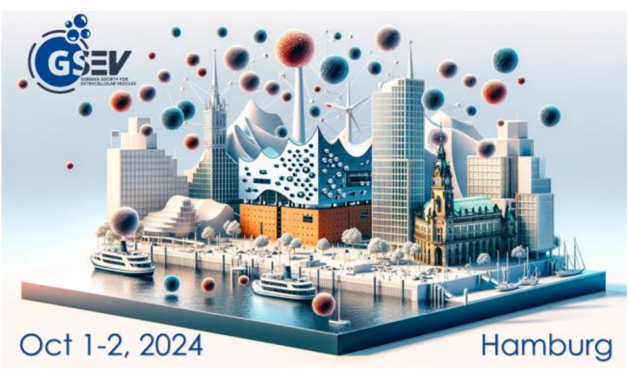
# Poster Abstract Book - GSEV Hamburg 2024




**Welcome to the GSEV Autumn Meeting 2024!**

Dear colleagues and friends,

It is with great pleasure that we welcome you to the Autumn Meeting of the German Society for Extracellular Vesicles (GSEV), taking place here in the beautiful city of Hamburg. With its rich history as a port city and cultural hub, Hamburg offers the perfect setting for fostering scientific exchange and collaboration. We hope that you will not only benefit from the scientific program but also take the time to explore this vibrant city, known for its historic Speicherstadt, stunning Elbphilharmonie, and welcoming atmosphere.

We are delighted to welcome more than 220 participants from over 10 countries, making this Autumn Meeting the largest EV meeting in Germany to date. We would like to take the opportunity to thank all our sponsors for making this possible! Our meeting will feature keynote lectures from pioneers and rising stars in EV research and beyond, offering us the chance to learn from those at the forefront of discovery. At GSEV, we believe in the interdisciplinary nature of EV research. We are therefore thrilled to welcome, among others, representatives of the European Liquid Biopsy Society, the German Society for Cell Biology and the German Society for Virology here at our congress. We are convinced that the connection with these neighboring fields will advance our understanding of EV biology, function and clinical potential.

The focus of this congress is on all of our talented junior researchers—who will play a vital role in shaping the future of this field. We encourage you to make the most of these two days—whether by forging new connections, sharing your latest findings, or finding inspiration for your next research endeavor. Together, we are contributing to a future where EVs hold the keys to new diagnostic, therapeutic, and technological breakthroughs. We wish you all a productive and enjoyable congress and thank you for your contribution!

Kerstin Menck (GSEV president)

Berta Puig (Local organizing committee)

# OCTOBER 1st

## EV Biology

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

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Mesenchymal stromal/stem cells (MSCs) possess significant therapeutic potentials due to their ability to modulate immune responses and promote regeneration, primarily through paracrine signaling, especially via extracellular vesicles (EVs). However, the specific mechanism by which MSC-EVs exert their therapeutic function remains unclear. Galectins are crucial players in various physiological and pathological processes by recognizing and bridging glycans on plasma membranes, thereby controlling diverse immune responses. Given that certain galectins, especially galectins 1, 3, and 9, are expressed in MSCs and their function has been linked to EV biology, they might essentially contribute to the therapeutic function of MSC-EVs. In our effort to effectively translate MSC-EVs as a new pro-regenerative agent into the clinics, we have established clonally immortalized MSCs (ciMSCs). These cells enable scaled manufacturing of MSC-EV products for the clinical setting and can be effectively genetically manipulated, providing an ideal platform for dissecting the mechanism of action of MSC-EVs. Here, we used CRISPR/Cas-9 technology to delete the coding regions of galectins 1, 3, and 9 in ciMSCs, exploring the impacts of the deletion on the biological properties of the cells and the immunomodulatory function of resulting EV products. In our ongoing experiments, deletion of galectin-1 did not notably alter the biological properties of the genetically engineered ciMSCs or their EV products. Currently, we explore the impacts of the Galectin 3 and 9 knock-outs, as well as various galectin knock-out combinations in future studies. In summary, the function of Galectin-1 appears dispensable for the immunomodulatory properties of ciMSC-EV products, as evidenced by the reduction of the activated T cell pool within the mdMLR assay. Further experiments are required to fully understand the role of Galectin-1 for the immunomodulatory potential of MSC-EVs and its contribution to the mechanism of action of ciMSC-EVs.

### 1.2 Dynamic changes of Extracellular Vesicles during zebrafish organogenesis

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Extracellular Vesicles (EVs) play a crucial role in cell migration and differentiation. Despite its role as a well-established vertebrate model, little is known about EVs during zebrafish embryogenesis. This study investigates EVs during zebrafish embryonic development. We discriminate between large (lEVs) and small EVs (sEVs) from whole zebrafish larvae (zfl), analyzing size and concentration changes over time. Wild-type AB strain zfl were collected at 24, 48, 72, and 96 hours post fertilization (hpf) and mechanically homogenized. LEVs and sEVs were isolated by differential centrifugation and characterized using flow cytometry, negative staining transmission electron microscopy (TEM), and nanoparticle tracking analysis (NTA). AxV-FITC and FM4-64 were used during flow cytometry to detect phosphatidylserine and a lipid bilayer. Flow cytometry showed a high amount of lEVS being double positive for AxV-FITC and FM4-64, which counts as purity control of the samples. The highest amount was detected at 72hpf. Two distinct populations (sEVs and lEVs) were differentiated using NTA, showing significant size differences at each time point. This not only indicates the quality of the isolation method but also shows that zfl uses different types of EVs during embryogenesis. It was observed that the total EV number increased significantly over the first 72hpf, but not proportionally to zfl length growth. Additionally, sEV size also increased significantly, with a maximum diameter at 72hpf. Based on the fact that during the first 72hpf most organs are formed and mainly maturation and growth occur afterward, the elevated number and larger size before 72hpf hints towards an important role of EVs during zebrafish organogenesis. Since EVs serve as intercellular cargo delivery platforms, larger EVs may reflect the need for a higher transport capacity. This study is the first to characterize whole zfl EV using NTA, TEM, and flow cytometry, suggesting an essential role for EVs in zebrafish organogenesis.

