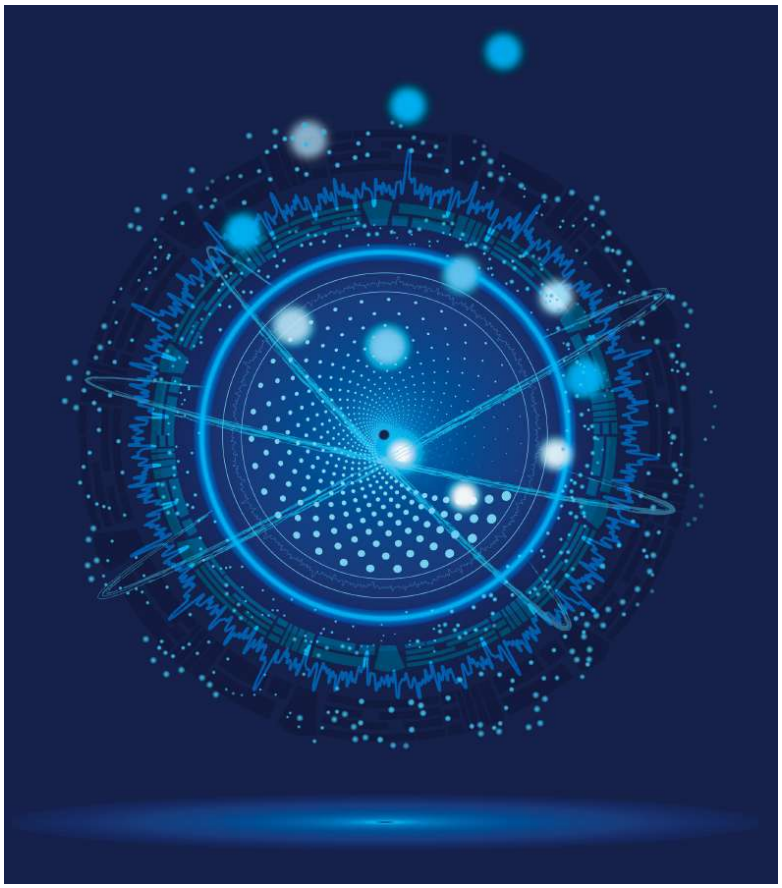
The aim of this study is to enrich and characterize EVs of the lymphatic endothelium from human blood plasma and pig lymph. After enrichment via either tangential flow filtration (TFF) or ultracentrifugation (UC), both plasma and lymph were purified through size exclusion chromatography (SEC). We use the fluorescence localization and colocalization features of nanoparticle tracking analysis (NTA) to investigate the presence of CD81, CD63, CD31 and podoplanin on vesicles labelled with CMDR lipid membrane stain. Furthermore, we investigate the presence of these signatures via fluorescence triggered flow cytometry (FT-FC). Moving forward, we plan to use to data from these experiments to compare healthy volunteer blood plasma with samples from patients with diagnosed vasculopathies, such as lymphedema.

Network session + MOVE

Tuesday, 5th September 2023

11:30 - 12:30

Chairs: Beate Rinner and Wolf Holnthoner



Presentation of organizing Societies

ASEV - Austrian Society of Extracellular Vesicles

Wolf Holnthoner

President of ASEV

Presentation of organizing Societies

HSEV - Hungarian Society of Extracellular Vesicles

[1]Edit Buzas and [2]Zoltan Giricz

[1]President of HSEV; [2]Vice-president of HSEV

Presentation of organizing Societies

SiN-EV - Slovenian Network for Extracellular Vesicles

Metka Lenassi

President of SiN-EV

Presentation of organizing Societies

SrbEVs- Serbian Society for Extracellular Vesicles

Maja Kosanović

President of SrbEVs

Presentation of ISEV

ISEV - International Society of Extracellular Vesicles

Edit Buzas

President of ISEV

Presentation of MOVE European Mobility Fellowships

MOVE news from Finland

Johannes Oesterreicher

ASEV

Presentation of MOVE European Mobility Fellowships

MOVE news from Sweden

Martin Wolf

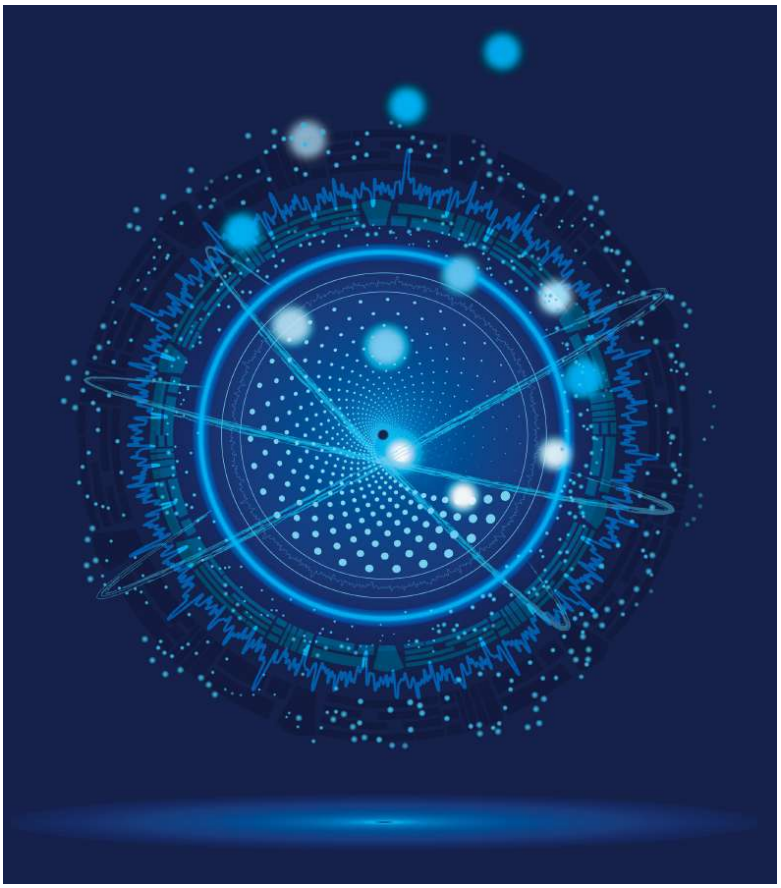
ASEV

Diversity of EV sources

Tuesday, 5th September 2023

13:45 - 15:15

Chairs: Edit Buzas (Hungary) + Djenana Vejzovic (Austria)

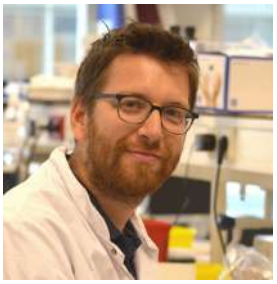


Keynote presentation

Extracellular Vesicle-mediated RNA Delivery: from Mechanistic Insights towards Therapeutic Applications

Pieter Vader

CDL Research and Department of Cardiology, Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands



• Pieter Vader graduated in Chemistry (B.Sc., 2005) and Drug Innovation (M.Sc., 2007) from the University of Utrecht. He earned his PhD degree in 2012 from the University of Utrecht on the subject of targeted delivery of siRNA to inhibit tumor angiogenesis. From 2012 to 2014, Pieter was employed as a (senior) postdoctoral fellow at the University of Oxford, UK, in the lab of Prof. Matthew Wood.