### 1.3 Dissecting the Mechanism of action of MSC-EVs through CRISPR/Cas9 mediated Gene Editing

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Mesenchymal stromal cells (MSCs)-derived extracellular vesicles (EVs) have demonstrated immunomodulatory and pro-regenerative capabilities in various disease models. For optimal clinical translation and to overcome challenges such as heterogeneity and reproducibility of MSC-EVs, a thorough understanding of their mechanism of action (MoA) is required. Results of previous studies have implied that certain enzymes and molecules inducing signal transduction pathways essentially contribute to the immunomodulatory activity of potent MSC-EV products. Upon deleting the coding region of selected proteins by CRISPR/Cas9 mediated gene editing, we aim to identify essential EV components required for mediating the MSC-EVs' immunomodulatory functions. Gene editing was performed on clonally immortalized MSCs (ciMSCs) using lentivirus-delivered CRISPR/Cas9 technology. Flow cytometry was used to identify successfully engineered

ciMSCs and to facilitate selection of knockout single cell clones, which were subsequently expanded. The cell surface phenotype of obtained ciMSCs is characterized via flow cytometry and their adipogenic and osteogenic differentiation potential explored in differentiation assays. EVs will be prepared from conditioned media applying an optimized polyethylene glycol precipitation followed by ultracentrifugation. MSC-EV preparations will be determined by nanoparticle tracking analysis, bicinchoninic acid assays, western blot and by imaging flow cytometry-based methods. Their immunomodulatory functions will be evaluated in a multi-donor mixed lymphocyte reaction assay. Upon utilizing the CRISPR-Cas9 technology, we have successfully deleted the coding regions of selected cell surface proteins, including CD49e, CD105 and CD166 and confirmed the absence of respective proteins. Our results reveled reduced proliferation capabilities of CD105 and CD166 knockout ciMSCs, while CD49e knockout ciMSCs impaired the osteogenic differentiation. Currently, we analyze impacts of the knockouts on the EV secretion behavior of the cells and the function of resulting EVs. Employing CRISPR-Cas9 technology to delete specific genes in ciMSCs appears to be promising methodology for unraveling the MoA of clinically applicable MSC-EV products.

### 1.4 The Link Between Cholesterol-Associated Plasma Membrane Lipid Raft Domains and the Shedding of Microvesicles Co-Expressing CD147 and Tissue Factor

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Microvesicle-like extracellular vesicles (mEVs) expressing CD147 and tissue factor (TF) have been associated with tumor progression and metastasis. This study investigates the role of plasma membrane lipid raft domains in the shedding of CD147 / TF positive mEVs. Standard adherent cancer cell lines were cultured in vitro and treated with the cholesterol-depleting agent methyl-β-cyclodextrin to disrupt the lipid rafts. For targeted analysis of coexpression, cells were transfected with constructs expressing tagged CD147 and TF prior to treatment. Disruption of lipid rafts was confirmed by staining with a fluorescently labeled cholera toxin B subunit, which binds to GM1 gangliosides. Serum-free conditioned media were collected to isolate mEVs, and vesicles expressing both CD147 and TF were quantified by flow cytometry using antibody staining or direct detection of the fluorescent tags. Reduced levels of lipid rafts in the plasma membrane correlated with a decrease in mEVs positive for TF but not CD147. However, it resulted in a significant reduction in CD147 / TF double positive mEV shedding compared to control cells. Our findings suggest that the reduction in cholesterol-associated lipid raft domains impairs the shedding of mEVs that co-express CD147 and TF. This study contributes to understanding the regulation of mEV biogenesis and may have implications for developing therapeutic strategies targeting mEV-mediated tumor progression.

### 1.5 Neutral Sphingomyelinase 1 regulates Cellular Fitness at the level of Secretory Protein Biogenesis

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Sphingomyelindiesterases (SMPDs) or sphingomyelinases (SMases) are a family of enzymes that hydrolyze the phosphodiester bond of the sphingomyelin to produce ceramide and phosphorylcholine. The sphingomyelinases are broadly divided into three major classes: acid sphingomyelinases, alkaline sphingomyelinases, and neutral sphingomyelinases based on their pH optima. In mammals, five neutral sphingomyelinase members have been identified, nSMase1 (Gene name: SMPD2), nSMase2 (SMPD3), nSMase3 (SMPD4) and mitochondrial nSMase (MA-nSMase; SMPD5). While nSMase 2 has been studied in the context of ILV budding and EV biogenesis, the molecular characterization and biological function of nSMase1 remain poorly studied. Using biochemical assays monitoring cellular and extracellular levels of secretory proteins, confocal microscopy and RNA sequencing we analyzed the effect of nSMase 1 inhibition by siRNA knockdown cellular fitness and the secretory pathway. Here, we discovered that nSMase1 plays an important role in maintaining overall cellular fitness, as SMPD2 KD cells are less viable and proliferate less than control cells. Two important cell growth pathways - PI3K/Akt and Wnt signaling - are altered and the overall protein translation rate is dramatically reduced by SMPD2 knockdown. Bulk RNA sequencing revealed SMPD2-dependent gene expression of specific secreted and transmembrane proteins. Among others, lysosomal-associated membrane protein 1 (LAMP1) mRNA as well as protein levels are downregulated in SMPD2 knockdown cells. Taken together, we propose a role for nSMase1 in modulating cellular fitness via modulation of secretory ER function.

## EVs in immunity & infection

### 1.6 Small Extracellular Vesicles Protein Cargo and Their Role in Microplotting in Long Covid

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In COVID-19 patients, it has been demonstrated that epithelial cells-derived extracellular vesicles (EVs) protein content play a significant role in the high coagulation rate in patients, as they can activate thrombin, leading to thrombo-inflammation. In parallel, the development of microclots are considered a signature of

long covid sequela. In this study, we aimed to investigate the role of EVs in the pathogenesis of microclots in long covid sequela. For this study we isolated sEVs (<200nm) form plasma of either healthy probands or long covid patients using size exclusion chromatography. The sEVs were then characterized using tunable recessive pulse sensing, electron microscopy and western blot. To investigate the role of sEVs in the micro-clots pathogenesis, we ran a mass-spectrometric analysis of sEVs derived/attached proteins. Data were further analyzed using Maxquant Perseus software and Expressanalyst.ca. online tool. The analysis of protein content of sEVs from both healthy probands and long covid patients has revealed 665 differentially expressed proteins. Principal component analysis clustered each groups of samples among each other confirming our data. Gene set enrichment analysis of the differentially expressed proteins has shown that different pathways related to micro-clotting are activated in long covid sequela patients including heparin binding, glycosaminoglycan binding, and cofactor binding. Furthermore, multiple key thrombosis and clotting proteins were upregulated including fibronectin (FN1), and Histidine rich glycoprotein (HRG). These findings suggest that sEVs from long covid patients may have the ability to regulate different clotting and thrombosis pathways and may lead to micro-clotting. More in vitro and ex vivo investigation is required to understand how sEVs can influence different components and players in the clotting system and can be used as a prognostic marker in long covid patients to anticipate micro-clotting in them.

### 1.7 Neutrophil-Derived Small Extracellular Vesicles and Their Role in Inflammatory Response and Angiogenesis Inhibition in Ischemic Stroke

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Neutrophils are the first responders to ischemic stroke, driving an inflammatory response after infiltrating the blood-brain barrier to the site of cerebral infarction. In addition to their known roles, neutrophils release extracellular vesicles (EVs), including small EVs (sEVs), which may contribute to the inflammatory process. However, the functions of neutrophil-derived sEVs in stroke are not well understood. We examined activation markers of peripheral neutrophils in stroke patients during subacute (2-7 days post-infarction) and recovery phases (>90 days post-infarction) using flow cytometry. Functional assays were conducted to assess the effects of neutrophil sEVs on angiogenesis and immune signaling. Neutrophil sEVs were analyzed using imaging flow cytometry (IFCM) to determine the expression spectrum of surface markers. To account for the potential effects of co-isolated cytokines, we also analyzed cytokine profiles that are isolated together with the EVs. Proteomic and multiplex analyses were performed to understand the composition and function of neutrophil sEVs across different stroke stages. Our study revealed distinct patterns in neutrophil activation markers: a decrease in CD62L, which normalized in the recovery phase. Functional experiments demonstrated that neutrophil sEVs attenuate angiogenesis, with a stronger inhibitory effect observed in the acute phase. Additionally, neutrophil sEVs displayed unique surface marker profiles that varied according to the disease stage. Analysis of co-isolated cytokines indicated they may also influence the observed effects of sEVs. This study demonstrates phenotypic changes in peripheral blood neutrophils in stroke patients and highlights the role of neutrophil sEVs in vascular regeneration and immune regulation. Our findings suggest

that neutrophil sEVs have significant potential as biomarkers and therapeutic targets, offering insights into their contributions to the inflammatory response and vascular pathology in stroke.

## 1.8 Placental Extracellular Vesicles As Messenger Of Placental Dysregulation In The Cross Talk With Maternal Immune Cells

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During pregnancy immunological factors (e.g. antibodies, maternal immune cells) are transferred from mother to fetus via the placenta promoting neonatal immunity. Stressors, such as infections, can lead to an impairment of this transfer. However, it remains unknown how this impairment is mediated. A growing body of evidence supports the idea that placental extracellular vesicles (EVs) play a major role in feto-maternal communication. Therefore, in this study we aim to characterize placental EVs in women with impaired placental transfer function upon SARS-CoV-2 infection and investigate their interaction with maternal immune cells. EVs were isolated either via cushion and differential ultracentrifugation from serum samples of third trimester healthy and SARS-CoV-2 infected pregnant women. Isolation was verified by Nanoparticle-Tracking-Analysis and Transmission Electron Microscopy. Placental EVs (pEVs) were enriched by immunoprecipitation and sorting with BD FACSAria Fusion Sorter with subsequent proteomic analysis by Liquid-chromatography coupled Tandem-Mass-Spectrometry. Placental proteomic and scRNAseq data were combined to extrapolate the specific cellular origin of placental EVs. Lastly, maternal Peripheral Blood Mononuclear Cells (PBMCs) were cultured and incubated with pEVs. Internalization of pEVs was verified by (Imaging) Flow Cytometry. After 24h incubation time, cells will be analyzed by Flow Cytometry and Seahorse RT Cell Metabolic-Analyzer. Infection with SARS-CoV-2 decreased overall EV levels, but increased the counts of pEV. Bioinformatics analysis indicates a shift in the pEV secretion by placental fibroblasts after infection. Further, alterations of metabolism-associated genes in pEVs of women with decreased placental transfer rates were detected by proteomic analysis. Incubation of maternal PBMCs with pEVs resulted in an uptake of pEVs, mainly by CD4+ effector memory T cells. PEVs of women with an impaired placental transfer show an altered origin and proteomic signature. Further analysis will unearth their effects on the functional status of maternal T cells.