In 2014 he moved back to The Netherlands to continue his work at the University Medical Center Utrecht. Currently, he is Associate Professor at CDL Research and at the Department of Experimental Cardiology. His main research interests are in the field of therapeutic applications of extracellular vesicles, including unraveling the mechanisms underlying extracellular vesicle-mediated cargo transfer. His research has been supported by a NWO Veni Grant (2014), ERC Starting Grant (2019), Dutch Heart Foundation Dekker Senior Scientist Grant (2019), and NWO Vidi Grant (2020). In 2021, Pieter was awarded the Prix Galien Research Award for his work on drug delivery. •

Extracellular vesicles (EVs) form an endogenous system for information transfer between cells. Since the recent discovery that EVs are also capable of functionally transferring RNA molecules, they are increasingly being considered as therapeutic RNA delivery systems. Despite extensive research into the engineering of EVs for RNA delivery, our understanding of the pathways and mechanisms regulating EV-mediated RNA delivery and processing is limited. Moreover, little is known about how their intrinsic RNA delivery efficiency compares to current synthetic RNA delivery systems.

We developed a novel CRISPR/Cas9-based reporter system in which eGFP expression is activated upon functional delivery of targeting single guide RNAs (sgRNAs) that allows study of EV-mediated RNA transfer at single-cell resolution. We employed this system to study EV internalization pathways and to compare the delivery efficiency of EVs to clinically approved state-of-the-art DLin-MC3-DMA lipid nanoparticles and several in vitro transfection reagents. We found that EVs delivered RNA several orders of magnitude more efficiently than these synthetic systems. This finding supports the continued research into EVs as potential RNA delivery vehicles.

To overcome challenges related to the difficulty of RNA loading into EVs, we prepared EV-liposome hybrid nanoparticles and evaluated them as siRNA delivery systems in terms of cellular uptake, toxicity, and gene-silencing efficacy. We show that hybrids combine benefits of both synthetic and biological drug delivery systems and might serve as future therapeutic carriers of siRNA.

Our data underline the potential of EVs as RNA delivery vehicles and highlight the need to study the mechanisms by which EVs achieve their efficiency. This may in turn contribute to the development of more efficient EV-based RNA delivery systems and accelerate clinical adoption of therapeutic EVs.

Oral presentation

Unraveling the pathogenic and pro-inflammatory potential of extracellular vesicles secreted by Bacillus cereus

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Bacillus cereus is a Gram-positive spore-forming bacterial pathogen that is well-known for its ability to cause food poisoning [1]. In recent years, this opportunistic pathogen has gained prominence as a causing agent of non-GI related illnesses, including systemic and local infections [2]. In addition, we recently showed that B. cereus also secretes biologically active extracellular vesicles (EVs) loaded with pore-forming toxins [3]. In our current project we evaluated systemic pathogenicity of B. cereus EVs in an in-vivo mouse model, of which first data will be presented.

In contrast to EVs of Gram-negative bacteria, EVs in Gram-positive bacteria are far less studied and their role in bacterial (patho)biology is largely unknown. To purify EVs, an ultracentrifugation-based protocol was used. Follow-up EV characterization included resin-embedding transmission electron microscopy, nanoparticle tracking analysis, immunoblotting, and in-vitro cytotoxicity assays using bone-marrow derived macrophages (BMDMs). The pro-inflammatory and pathogenic potential of EVs was then determined in a murine in-vivo model.

In this study, we show for the first time that the presence of B. cereus EVs without its bacterial cells is sufficient to cause systemic inflammatory response symptoms. Therefore, it is anticipated that this research will provide new ways for understanding EV-contribution of pathogenic Gram-positive bacteria to systemic bacterial infections, as well as aid in the development of innovative defense mechanisms and novel therapeutic approaches. First results from an ongoing study will be presented.

Oral presentation

Proteomic analysis of ascitic extracellular vesicles describes tumor microenvironment and predicts patient survival in ovarian cancer

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Introduction: High-grade serous carcinoma of the ovary, fallopian tube and peritoneum (HGSC), the most common type of ovarian cancer, ranks among the deadliest malignancies. Many HGSC patients have excess fluid in the peritoneum called ascites. Ascites is a liquid tumor microenvironment (TME) containing various cells, proteins and extracellular vesicles (EVs). Ascites is therefore an underrated experimental system and a feasible source of new potential biomarkers.

Material & Methods: We isolated EVs from ascites of eleven patients by two orthogonal methods (ultracentrifugation coupled to sucrose cushion and size-exclusion chromatography) and analyzed them by label-free tandem mass spectrometry.

Results: We identified not only a set of "core ascitic EV-associated proteins" but also defined their subset unique to malignant ascites in HGSC. Using single cell RNA sequencing data, we mapped the origin of HGSC-specific EVs to different types of cells present in ascites. Surprisingly, EVs in ascites did not come predominantly from tumor cells, but from a variety of non-malignant cell types. Next, we performed flow cytometry of ascitic cells in combination with the analysis of EV protein composition in matched samples and showed that analysis of cell type-specific EV markers in HGSC has a stronger predictive potential than analysis of ascitic cells.

Discussion: To conclude, we provide evidence that proteomic analysis of EVs is capable to define status of the cellular composition of HGSC tumor microenvironment. This finding opens numerous avenues both for a better understanding of EV role in tumor promotion/prevention and for improved HGSC diagnostics.

Oral presentation

Effects of Placental Extracellular Vesicles on Maternal Hematopoiesis

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Introduction: During pregnancy major adaptations of the maternal blood profile take place in order to successfully overcome the immunological and hemostatic challenges. There is a lack of evidence regarding the effects of pregnancy on maternal hematopoiesis. The alterations could be the result of an interaction between maternal hematopoietic stem cells (HSCs) and extracellular vesicles (EVs) secreted from the placenta.

Methods: One-sided human placenta perfusion was performed as a source of placental EVs. Different subpopulations of EVs were enriched by ultracentrifugation and characterized in regard to protein content, marker expression and particle size. CD34+ HSCs were obtained from healthy female donors. Uptake of EVs by HSCs was analyzed. HSCs were treated with EVs for 7 days. The ratio of different marker-expressing cell populations determined by flow cytometry was compared between different EV-treatments and time points.

Results: Placenta perfusions were successfully performed and distinct populations of EVs enriched. EVs were taken up by HSCs. A time-dependent decrease of HSCs was concomitant with an increase of mature blood cells. Placental EV-treated HSCs differentiated into CD235a+ erythrocytes and CD14+ monocytes in a higher ratio compared to the non-treated control.

Discussion: HSCs are able to internalize placental EVs. Our results are consistent with the reports in the literature showing that the maternal blood cell composition in pregnancy is characterized by an increase in erythrocytes and monocytes, both executing crucial functions in pregnancy. These findings indicate that maternal hematopoiesis might be reprogrammed by the EVs of fetal origin secreted to the maternal circulation.