## 1.9 Origin and Regulation of Antiviral Phosphatidylserine-Exposing EVs

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EVs in body fluids provide a barrier to transmission by viruses applying the strategy of viral apoptotic mimicry, in which they display phosphatidylserine (PS) on the viral membrane to attach to and enter cells. High abundancies of PS-exposing EVs in semen and saliva outcompete virus particles for cell binding and thereby limit e.g. sexual or oral transmission by Zika virus. Their origin and whether PS-EVs can be induced as part of the immune system remain unknown. We collected EVs from conditioned medium from cells of different origin such as lung (A549), liver (Huh-7), prostate (LNCaP, PC3), astrocytes (1321N1), and neuronal cells (SH-SY5Y) as well as those commonly used for infection (Vero E6) or transfection (HEK293T) experiments. In addition, we stimulated cells with inactivated viral particles or transfected them with immune regulatory factors. The resulting EVs were analyzed for anti-Zika virus activity and correlated to EV quantity and cellular origin. We found that both, the antiviral activity and the quantity of EVs released from different cells varies and seems to be cell specific. High potencies were detected for LNCaP EVs, suggesting that prostate EVs contribute to the antiviral activity of semen. After viral stimulation of macrophages and dendritic cells, we observed an increase of EV release, however, with unchanged antiviral potencies. Finally, transfecting cells with SERINC3, a known viral restriction factor and 'serine incorporator', resulted in the release of EVs with increased antiviral potency. This poses the question whether a SERINC3 response is involved in the antiviral defense by EVs. The release and activity of antiviral EVs appears to be conserved but specific for cells of different origin. Antiviral responses followed by immune stimulation or gene expression might increase the number or antiviral potency of released EVs.

## 1.10 Extracellular vesicles released from apoptotic cells impact macrophage response and S. mansoni-induced pathology

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Sensing of dying cells is critical for macrophages to acquire a tissue remodelling function. Therefore, we wanted to investigate whether Extracellular Vesicles (EVs) released by apoptotic cells can contribute to this by influencing macrophage behaviour. Firstly, EVs released by living or apoptotic T cells were analysed for differences in their morphology and cargo composition via transmission Electron Microscopy, Nanoparticle Tracking Analysis, Proteomics and Flow Cytometry. Secondly, the functional impact of EV uptake on bone marrow-derived macrophages was analysed via transcriptomic analysis and flow cytometry. Finally, adoptive transfer of EVs into S. mansoni-infected mice was performed to evaluate their impact on the disease outcome, in which the interplay between dying cells and macrophages plays a crucial role. EVs released by apoptotic T cells highly differ in their protein cargo compared to EVs released by living T cells, for instance, they exclusively contain the active form of the apoptosis executioner caspase: cleaved caspase-3. Macrophages treated with EVs from apoptotic T cells show higher expression of markers associated with an anti-inflammatory phenotype, such as inducible nitric oxide synthase (iNOS) compared to macrophages treated

with EVs from living T cells. Adoptive transfer of EVs released from apoptotic T cells into S. mansoni-infected mice reduced liver damage, as measured by significantly lower ALT levels in the serum of these mice, compared to mice receiving PBS as control. Altogether, the presented results demonstrate that EVs released by apoptotic T cells possess distinct characteristics from EVs released by their living counterpart and can differentially impact macrophage immune response as well as magnitude of tissue damage in the context of a helminth infection. These findings highlight that cell fitness dictates the quality of the EVs released and in turn they can differentially affect the surrounding environment. Investigation of differences between EVs released by living cells or cells undergoing apoptosis and their impact on the interacting cells is crucial for increasing the knowledge about communication of dying cells as well as EV target specificity. Directing EVs towards a specific target cell subset might in the long run contribute to the development of novel drug delivery and therapeutical strategies.

## Liquid biopsy

### 1.11 Extracellular vesicles in pneumogenic ARDS

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Acute respiratory distress syndrome (ARDS) is a major global health problem with a mortality rate of 35% to 45%. It is commonly associated with pneumonia and is characterized by noncardiogenic pulmonary edema, acute hypoxemia, and decreased pulmonary compliance, resulting in shortness of breath. ARDS presents a complex clinical scenario marked by the breakdown of lung epithelial and endothelial barriers. Diagnostic options are limited due to poorly defined symptoms, and treatment efficacy is hindered by ARDS' heterogeneous etiology. In the pathophysiological cascade of ARDS, extracellular vesicles (EVs) emerge as crucial mediators, released from cells into body fluids such as blood. These membrane-enclosed structures, ranging from nanometers to micrometers in size, carry a diverse cargo of proteins and lipids central to various biological processes in health and disease. EVs are being investigated as potential diagnostic and prognostic biomarkers for infectious diseases. This research aimed to explore EVs' role in lung diseases, particularly ARDS, by analyzing EVs from ARDS patients' blood plasma. Comparative analyses involved EVs from severe pneumonia patients and healthy controls. By analyzing the physical, immunological, and compositional properties of these EVs, potential disease progression indicators were identified. The isolated EVs

were analyzed by mass spectrometry proteomics to investigate their protein composition. This comprehensive investigation uncovers EVs as promising biomarkers for diagnosing and prognosing ARDS, while also paving the way for novel EV-based therapeutic approaches to treat ARDS.

## 1.12 Improved Proteomic Identification of Hepatic Metabolic Pathways in Liver-Specific EVs Isolated from Liquid Biopsy by a Combination of SEC, Ultracentrifugation and Immunocapture

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Proteomic analysis of liver-derived extracellular vesicles (EVs) isolated from human plasma holds significant potential as minimal-invasive approach for characterizing organ-specific pathways, including drug metabolism and transport. However, isolating EVs from plasma is challenging due to the co-isolation of abundant contaminants such as lipoproteins and protein corona components, which reduce LC-MS/MS ionization efficiency and hinder the identification of less abundant target proteins. Global EVs were isolated from human plasma either by size exclusion chromatography and ultrafiltration (SEC) or in combination with ultracentrifugation (SEC-UC), followed by immunocapture of liver EVs using anti-ASGR1-coated magnetic beads. Nontargeted proteomic profiling was performed with a nanoflow LC system coupled to an Orbitrap Exploris 480 utilizing optimized acquisition and processing methods for label-free quantification. Enrichment of liver-specific proteins was assessed by tissue mapping using data from the Human Protein Atlas. Comparative analysis revealed that SEC-UC provided approximately twice as many protein identifications for both global (>3,000) and liver-specific EVs (>1,000) than SEC alone. The relative abundance of over 70 common plasma proteins was significantly lower in both global and liver-specific EVs isolated by SEC-UC, indicating reduced contamination. Proteins identified exclusively by SEC-UC included EV markers and, importantly, drug-metabolizing enzymes such as members of the Cytochrome P450 family. Overrepresentation analysis showed significant enrichment of several KEGG metabolic pathways in SEC-UC isolated EVs. The optimized isolation method identified a higher number of liver cell type-specific proteins, including those related to Kupffer cells involved in inflammation and tissue repair. Our results suggest that SEC-UC provides a more comprehensive proteomic profile of liver-specific EVs, covering characteristic metabolic pathways of the liver, including detoxification via CYP enzymes. The method will improve the characterization of proteomic signatures of liver-specific EVs from liquid biopsies under different physiological and pathological conditions, thus facilitating investigations of their use as diagnostic tools.

## 1.13 Translational EV diagnostics: optimized protocol for analysis of EV-derived proteomic signatures from peripheral blood samples in a clinical setting

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Liquid biopsy has emerged as a promising possibility in the clinic to direct individually targeted treatments. Among the components of liquid biopsy, extracellular vesicles (EVs) carry manyfold molecular cargo and are therefore increasingly being studied for biomarker identification. Assessing the impact of preanalytical variables that arise in a clinical setting, is crucial to identify potential confounding factors and determine optimal conditions to take into consideration when studying blood-derived EV contents for biomarker implementation. We established an EV purification and proteomic analysis workflow in a real world clinical setting in which we evaluated different variables, from blood collection through protein preparation for mass spectrometry (MS). We compared blood collection tubes, sample transportation and storage conditions; as well as EV enrichment methods, size exclusion chromatography columns, lysis and protein preparation conditions. For each parameter tested, we assessed hemolysis, particle concentration and size, protein quantity, protein markers (EV-related, cell-specific, co-isolates) and for some, MS analysis was performed. The selection of blood collection tubes affected the stability of blood cells and the characteristics of nanoparticles recovered, showing superiority of citrate tubes. Transportation of blood samples from the hospital to the lab increased particle numbers and, depending on the temperature, also showed differences in cell-specific markers. Delayed processing of the sample resulted in co-isolation of non-physiological particles. In terms of EV enrichment strategy, the use of density gradient, followed by size exclusion chromatography (SEC) recovered high particle numbers with little co-isolated molecules. For SEC, the resin Sepharose CL-4B showed good particle enrichment. For MS, TCA protein precipitation had the highest numbers of identified proteins. We developed an optimized protocol for analysis of plasma EV-derived proteomics, evaluating pre-analytical variables relevant for implementation in a clinical setting.

### 1.14 Pre-Analytical Framework for Routine Clinical Use of Liquid Biopsies: Combining EVs and cfDNA

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Liquid biopsies hold significant potential for the non-invasive management of tumors and other diseases. While the clinical application of cell-free DNA (cfDNA) methodologies is emerging, the implementation of

tumor-derived extracellular vesicles (EVs) as validated biomarkers is hindered by substantial pre-analytical variations. In this work, we are taking a step towards standardizing the pre-analytical procedures of blood collection for subsequent coisolation of plasma EVs and cfDNA with high yield and purity from a single blood collection tube. We compared effects of blood preservation tubes, storage time and subsequent isolation protocols to enable proteomic profiling of resulting EVs next to cfDNA sequencing. Following a stringent method of large and small EV isolation, consisting of differential ultracentrifugation and size exclusion chromatography, we evaluate concentration, quality and integrity of the isolated EVs, EV protein content, the quality of the obtained proteomic datasets as well as possible contaminations by platelets, erythrocytes or lipoproteins. Quantitative and qualitative measurements of EVs indicated that Norgen® tubes are not suitable for EV preservation. While ACD and Citrate tubes demonstrated good results in preserving EVs, only Streck RNA®, Streck DNA® and PAX® tubes showed superior performance in preserving both EVs and cfDNA for up to 7 days. Nevertheless, quality of isolated EVs quickly deteriorated in all tubes when stored for 7 days instead of direct processing. We observed changes in EV size, quantity and protein composition over time potentially originating from blood cell contaminations. Observing significant more variations of protein composition in small than in large EVs after 7days, small EVs might present a greater susceptibility to storage effects. Aiming for fast clinical implementation, our unique workflow provides the basis for informed choice of liquid biopsy tubes along with ready-to-use protocols to retrieve both genomic and EV proteomic biomarker information for multi-omics biomarker-based clinical studies.

## 1.15 Profiling of circulating extracellular vesicles in Bungee-Jumping humans as a model of mental stress

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Brain-derived extracellular vesicles (EVs) potentially cross the blood-brain barrier (BBB) and reach the circulation. Circulating EVs could possibly be used as minimal-invasive liquid biopsy biomarkers of neurological conditions, since they may carry disease-associated molecular cargo or their overall profile could be altered. The multiparametric profiling of circulating EVs from body fluids could thus inform about distinct physiological and psychological states of the CNS, including stress-related conditions. Physiological stress in form of physical exercise is known to trigger the release of EVs into the circulation possibly contributing to adaptive signaling and facilitating homeostasis. Here, we assessed the dynamics of circulating EVs in plasma samples of a Bungee-Jumping cohort as a model for acute mental stress. For profiling of circulating EVs, we used two multiplex bead-based flow cytometry platforms (EV-Immuno and EV-Neuro) for phenotyping of EVs in plasma samples collected from individuals undergoing Bungee-Jumping for the first time. Plasma samples were collected longitudinally at six different timepoints prior and after the jump. CD63+ EVs were separated from plasma and profiled using the EV-Immuno and EV-Neuro assay to assess EV dynamics during an acute stress situation. The longitudinal EV profile of Bungee-Jumpers revealed enhanced signal intensities over four hours post-jump for the majority of the assessed EV-associated markers, e. g. CD45 (lymphocytic), CD49 (platelet) or CD105 (endothelial). Signal intensities were back to baseline one

week after the jump. Interestingly, CD107a+-EVs were also increased after the jump (late endosomal marker), while the tetraspanin and genuine EV marker CD81 remained at baseline over the course of time. This Bungee-Jumping stress model allows for the longitudinal assessment of peripheral EV profiles, including CNS-EV markers. The EV profiles indicate a sustained increase of circulating EVs during stress originating largely in the periphery. Future studies will address their potential functions in stress adaptation.