Oral presentation

Mechanisms of formation of extracellular particles in diverse samples from human, animal, plant and microalgae

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Introduction: By using different microscopic techniques, we have imaged the shape of particles enclosed by bilayered membrane (colloidal vesicles) from different systems (blood, tomato homogenate, spruce needle homogenate, cell culture) and the shapes of artificial vesicles (liposomes) in the last 25 years. The experimental evidence shows that these vesicles can obtain diverse shapes (globular with different elongations, dumbbell, discocyte, stomatocyte, starfish, cubosome, etc).

Methods: Considering that the extracellular vesicles are composed of a lipid bilayer-based membranes and fluid interior, we have predicted their shapes theoretically by considering that the shape of the vesicle is determined by the minimal membrane free energy at relevant constraints.

Results: We found good agreement between the experimentally observed and theoretically predicted shapes which indicates that the same physical mechanism rules the membrane shape over three orders of magnitude, regardless of the chemical composition of the membrane. Staurosporine-induced apoptosis in cultured cells created fragments of different sizes (from 50 nm to micrometers). In plant and microalgae samples, cryogenic electron microscopy revealed presence of different types of cell-engineered particles while mass spectrometry revealed that some of these particles were viruses. In microalgae, different processes of extracellular particle shedding were observed, depending on whether the microalgae contained the cell wall or flagellae.

Discussion: Regarding the mechanisms of extracellular particle formation we distinguish colloidal vesicles and cell-engineered particles. Imaging of vesicles is important in determination of their identity. If the observed shapes agree with the theoretical shapes of minimal membrane free energy, they are vesicles (membrane-enclosed fluid).

Diversity of EV sources

– poster presentations –

Poster presentation P61

Specific qualities of different placental small extracellular vesicles

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Introduction: During pregnancy, the placenta releases small extracellular vesicles (sEVs) to the maternal and fetal circulation. Since placental sEVs may act immunomodulatory to the pregnant mother, we hypothesize that a similar crosstalk exists between the placenta and the fetus. To explore this, we isolate and characterize sEVs from various placental sources.

Materials and Methods: sEVs were obtained either from the media of perfused placentae (trophoblast-derived, T-sEVs) and from the supernatant of primary feto-placental endothelial cells (EC-sEVs). sEVs were enriched by differential ultracentrifugation; size (in range of 0-1000 nm) and concentration of sEVs were determined using nanoparticle tracking analysis (NTA), and characteristic protein of sEVs were analysed.

Results: The yield of sEVs (50-150 nm) relatively to the number of total particles, varied between EC-sEVs (~80%) and T-sEVs (~30%). The protocol for isolation of T-sEVs was optimized by extending the centrifugation-times, due to the high viscosity of the perfusion media. This results in an increased yield of T-sEVs (~30% to ~70%). Interestingly, different levels of TSG101, an ESCRT-associated protein and Syntenin-1, a mediator of the ESCRT-independent pathway were found in both sEV fractions. While both TSG101 and Syntenin-1 were highly expressed in T-sEVs, only Syntenin-1 was significantly expressed in the EC-sEVs fraction.

Discussion: sEVs from different placental sources require refined isolation protocols, considering the characteristics of each collection media. Additionally, differences in the levels of proteins linked to the biogenesis of sEVs provide preliminary evidence that the secretion of sEVs from the placenta varies between the trophoblast layer and the fetal endothelium.

Poster presentation P62

Characterization of EVs in the developmental stages of the apicomplexan parasite Cystoisospora suis

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Introduction: Cystoisospora suis, a porcine apicomplexan parasite, is characterized by a complex life cycle, and an environmental phase the oocyst. Due to the complexity of the parasite's development we hypothesize that the cargo transported by parasite EVs varies with the life cycle stage. Hence, this study aimed to characterize EVs of all developmental stages of C. suis.

Methods: C. suis parasite stages were obtained from intestinal porcine epithelia cell cultures (IPEC) during their respective expression and incubated for two hours in a host cell-free environment. All EVs were obtained by several centrifugation steps, and particle numbers and size distributions of stage-specific parasite EVs were analysed by NTA. TEM and SEM were used to visualise the respective EVs locations on the developmental stage. Furthermore, correlations between the lipidomic profile of EVs derived from C. suis asexual and sexual stages and their FT-IR-spectra were compared.

Results: Asexual, sexual and transmissible stages of C. suis expressed different EVs during the parasite's life cycle. EVs of asexual and sexual stages, which occur in the host animal, are more similar to each other than to the transmissible environmental stage, the oocyst. We could also show a cargo of polysaccharides, which are known to influence the conversion of parasite stages, and the occurrence of fatty acids in EVs, which presumably down-regulate the toxic activity of parasites.

Discussion: This study presents the first characterization of C. suis EVs and links them with the respective developmental stages of the parasite and putative functions.

Poster presentation P63

Proteomic analysis of different EVs sources to access the molecular signature of enhanced turbulence EVs production

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Advances in large-scale EV production technologies using bioreactors made it possible to rapidly produce large quantities of EVs from cultured cell lines or primary cells for study purposes. 3D cultures allow greater control of biochemical and biophysical conditions than 2D cultures and offer many advantages for downstream applications including purification of specific types of EVs, production of large amounts of clinical-grade EVs, and improved yield compared to standard culture methods. Another aspect is that shear force (turbulence) using stirred tank bioreactor can also increase the number of EVs secreted by cells. We used cells from different sources (adipose stem cells (ASCs), fibroblast (FB) and monocytes (THP-1)) and expanded cultures using conventional cell culture flasks (2D). EVs from all cell sources were prepare for mass spectrometry. We therefore asked if the extracellular vesicles from different cell sources like adipose stem cells (ASCs), fibroblast (FB) and monocytes (THP-1) could secrete EVs with distinct secretome signature depending on the culture method using conventional cell culture flasks (2D) or using microcarriers in spinner flasks (3D). We found that the proteomic profile of EVs secreted by different cell types and culture method have a distinct molecular signature by comparing the proteins identified by principal component analysis (PCA). We also identified several biological process and metabolic pathways related to these common proteins that can regulate cell growth, cytoskeletal reorganization, and the activation of protein kinases. These unique features presented in our work are an evidence that shear stress can affect the molecular signature of different cell lines. Now we planned to identify shear stress related proteins among the common proteins differentially express on all compared cell lines. Also with the list of common proteins we aim to identify pathways responsible for the increase of EV under turbulence.

Poster presentation P64

Extracellular vesicles as a next-generation drug delivery vehicles for precision cancer treatment

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Introduction: Despite the remarkable improvements achieved in cancer nanomedicine, tumor selective delivery of diagnostics and therapeutics is still considered a critical issue in successful cancer therapy. Among the nanoparticle-driven strategies extracellular vesicles (EVs), which are nano-to-micron-sized lipid membrane-bound vesicles, represent a promising drug delivery tool for anti-cancer therapies. Nevertheless, the limited knowledge about their in vivo tropism still hinders their therapeutic applications.