### 1.16 Detection of Bacterial Membrane Vesicle-associated RNA in Sepsis Patient Samples for Diagnostics

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Even in 2024, 20% of global deaths are still caused by sepsis with the majority being induced by bacteria. So far, specific antibiotic treatment relies on time-consuming identification of bacterial strains by blood culture, making the development of faster and more reliable diagnostics of bacterial infections of the blood stream (bacteremia) a high priority in the battle against sepsis. To further this end, we want to detect bacterial membrane vesicle (bMV)-associated RNA in patient serum samples as a novel approach to identify bacterial strains. The RNA of bMVs from five bacteria strains commonly causing sepsis in humans from three different time points as well as 25 patient serum samples were isolated using a combination of ultracentrifugation and size exclusion chromatography. Samples were analyzed by nanoparticle tracking analysis before RNA isolation and subsequent total RNA sequencing. The obtained sequences were mapped against a representative genome obtained through a meta-analysis cohort. Preliminary results show that we are able to identify several unique genes in the transcriptome obtained from bMV samples allowing for correct identification of bacterial strains.  Although EV preparations from patient serum samples contained mostly human RNA as expected, potential bacterial biomarker sequences could be partially detected in sepsis samples while being absent from healthy volunteers. Further analyses will reveal if these bacterial sequences can be combined to an universal detection profile allowing for unbiased identification of bacterial strains, e.g. in the context of multiple infections. Our findings suggest that it is possible to identify bacterial RNA in human serum samples and therefore opens the possibility of developing a RT-qPCR-based rapid test utilizing bMV biomarkers for a faster sepsis diagnostic enabling to save the lives of thousands of patients worldwide.

### 1.17 Huntington Disease Alters the Actionable Information in Plasma Extracellular Vesicles

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Peripheral disease signatures can be used to monitor the effects of disease-modifying interventions in neurodegenerative diseases such as Huntington disease (HD). Transcriptome and proteome analyses at the tissue and cellular levels suggest alterations in the homeostasis of extracellular vesicles (EVs). Comprehensive and unbiased analyses of EV biology can therefore provide insights into their biomarker potential. We analyzed plasma EVs from a large cohort of control individuals and matched HTT mutation carriers from two different HD clinical stages (control n = 24; pre-HD n= 22; early-HD n = 20). We developed a robust, highly scalable method to isolate EVs from human plasma. We analyze the purified EVs with western blotting, particle size determination and electron microscopy to ensure consistent and robust isolations. For our full cohort, we analyze the EVs by proteomics and RNAseq. We show that the HD EV population contained more small particles, and the 'omics signatures suggested involvement of the innate immune system and REST, a multifunctional gene expression regulator. A supervised machine-learning based classifier enabled accurate assessment of molecular signatures corresponding to the clinical disease stage. Moreover, EVs from HD individuals transmitted actionable information to other cells. EV biology is altered in HD highlighting its biological relevance and potential use as a biomarker in clinical trials. Our approach can be generalized to other sample sources and diseases.

### 1.18 Glycan mapping of tumor-derived extracellular vesicles: a multiplex lectin bead approach

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The glycosylation profiles of lipids and proteins play crucial roles in cell signaling and immune response. In cancer, these patterns shift, driving tumor progression, metastasis, and immune evasion. Tumor cells release extracellular vesicles (EVs) containing various biomolecules that can serve as liquid biopsy biomarkers. However, the variation in glycan profiles of tumor-derived EVs (tEVs) across different cancer stages remains unclear. Currently, there are limited quick and affordable technologies for glycan profiling. Our goal is to develop a rapid and cost-effective technique to characterize these glycan profiles. We isolated EVs from non-cancerous and cancerous cells using size exclusion chromatography and quantified particle numbers with a Zetaview analyzer. To profile glycans, we developed an in-house bead-based protocol utilizing lectins. EVs were pulled down using lectin-conjugated beads and analyzed via flow cytometry. Our findings reveal distinct glycan expressions on HEK EV surfaces. Using lectins such as ConA, WGA, and UEA, which target mannose, sialic acid, and fucose residues respectively, we noted higher sialic acid levels on HEK EV surfaces with minimal mannose detection. Fluorescent-tagged tetraspanin antibodies confirmed EV-lectin binding. Interestingly, EVs from breast cancer cells showed altered glycan profiles with reduced sialic acid and fucose residues compared to non-cancerous cells. Our in-house lectin-based EV profiling

technique is faster (<24 hours) and more cost-effective than traditional glycan profiling methods like microarrays and mass spectrometry. We aim to develop a multiplex lectin-based bead panel to fingerprint cancer cell glycan profiles and identify potential glycan-based biomarkers for future research.

## EVs in cancer

### 1.20 Treatment dependent effects of plasma-derived exosomes from head and neck cancer patients on epithelial-to-mesenchymal transition

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Epithelial to mesenchymal transition (EMT) is a key process in carcinogenesis of head and neck squamous cell carcinoma (HNSCC), contributing to tumor invasiveness, distant metastasis, and recurrence. Exosomes are known mediators and regulators of EMT. Here, we analyze the impact of exosomes that were primed by conventional therapy on EMT modulation. Plasmas of n = 22 HNSCC patients were collected before and after standard of care surgery and prior to adjuvant or primary (chemo)radiotherapy. Exosomes were isolated by size exclusion chromatography. Upon co-incubation of exosomes with HNSCC cells, the cellular EMT profile was analyzed by flow cytometry and RT-qPCR. Wound healing assays were performed to evaluate migratory potential of exosome-treated cells. Reduction of total exosome protein after therapy and in vitro exosome induced EMT profiles were dependent on the type of treatment. Exosomal TFG-β and miRNA cargo were partly responsible for observed exosome induced EMT changes. Exosomes from recurrent patients induced higher tumor cell migration after therapy than exosomes from disease-free patients. HNSCC patients' exosomes from timepoints before and after therapy were able to confer therapy induced EMT modulation in vitro and have the potential to monitor the EMT process. Exosome induced changes in migratory potential emerged as discriminants of therapy outcome.

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

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Atezolizumab and bevacizumab (AB) is the new standard of care for advanced hepatocellular carcinoma (HCC). However, with only 30% objective response, predictive biomarkers are urgently needed. Tissue expression of PD-L1 is associated with better response (Zhu Nat Med 2023), but with limited access to tissue specimens, blood-based biomarkers would be preferable. The aim was to correlate immune checkpoint (IC) expression of tumor-derived circulating extracellular vesicles (EV) with response to AB. This multicenter study included 4 independent cohorts with 158 HCC patients with 414 sequential blood samples and 50 tissue specimens (2 more cohorts collected: analysis of another 104 patients and 284 samples pending). Tissue specimens were stained for PD-L1 and PD-1 (CTLA-4 pending). EV were extracted from serum using differential ultracentrifugation and quality control of isolates was performed using nanoparticle tracking analysis and electron microscopy. IC were quantified using bead-based multiplex immunoassays, normalized to total protein input, and presented as pg/µl. Membrane-bound immune checkpoints (ICs) were significantly enriched in extracellular vesicle (EV) isolates compared to whole serum or EV-depleted serum in HCC patients (Cohort 1, n=40). There was a strong correlation between IC levels in tumor tissue and paired serum EVs (Cohort 2, n=50). Baseline EV-IC levels were higher in non-responders compared to responders in HCC patients receiving AB (Cohort 3, n=49), with distinct EV-IC dynamics over time showing increases in non-responders and decreases in responders. These findings were validated in an independent cohort (Cohort 4, n=43), where baseline IC levels predicted response with high accuracy. Immune checkpoint levels on circulating extracellular vesicles from blood samples are able to predict response to atezolizumab and bevacizumab in two independent cohorts, both before initiation of therapy and based on early dynamics after initiation. Our results hold promise for the development of liquid biopsy-based biomarkers for treatment prediction in HCC.

## 1.22 Extracellular Vesicles from Primary Tumor Spheroids: A Proteomic Approach to Understanding Ovarian Cancer Microenvironment

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Extracellular vesicles (EVs) play a crucial role in modulating the tumor microenvironment during tumor progression. However, targeted analysis of EVs directly derived from primary tumors remains limited across various entities. In ovarian cancer, often diagnosed at a late stage, research primarily focuses on EVs from peritoneal fluid (ascites) which are released by a mixture of cell types including tumor cells and immune cells. This study is the first to our knowledge to specifically isolate and analyze EVs secreted by ovarian cancer spheroids, utilizing a 3D cell culture model derived from primary ovarian cancer cell lines. Primary ovarian cancer spheroids were isolated and purified from patient ascites, and cultured in a chemically

defined medium, followed by EV isolation using a combination of differential ultracentrifugation and size exclusion chromatography. After characterization, EVs were subjected to proteomic mass spectrometry analysis. We successfully isolated EVs from primary-derived tumor spheroids. Proteomic analysis identified several known proteins, such as ATP2B4 and FKBP4, which are reported to be highly expressed in ovarian cancer and associated with poor prognosis. Additionally, we identified numerous proteins that not have been previously linked to ovarian cancer. The study of primary tumor-derived EVs can significantly enhance our understanding of tumor progression and immune modulation within the tumor microenvironment. Overall, the data suggest that certain proteins may serve as critical biomarker for the prognosis and treatment of ovarian cancer.

### 1.23 The Effect of Melanoma-derived Extracellular Vesicles with Different p53 Status on the Formation of Lung Metastasis

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Previously, it has been demonstrated that tumor-derived extracellular vesicles (T-EVs) can promote pre-metastatic niche formation and affect immune responses, yet the specific molecular mechanisms of their protein packaging and impact on metastasis remain unclear. Thus, our study focuses on the influence of p53 status on the molecular composition and functional activity of T-EVs, and their subsequent effects on immune cell plasticity within the melanoma tumor microenvironment and pre-metastatic niche (PMN) in the lungs. Using the B-16V mouse melanoma model, we generated B-16V cells with different p53 statuses via the Sleeping Beauty transposon system. Then, T-EVs were isolated from these cell lines using differential ultracentrifugation (dUC). Also, Nano-flow cytometry, Nanoparticle tracking analysis (NTA), and proteome analyses were employed to determine the morphology and molecular composition of different T-EVs. Our observations revealed that the T-EV samples had similar sizes and concentrations but differed significantly in protein composition. Preliminary data suggest that T-EVs with different p53 statuses may induce distinct effects on pre-metastasis niche formation. Isolated EVs may induce considerable effects on PMN formation and the activity of immune cells in the lungs. However, Future in vivo assessments will be performed to focus on the functional activity of T-EVs to identify their effects on immune cell regulation and lung metastasis. The role of identified EV candidates will also be analyzed in patient samples, correlating findings with clinical data to explore potential diagnostic or therapeutic approaches.