Materials and Methods: Herein, cancer derived-EVs were loaded with therapeutic agents such as paclitaxel alone or in combination with oncolytic adenoviruses, and with fluorescent dyes such as DiIC18(5); 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenz- enesulfonate salt (DID) or indocyanine green (ICG). Finally, optical imaging was extensively applied to study the in vivo and ex vivo biodistribution of different EV-formulations in different mouse models of cancer, including syngeneic graft and patient-derived xenograft.

Results and Discussion: We proved the existence of tumor-specific EVs in the blood of patients with stage IV (metastatic) colon cancer undergoing surgical tumor resection. The isolated EVs can be delivered to the same tumor from which the EVs were derived thus they can be used for an innovative autologous delivery approach for theranostic agents. Moreover, we demonstrated a selective targeting of cancer-derived EVs to the neoplastic tissues thus opening new avenues for the selective delivery of both diagnostics and therapeutics. Altogether, our findings strongly suggest that EVs represent an efficient and highly biocompatible way to deliver molecules directly to the tumor with great precision.

Poster presentation P65

Setting up large scale production of exosomes

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Introduction: The FH-Campus Vienna plans to enter the production of exosomes. For this purpose, different strategies are tested to grow cells in high densities. Possible cultivation methods are e.g. fixed bed reactors or stirred tank fermentors in which cells are cultivated on microcarriers.

Material and Methods: As a proof of principle, a system is established in which human dermal fibroblasts are cultured on microcarriers in spinners. We will try to stimulate the exosome production by different stimuli like hydrogen peroxide, ATP, or induction of senescence. Concentration is done by cross flow filtration followed by PEG precipitation. The exosomes are characterized by western blot (CD9, CD63, Alix), total protein and total RNA and DNA measurements.

Results: The fibroblasts are well cultivable on microcarriers, cellular senescence can be induced, and exosomes are produced.

Discussion: The cultivation of fibroblasts should only be seen as a first step to establish the methods. It is planned to switch to cells, like mesenchymal stem cells, and to produce valuable exosome material on a large scale.

Poster presentation P66

Examination of the interaction between oral squamous cell carcinoma and Candida species at the level of extracellular vesicles

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In the European Union, especially in Central Europe, including Hungary has the highest incidence of oral cavity tumors. 90% of the mentioned tumor type is oral squamous cell carcinoma (OSCC). A previous study of our laboratory showed that the presence of Candida albicans increases the progression of OSCC in vitro and in vivo. During our work, we aimed to investigate the effect of Candida derived extracellular vesicles (EVs) effect to the progression of OSCC furthermore to other Candida species.

To investigate the interaction between oral squamous cell carcinoma (OSCC) and Candida cells we used HSC-2 cell line and EVs derived from C. parapsilosis and the yeast and hyphae forms of C. albicans. We examined the uptake of the fungal EVs by the OSCC cells with confocal microscopy. Live cell imaging system was used for the examination of the effects of fungal EVs on the migration of OSCC cells. The activity of MMPs after EV treatment was also measured. Moreover, we investigated the effect of C. albicans derived EVs to the growth and biofilm formation of C. tropicalis and C. parapsilosis.

Our results showed that OSCC cells are able to take up the fungal EVs and Candida derived EV treatment affect the migration of the cells and significantly increase the activity of secreted MMPs. Furthermore, the C. albicans EV treatment significantly decrease the biofilm formation of C. parapsilosis.

These results suggest the presence of an active interaction between OSCC cells and Candida species at the level of extracellular vesicles.

Poster presentation P67

Investigating the effect of extracellular vesicles on proliferation and migration on syngeneic colorectal cancer cell lines

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Introduction: Metastasis, the dissemination of tumor cells from their primary site of origin to anatomically distant tissues and cavities, can be identified as the most defining and lethal characteristic of malignant cancer. Emerging evidence suggests that extracellular vesicles (EVs) contribute to a variety of biological processes of tumor cells such as immune modulation, proliferation and migration. Our aim was to explore the biochemical properties as well as physiological functions of EVs, particularly those pertaining to cell proliferation and migration, in the context of colorectal cancer.

Methods: EVs produced by a syngeneic colorectal cell pair were isolated from cell-supernatant after 72hs incubation using ultracentrifugation. Isolated EVs were characterized with dynamic light scattering (DLS), Nanoparticle Tracking Analysis (NTA) lipid assay (Sulfo-Phospho-Vanillin Assay - SPV) and protein assay (Qubit Protein Assay Kit). The commonly used EV markers were measure by bead-based flow cytometry. The pair of cell line were treated with the EVs originated from both cell lines and proliferation (SRB assay) and migration (videomicroscopic imaging) were investigated.

Results and Conclusion: SW480-derived EVs had higher lipid and protein content compared to SW620 EVs. Interestingly, an inhibitory effect on cell migration was observed when the cells were treated with their own EVs. Generally, the treatments showed that EVs had inferior impact on proliferation as on migration.

Poster presentation P68

Fractions of cell culture supernatant show distinct effects on cell proliferation

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Introduction: Extracellular vesicles are involved in several stages of cancer progression. Studies often use the supernatant of cultured cells as EV sources and investigate the effects of the EV-enriched fractions. However, other soluble components can modify and work as synergies in the investigated features and cells with different aggressivity could produce different components.

Methods: The supernatants of a syngeneic pair of melanoma cell lines corresponding to different stages of the malignancy was sampled (TS) and differential centrifugation was performed for EV isolation, which resulted in two fractions: EV-depleted supernatants (S) and the EV-enriched (EV) isolates. DLS verified the presence or absence of particles in the fractions. EVs' size, size distribution and particle concentration were measured with NTA. Protein and lipid content were also determined. The commonly expressed EV markers presence were investigated with flow cytometry. The fractions effect on cell proliferation was measured by SRB cell viability assay after 72 and 96 hours' of treatment.

Results: The EVs' particle number, protein and lipid content were similar for both cell line. After 72 hours WM983B-derived EVs could promote the proliferation of the parental cells, but did not affect WM983A cells. When both of the cell lines were incubated with WM983A-derived EVs for 96 hours their cell viability was significantly increased. The TS and EV-depleted S fractions did not promote cell proliferation.

Conclusion: In summary, our results show that the experienced effects of the cell culture supernatant are specific for the EV-enriched fraction. Additionally, the effect of EV treatment might depend on the incubation time, suggesting different underlying molecular mechanisms.

Poster presentation P69

Extracellular vesicles can promote migration and transfer vemurafenib resistance among melanoma cells

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Introduction: Primary cause of cancer-related deaths is metastasis formation and one of the most metastatic tumor is melanoma. BRAF V600E mutant proteins are present in 40-60% of melanoma cases and can be selectively targeted with vemurafenib however resistance accompanied with increased metastasis formation is observed in the majority of patients. More and more evidence suggest that extracellular vesicles (EVs) can play crucial roles in cancer progression and therapy resistance. Hence aim was to investigate if migration, as an important step of metastasis formation, and vemurafenib resistance can be modulated by EVs.

Methods: Syngeneic pairs of melanoma cell lines – established from specimens before and during vemurafenib treatment – were used. SRB assay was used to measure vemurafenib resistance. EVs were isolated from each cell cultures supernatant by differential centrifugation. Size, size distribution and particle concentration were measured by NTA analysis, the isolated fractions protein and lipid concentrations were also determined. The commonly used EV markers presence in our isolates were analyzed with bead-based flow cytometry. To investigate EVs effect on cell migration and vemurafenib resistance mean squared displacement (MSD) total travelled distance (TTD) were determined using videomicroscopy and single-cell tracking.

Results and Conclusion: EVs could promote single cell migration, best seen in the parameter MSD. Furthermore, vemurafenib could not diminish the migration promoting effect of the resistant cell lines derived EVs, suggesting EVs' role not only in metastasis formation but also in the transfer of vemurafenib resistance.

Poster presentation P70

The effect of inflammation level on the production of osteoarthritic tissues-derived extracellular vesicles

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Introduction: Osteoarthritis (OA) is a degenerative disease of the musculoskeletal system that affects many people worldwide. Research of the production and composition of extracellular vesicles (EVs) and the clarification of their functions in OA may help to understand the pathogenesis of OA and help in the field of the diagnostic process of this disease in the future.

Material and Methods: This work focuses on the comparative analysis of conditioned media (CM) prepared from OA tissues (synovial membrane, Hoffa's fat pad and cartilage) and EVs isolated from these CM. Levels of inflammation in CM were confirmed by multiplex analysis using Magpix and flow cytometry. NTA was used to determine the concentration and size of EVs. Phenotypic characterization of EVs was performed by western blot analysis and flow cytometry.

Results: The results of this study revealed the highest levels of inflammatory mediators in CM from synovial membranes compared to CM from Hoffa's fat pad and cartilage tissues. EVs isolated from CM of OA synovial membrane produced the highest number of EVs compared to other tissues. Phenotypic characterization of EVs showed the expression of surface markers CD9, CD63, and CD81 and also of HLA-DRPDDQ, CD62p, MCSP, CD42a and CD29 in all analyzed samples.

Discussion: This work indicates that the synovial membrane is most significantly involved in inflammation in the OA process compared to other studied tissues. The inflammation present in the synovial membrane could have a direct effect on the increased production of EVs, which directly affect the microenvironment of the joint.

Poster presentation P71

Morphology of extracellular particles from equine colostrum

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Introduction: Milk is a source of variety of particles including molecules, their complexes and extracellular particles (EPs). It contains bioactive components which are derived from various cell sources transporting nutritional components and immune system cells. Recently, the molecular mechanisms underlying the mother-to-child information transfer have been further studied, more recently, a pivotal role in these processes has been linked to EPs, which are enriched in milk. Here we considered EPs from equine milk at day 1 (colostrum) and two consecutive days. The samples were taken from two mares.

Methods: Samples were observed under Nikon Eclipse TE2000S inverted microscope (Nikon Instruments Inc., Tokyo, Japan) with CCD 512 Digital Camera System SPOT BOOST (Visitron Systems, Puchheim, Germany). Samples were fixed with OsO4, dried in the air, sputtered with gold/palladium and examined under JSM-6500F Field Emission Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan). The concentration of EPs and their average hydrodynamic diameter were determined by using the interferometric light microscope (Myriade, Paris, France).

Results: Images showed globular particles of different sizes (from tens of micrometers downto 50 nm). Measurements of objects on images agreed with determination of the hydrodynamic diameter by interferometric light microscopy. EPs in colostrum were found larger and their number density was lower than the two consecutive days.

Discussion: We observed population of globular EPs heterogeneous in size. Interferometric light microscopy is a convenient method to assess EPs in milk. Some samples could be measured directly without dilution or filtration of the samples.

Poster presentation P72

Exosomes are among the vesicles shed from human embryo during fragmentation

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Introduction: Developmental arrest of human embryos and fragmentation are serious complications in assisted reproduction. Around 15% of IVF embryos permanently arrest in mitosis at the early cleavage stage, while >80% displaying some degree of fragmentation e.g. shedding various membranous extracellular vesicle (EV). Although fragmented embryos may produce term pregnancies, the capability to reduce or prevent fragmentation clinically would be highly required. Namely, removal of fragments enhances embryo survival leading to idea that avoiding fragmentation should be beneficial, and that interventions could help embryo survival. The underlying cause of fragmentation has remained unknown and the quantity of isolated fragments is extremely low. Hence, we aimed this study to examine ultrastructure of arrested, fragmented human embryo.

Material & Methods: Total of 6 embryos stopped at Day 3 with various degrees of fragmentation were examined. Transmission electron microscopy (TEM) was used to study presence, distribution and ultrastructural details of extracellular vesicle (EVs) in arrested embryo. EVs further characterized via morphology and immunogold labeling.

Results: Using TEM on arrested human embryos, we observe presence, distribution and ultrastructural details of various EVs. Blastomeres in various degree of fragmentation shed various EVs. Among them we found exosomes and multivesicular body.

Discussion: It is known that fragmentation is one of the mechanisms responsible for the elevated levels of embryo demise during the first week of in vitro development. Potential role of EVs in fragmentation and early embryo arrest are still matter of debate. Our ongoing work is focused on establishing new methods for ultrastructural demonstration of exosome content due to their low abundance.

Poster presentation P73

The crosstalk between circulating exosome carried miRNAs and ferroptosis related genes in multiple sclerosis

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Background: Ferroptosis is one of the processes that could drive immune-mediated neurodegeneration in multiple sclerosis (MS). Exosomes as biologically active extracellular nanovesicles can carry miRNAs and are easily delivered across blood - brain barrier. Our aim is to provide data about the difference in network interplay of exosome carried miRNAs and PBMC mRNA between mild and severe MS phenotypes, in context of ferroptosis process regulation.

Methods: Targeted mRNA sequencing of selected ferroptosis related genes was performed in PBMC of two MS phenotypes on Illumina iSeq100 NGS instrument. DESeq2 algorithm was used for obtaining DE genes and miRNA/mRNA interplay was woven using miRNET platform. Purification of exosomes from plasma and extraction of total RNA was performed using Plasma/Serum Exosome RNA Isolation Kit (Norgen Biotek). Exosomes were evaluated on the ZetaView instrument and the presence of brain derived fraction was evaluated with L1CAM and MOG antibodies. Expression profiles of the bioinformatically defined miRNA hubs was evaluated in circulating exosomes containing fraction of the brain-originating ones, using TaqMan technology.

Results will provide insight if the expression of ferroptosis related genes observed in PBMCs reflect miRNA levels in exosomes, with regard to MS severity.

Discussion: Exosome cargo could serve as easily accessible biomarker for monitoring MS course/severity. Detection of brain fraction exosomes in circulation will provide new data on exosome signature and miRNA content in MS patients. Validation of miRNAs in exosomes and their regulatory background of ferroptosis in MS will have impact on research toward diagnostic/therapeutic application of both, miRNAs and exosomes.

Poster presentation P74

Exploring the interaction of Outer membrane vesicles (OMVs) produced by Paraburkholderia phytofirmans PsJN with Arabidopsis thaliana roots

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Outer membrane vesicles (OMVs), extracellular vesicles (EVs) produced by Gram-negative bacteria, are increasingly recognised as promising tools in biomedicine due to their innate ability to interact with human cells and trigger immune responses. The interaction of OMVs of plant growth-promoting bacteria (PGPB) with plants, as well as with plant-pathogenic microorganisms, is far less explored. Considering the great importance of PGPBs for the development of sustainable, environmentally friendly solutions in agriculture, the study of the role of OMVs in PGPB-plant and PGPB-phytopathogen interactions holds valuable application potential.

To investigate PGPB OMVs, we isolated and characterised OMVs produced by Paraburkholderia phytofirmans PsJN, a PGPB strain known to enhance plant resistance to various abiotic and biotic stresses. After testing different methods for isolating and purifying OMVs, a commercially available affinity-based column system was selected as the most efficient. Outer membrane origin of isolated OMVs was confirmed using an essay for detection of lipopolysaccharide (LPS).

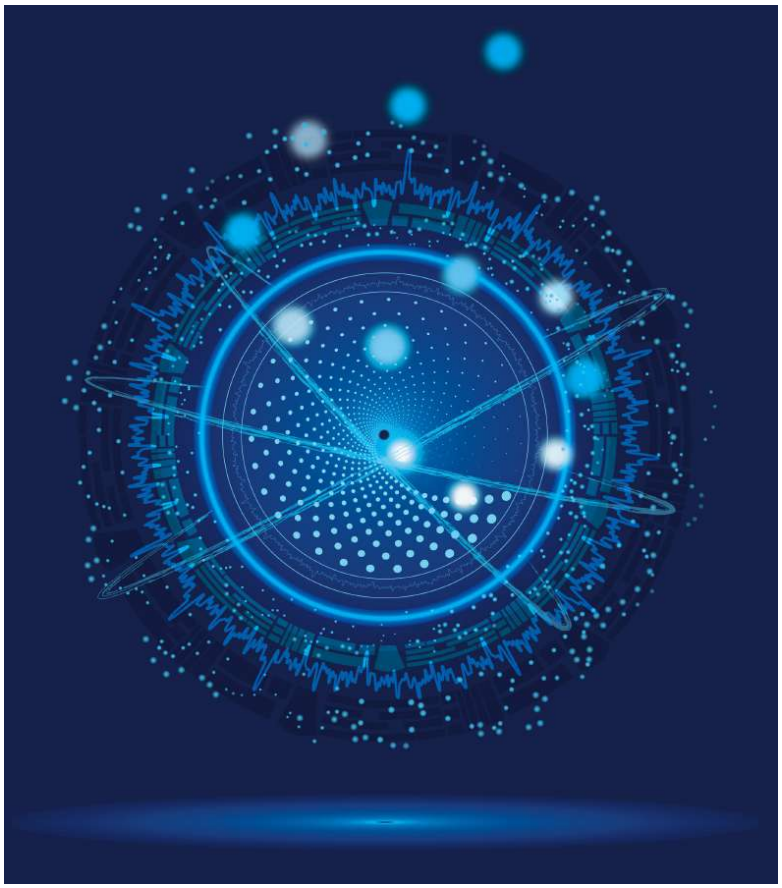
To examine the interaction of OMVs with plant cells, Arabidopsis thaliana roots were incubated with isolated P. phytofirmans PsJN vesicles, previously labelled with lipid binding fluorescent dye Vybrant™ DiD. Red signals were observed, under confocal laser scanning microscope, in root hairs and root surface in DiD-OMV treated plants, while in control-treated roots the same signals were missing. The results suggest direct contact of OMVs with root hairs, which are necessary for nutrient acquisition and plant-microbe interactions in rhizosphere. Our further research is focused on the characterization of OMV-associated RNA and its potential delivery into host plant cells.

Purity meets function

Tuesday, 5th September 2023

15:45 - 17:00

Chairs: Metka Lenassi (Slovenia) + Krisztina Nemeth (Hungary)



Keynote presentation

To EV, or not to EV: that is the question

Saara Laitinen

Finnish Red Cross Blood Service (FRCBS), Helsinki, Finland

• Saara Laitinen, R&D Manager in Finnish Red Cross Blood Service, Saara Laitinen, PhD has over ten years of research experience on extracellular vesicles (EV). Her interest towards EVs started from the mesenchymal stromal cells (MSC) and their paracrine mechanisms in immunomodulation. Her focus as a researcher has long been in the functionality of the membrane lipids, particularly fatty acids. Thus, the role of the membranes in cell and EV functionality has triggered her interest at the beginning and her team has developed lot of functionality models especially from immunological perspective.

Saara Laitinen has led the Finnish National EV Ecosystem, EVE – Extracellular Vesicle Ecosystem for Theranostic Platforms aiming for better analytical performance and development of EV based diagnostics and therapies. As one of the results from that consortium emerged Finnish Society of Extracellular Vesicles (FISEV). During recent years Laitinen' EV team has developed isolation and purification methods for blood derived EVs, but also blood cell derived EVs as drug delivery vehicles. Based on the results obtained FRC Blood Service took important first step towards manufacturing of EVs. In collaboration with HansaBioMed Life Sciences, FRC Blood Service will aim for product development on extracellular vesicles from blood and for life science market (https://www.bloodservice.fi/news/the-blood-service-and-hansabiomed-life-sciences- sign-a-letter-of-intent). Saara Laitinen is a president of the Finnish Society of Extracellular Vesicles (www.fisev.fi) has published over 50 EV / membrane biology related publications. •

As in higher organisms, all cells in different tissues have their special function, they need to work in concert. Interest in blood as connecting, fluid tissue consisting different highly differentiated cell types, long known from their vesiculation as part of their function it is evident that we need to learn more of these EVs as potentially, they can be used as tools to pave the way in EV field.

Currently in EV research there are several parallel processes moving forward same time. First and still needed are the research initiatives that aim to understand fundamentals in EV biology. Second is the development of the reliable tools to perform EV analyses. Third, the development of various applications EVs can be part: monitoring, diagnose, and therapy. Sometimes the applied research is in the frontline although in parallel we need to take care of deeper understanding on EV biology. From isolation to characterization and nomenclature, the latest trend in EV field has been the appreciation of the functionality. To assess EV functionality, reliable tools and assays need to be set in place. Although in cellular therapy development many assays are developed, they may not be directly transformable for EV research. We also need to understand that as cells, so does the EVs derived from them, have different functionalities. When we are aiming to set some EV functionality measure per se, we might end up wrong. Functionality is a challenging issue not only from this cell specific perspective but also from the perspective of assays and tools used. We only get the information we can measure in a set limitation of an assays: in vitro, ex vivo or in vivo. Finally, our physiology and biology will bring the extra twist to everything. I will try to open this challenging world of EV functionality in the context of an EV source and process in my presentation in SmallNewWorld.

Oral presentation

Functional implications of protein EV corona

Martin Wolf[1]; Rodolphe Pupardint[1]; Anna Raninger[1]; Patricia Ebner[1]; André Cronemberger Andrade[1]; Nicole Maeding[1]; Fausto Gueths Gomes[2]; Balazs Vari[1]; Essi Eminger[1]; Astrid Obermayer[3]; Thomas Heuser[4]; Michaela Öller[2]; Hans-Dieter Volk[5]; Katharina Schallmoser[2]; Dirk Strunk[1]

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

Material & Methods: EVs were harvested from placenta-derived stromal (PLX) cells and enriched by tangential flow filtration (TFF) optionally followed by ultracentrifugation. EV preparations were characterized using western blot, tunable resistive puls sensing (TRPS) and super-resolution microscopy following MISEV2018 guidelines. T cell proliferation and matrigel network formation served as in vitro functional assays.

Results: Single particle analysis revealed a typical sEV mode size of 107 nm via TRPS and highly heterogenous tetraspanin distribution by super-resolution microscopy. Half of PLX-EVs expressed one single tetraspanin, <15% all three tetraspanins CD9/63/81 simultaneously. Electron microscopy revealed the presence of a protein corona around EVs that was depleted by ultracentrifugation. TFF-enriched EVs bearing a protein corona inhibited T cell proliferation and promoted angiogenesis. Removing/reducing the EV protein corona via ultracentrifugation or size exclusion chromatography diminished T cell proliferation-inhibition and depleted their angiogenesis-supporting function. Re-loading the EVs with a cocktail of three proangiogenic proteins reestablished angiogenesis significantly superior to free factors. Super-resolution microscopy with fluorescently labeled corona proteins revealed outside association of these proteins with the EVs.

Discussion: These findings show the important role of corona proteins for EV function. Recent calculations indicating a 'surface-to-bulk partition of EV cargo', for EVs < 180nm, in favor of surface cargo loading support the new concept of a functional EV corona.

Oral presentation

The good in the bad and the good: From cellular senescence to recombinant therapeutic EVs for tissue engineering

Johannes Grillari[1]; Madhusudhan Reddy Bobbili[1]; Stefan Vogt[2]; Johannes Österreicher[1]; Gordana Wozniak-Knopp[3]; Florian Rüker[3]; Matthias Wieser[4]; Wolfgang Holnthoner[1]; Nuria Gimenez[3]; Matthias Hackl[5]; Regina Grillari[4]

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Introduction: Cellular senescence has evolved from an in vitro model system of aging research to a therapeutic target in age-associated diseases. In this context, we recently identified extracellular vesicles and their miRNA cargo as a factor contributing to detrimental effects of senescent cells on organ function. However, senescence is also a roadblock to generating human cell factories, as it limits the replicative life span of normal human cells like mesenchymal stem cells and thus the possiblity to genetically engineer the cells and to produce the biomass necessary for biotechnological processes.

Methods: EVs of senescent cells have been tested for influencing differentiation and proliferation of recipient cells resulting in a general pattern of decrease differentiation versus increase proliferation. In addition, to circumvent senescence as a roadblock, we have used reactivation of telomerase in normal human cells resulting in continuous growth of MSCs without these cells losing their primary cell like characteristics. Thus, we genetically engineered CD81 to carry an additional artificial transmembrane domain, a 'snorkel' tag, resulting in its C-terminus being presented on the outside of EVs. A series of features added to this snorkel including affinity tags and a prescision protease cleavage site generates a system for purification and visualization of CD81 positive EVs. In addition, randomization of amino acids within the large extracellular loop domain of CD81 resulted in a combinatorial library allowing to identify and isolate specific binders to any surface molecule.

Results: Indeed, recombinant CD81 binders to laminin or EGF receptor have been identified and increase EV uptake by target cells.

Discussion: Summarized, secreted miRNAs in and outside of EVs can be used as a biomarker in age associated diseases. the combination of telomerized cells as production hosts or for potency assays, together with recombinant tetraspanins result in a versatile EV toolbox with the potential to boost research and development in an area with high hopes and promises for combating a series of major challenges, especially in the space of inflammatory and/or age associated diseases.

Oral presentation

Alternative Mechanisms of mitochondria quality control elicited by EVs in skin aging and disease

Maria Cavinato

University of Innsbruck / Research Institute for Biomedical Aging Research

Introduction: Accumulation of dysfunctional mitochondria due to impaired mitophagy is a major characteristic of senescent cells as well as of several human age-related conditions and cancer. Mitophagy receptor NIX has been shown to drive epidermal differentiation through the control of mitochondrial fragmentation. Furthermore, NIX epidermal expression is decreased during aging and leads to the accumulation of dysfunctional mitochondria in this tissue, suggesting that NIX-dependent mitophagy is an important mechanism for the maintenance of skin homeostasis. Recently, the release of extracellular vesicles (EVs) containing damaged mitochondria has been proposed to act as an alternative mechanism of mitochondria quality control to outsource mitophagy. However, it is unknown if such mechanisms are involved in the process of senescence of human dermal fibroblasts (HDF) as well as if they participate in aging and disease of human skin.

Materials and Methods: We have thus investigated the role of NIX-dependent mitophagy in cellular senescence, skin aging, and skin tumorigenesis, the release of EVs containing mitochondria as a compensatory mechanism to outsource mitophagy and explored the implication of these mechanisms in skin homeostasis and disease.

Results and Discussion: Based on our preliminary data we have observed that NIX-dependent mitophagy plays a crucial role in HDF homeostasis. Impairment of this mechanism induces senescence of HDF, even in the absence of UVB, characterized by secretion of different SASP factors, including EVs containing mitochondria. Work with 3D skin equivalents demonstrated that the absence of NIX in dermal fibroblasts induces hyperproliferation and impaired differentiation of epidermal keratinocytes suggesting that factors secreted by NIX-depleted fibroblasts can modulate skin homeostasis and induce skin carcinogenesis.

Oral presentation

Outer membrane vesicles of the probiotic E. coli O83 activate innate immunity and prevent allergic airway inflammation in mice

Irma Schabussova
Medical Universiity of Vienna

Introduction: Probiotic bacteria reduce allergic sensitisation in mice and humans. Oral administration of the probiotic bacterial strain E. coli O83 (EcO83) to neonates reduced allergic sensitisation later in life but had no beneficial effects on allergic lung disease. Bacterial extracellular vesicles are known to be efficient carriers for the delivery of biomolecules to recipient cells efficiently regulating host pathophysiology. The potential of outer membrane vesicles (OMVs) from probiotic bacteria for the prevention or treatment of allergy is unclear.

Material and Methods: We isolated OMVs from EcO83 (EcO83-OMVs) by ultracentrifugation and investigated their effect in a mouse model of ovalbumin-induced experimental allergic airway inflammation (AAI). HEK293 cells expressing NOD1, NOD2, TLR2, and TLR4 and bone marrow-derived dendritic cells (BMDC) from wild-type (WT) and TLR4KO-BALB/c mice were stimulated with EcO83-OMVs.

Results: EcO83-OMVs are spherical nanoparticles with a size of approximately 110 nm. They contain lipopolysaccharide and protein cargo. Stimulation of HEK293 NOD1, NOD2, TLR2, and TLR4 cells with EcO83-OMVs increased the production of IL-8, indicating the involvement of these receptors in signal transduction by EcO83-OMVs. Stimulation of WT BMDC with EcO83-OMVs increased the production of IL-23, IL-12, TNFα, IL-1β, and IL-6, whereas BMDC from TLR4KO mice showed decreased production of these cytokines. Intranasal administration of EcO83-OMVs reduced allergic inflammation, lung eosinophil levels and type 2 cytokines compared with sham-treated controls.

Discussion: We demonstrate for the first time that intranasally administered vesicles from probiotic Gram-negative bacteria have an anti-allergic effect suggesting that probiotic-derived OMVs may be a novel treatment option for allergic diseases in humans.

Purity meets function

– poster presentations –

Poster presentation P75

Characterisation of extracellular vesicles from human cerebrospinal fluid after enrichment by size-exclusion chromatography

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Introduction: Extracellular vesicles (EVs) are membrane-like nanostructures secreted by almost every cell. They represent promising material for liquid biopsy applications and diagnostics. However, to ensure this, EVs should first be identified and quantified. The aim of this work was to determine the size and concentration of EVs from human cerebrospinal fluid (CSF) enriched by size-exclusion chromatography (SEC) and reveal their morphology by atomic force microscopy (AFM).

Materials and Methods: A total of 55 samples of the CSF were collected, each of 500 µl in volume and pooled for SEC. The enrichment of EVs after SEC was tested by immunoblotting for the transmembrane protein CD9 and CD81, whilst particle concentration and mean size were determined by dynamic multi-angle light scattering (DLS) in different fractions and in the native CSF pool. AFM in liquid was used to image the near-native morphology of EVs.

Results: The enrichment of EVs in SEC fractions 8-10 was confirmed only for the transmembrane protein CD9 but not for CD81 on immunoblots, and the effective elimination of contaminants was shown by immunoblotting for albumin. EV`s diameter ranged from 59 to 197 nm, and the concentration of particles in EV-positive fractions decreased by approximately 107 compared to the CSF pool. AFM in liquid revealed various morphological features of EVs: round, concave, single-lobed, multilobed and flat.

Discussion: The obtained results could make an important contribution to the comprehensive understanding of the observed EV subpopulations in human CSF. To correlate the different EV morphologies with their biological functions, further research should integrate other methods of nanotechnology.

Poster presentation P76

Extracellular vesicles from blood plasma as mediators of anti-inflammatory effects, oxidative stress and angiogenesis in HUVEC

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Introduction: Blood plasma is used in regenerative medicine for more than 30 years as it was found to have impact on angiogenesis and proliferation of endothelial cells. This effect was initially ascribed to growth factors and cytokines, however, particularly in the last decade researchers are focusing on platelet-derived extracellular vesicles (PEVs) that are also present in the platelet rich plasma.

Methods: We prepared platelet and extracellular vesicles-rich plasma (PVRP) by centrifugation of the human blood, and differential (ultra) centrifugation of PVRP to isolate PEVs. We exposed Human Umbilical Vein Endothelial Cells (HUVEC) to 5% PEVs for 24 hours and assessed inflammation markers (Interleukin(IL)-1beta, IL-6 and Tumor necrosis factor (TNF)-alpha using ELISA tests), oxidative stress markers (Cholinesterase (ChE) and glutathione S-transferase (GST) activity by spectrophotometry, as well reactive oxygen species (ROS) and lipid droplets (LD) by flow cytometry). We observed morphological changes in HUVEC indicating angiogenesis by using optical microscopy.

Results: We found that after 24 hours caused a decrease of the concentration of IL-6, IL-1beta and TNF-alpha. Also, ChE and GST activity and ROS count were decreased. LD production, which is triggered in need to protect the cells from free radicals and oxidative stress damage, was however higher. Treatment of the cells had impact on cell morphology with progressed formation of the tubes and cell connecting, which is regarded as the beginning of HUVEC angiogenesis process.

Discussion: Beneficial effect of PVRP in healing and regeneration may include suppression of inflammation and oxidative stress by PEVs.

Poster presentation P77

Influence of SEC to resistance of EVs to purity control treatments and stability in different handling/storage conditions

Sofija Glamočlija, Marina Bekić; Alisa Gruden-Movsesijan; Sergej Tomić; Maja Kosanović
Institute for the application of nuclear energy, INEP, Serbia

Introduction: To utilize diagnostic/therapeutic potential of extracellular vesicles (EVs), their integrity should be kept throughout the separation, purification and storage process. However, some techniques, such as size exclusion chromatography (SEC), can affect the corona of EVs and influence both diagnostic/therapeutic molecules and stability of EVs. Therefore, the aim of this work was to investigate whether SEC affects EVs' resistance to purity control treatments and stability in different handling/storage conditions.

Materials & Methods: We separated EVs by ultracentrifugation (UC) on 17000xg (T17) and 100.000xg (T100) and subjected half of obtained EVs to SEC (Sepharose 2B CL, 11 x 1.2 cm column). In each fraction, the number of particles was determined by NTA, protein concentration by BCA and EVs' marker profile by dot-blot. Fractions containing peak of EVs were pooled. EVs before and after SEC were incubated with different detergents, buffers and were subjected to different temperatures after which the median size and concentration were determined by NTA. Tripsin was used for direct removal of corona.

Results: Most detergents had high number of detected particles and could not be used in NTA/scatter mode. EVs separated by UC only and EVs further purified by SEC were differentially sensitive to temperature and differentially stable in different buffers. Notably, tripsin treatment caused increase in the total number of particles but reduction in median size, possibly indicating disruption of EVs or .

Discussion: Obtained results point out to the importance of quality controls following each step of separation and purification processes and handling/storage.

With the ability to remove bovine serum EVs without starvation, CELLine bioreactors offer improved efficiency in extracellular vesicle production

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Introduction: Low efficiency and FBS-induced vesicle contamination are two major challenges in efficient EV production. The CELLine AD 1000 bioreactor consisting of two chambers (cell and medium compartment) separated by a 10 kDa semipermeable, silicon membrane, eliminates the need for a pump, as gas as well as nutrient and waste exchange can take place passively through the membrane.

Methods: To reduce vesicle contamination from FBS, researchers working with bioreactor appear to apply two methodologies: (i) replacement of the growth factor with CDM-HD or exosome-depleted FBS (handmade/commercial), or (ii) change of the media to advance instead of normal. The media are changed 1-2 times a week in cell compartment and once a week in medium compartment. Vesicles are isolated by UC, UF, SEC and characterized by NTA, BCA, TEM, WB.

Results: We expect improvement of EV production in measure of 8-10x more particles and protein content, as reported in the literature. When bioreactor and classical production methods (T75/175) are compared, it is observable that semi-permeable membrane in bioreactor structure prevents FBS vesicle contamination in media, confirming an increase in both yield and purity.

Discussion: In bioreactor, there is only physical separation. However, more descriptive information is needed for knowledge about origin (cell culture/FBS) in analysis of EVs which we plan to study in next project. The number of cells obtained from EV will be calculated in line with conditions of MISEV 2018. More experiments are also required to evaluate the effects of FBS use on the vesicle number/structure.

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