### 1.24 Cargo and Functionality of Plasma-derived Small Extracellular Vesicles in Head and Neck Cancer

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Head and neck cancer (HNSCC) is known as a particularly immunosuppressive type of cancer. Small extracellular vesicles (sEVs) contribute to the immunomodulatory capabilities of HNSCC as part of the tumor microenvironment. The aim of this study was to characterize the cargo of plasma-derived sEVs from HNSCC patients. In addition, the direct and indirect effect (via sEVs-treated macrophages) of sEVs on tumor cells was evaluated to assess the role of sEVs in HNSCC. A MACSPlex EV and a Luminex® multiplex assay were performed to characterize the cargo of plasma-derived sEVs. To evaluate functionality, uptake, proliferation and migration assays were performed and the expression of three EMT markers was measured by qRT-PCR. Here, not only the direct effect was assessed, but also the indirect effect via macrophage supernatants, which were previously generated from primary macrophages treated with sEVs. Between HNSCC patients and healthy donors (HDs) 13 different surface markers were identified with different levels on sEVs. It could be shown that higher concentrations of five immune checkpoint proteins were present on sEVs from HNSCC patients than on sEVs from HDs. Plasma-derived sEVs had no effect on the proliferation of tumor cells but reduced their migratory behavior. sEV-treated macrophages led to a decreased proliferation of tumor cells and an increase in migratory behavior. sEVs of HNSCC patients were taken up into the cells faster than those of the HDs. In addition, supernatants of treated macrophages led to a downregulation of EMT markers. The results showed that plasma-derived sEVs have a limited direct effect on tumor cells but influence them indirectly via macrophages. Characterization of surface markers can be used as a further basis for the identification of biomarkers for the diagnosis and monitoring of disease progression.

### 1.25 Large extracellular vesicles from colorectal cancer cells support metastasis formation by promotion of tumor invasion and adhesion

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The formation of metastasis in patients suffering from colorectal cancer (CRC) leads to poor prognosis. Although the involvement of extracellular vesicles (EVs) for pre-metastatic niche formation has been proven, the underlying mechanisms remain mostly unclear. Intracranial injection of murine CRC cells (CMT93) into syngeneic BALB/c mice was performed to model metastatic colonialization of the brain. Large and small

tumor-derived extracellular vesicles (lEVs / sEVs) from CMT93 cells were isolated via differential ultracentrifugation and comprehensively characterized by electron microscopy, NTA, immunofluorescence imaging and immunoblots. Using functional assays, cells and EVs were analyzed for their tumor-supporting effects with potential implications for brain metastasis. Our mouse model revealed two variants of CMT93 cells that differed in their potential for metastatic colonization (CMThigh/low). Subsequent analysis of the proliferation rate, migratory and invasive behavior and the expression of markers for epithelial-to-mesenchymal transition exhibited no major differences in cell-intrinsic characteristics. Instead, stimulation with supernatant from CMThigh cells caused a higher invasion rate of the more benign CMTlow cells. This effect was abolished after depletion of EVs from the supernatant indicating that EVs are the active component in the CMThigh secretome. Transwell assays confirmed that in particular the lEVs of CMThigh cells increased cancer cell invasiveness. We observed no alterations in EV size or secretion rates between CMThigh and CMTlow cells, but the pro-invasive phenotype of the lEVs was antagonized by heat-inactivation. As this suggested that lEV-associated proteins are responsible for supporting tumor invasion, we profiled the lEVs from both variants by mass spectrometry and detected major differences in their cargo. The identified two model cell lines will help to identify the molecular factors involved in CRC brain metastasis and have highlighted the role of lEVs in this process.

### 1.26 Therapy Response Monitoring in Anaplastic Thyroid Cancer Cells Using Extracellular Vesicle-associated RNA

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Anaplastic thyroid cancer (ATC) is a rare but highly aggressive form of thyroid carcinoma with a tendency for early metastasis and poor prognosis. Because there is a lack of targeted therapeutic options for ATC, repurposing of drugs approved for the therapy of other (thyroid) carcinomas, e.g. vandetanib, offers promising treatment alternatives. We treated the ATC cell line Cal62 in triplicates with 8.8 (EC50) and 20 µM vandetanib as well as a 0.1% DMSO control for 48 h in EV-depleted medium and characterized the EVs enriched from the cell culture supernatant. Subsequently, we established several RT-qPCR assays to evaluate the cellular and EV-associated transcriptomic response to the treatment and investigated the dynamic change of transcription levels at different time points after treatment. Nanoparticle tracking analysis and flow cytometry confirmed the successful enrichment of Cal62 EVs from cell culture supernatant. Cal62 cells responded to the vandetanib treatment with significant transcriptional regulation of several transcription factors involved in the MAPK-pathway (FOS, STAT3, MYC). Consequently, we also observed significant up- and downregulation of multiple of their target genes (EGFR, ERRFI1, HSP90AA1, CDKNA1, CDC25A), corresponding to cell-cycle arrest and negative RTK signaling feedback regulation, however, the time-specific expression patterns differed between genes. This transcriptomic response was also reflected in the extracellular RNA isolated from the Cal62 EV-enriched supernatant. Our results demonstrate that vandetanib induces transcriptional changes in Cal62 cells, which is also reflected in Cal62 EVs. This is further evidence

that vandetanib is a good candidate for drug repurposing in ATC and EV-associated RNA might facilitate therapy assessment through liquid biopsy. As a next step, we will analyze the cellular and EV-associated transcriptomic profile of Cal62 cells in-depth, using next-generation sequencing to identify an EV-associated transcriptomic biomarker signature for the prediction of vandetanib response in vitro.

### 1.27 Extracellular Vesicle-mediated Wnt Signaling in Health and Disease

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Wnt signaling is pivotal for cell proliferation and differentiation. Mutations in Wnt pathway components are associated with several cancers, e.g. colorectal cancer (CRC). Wnt ligands secreted on extracellular vesicles (EVs) have been shown to promote recipient cells' proliferation and invasiveness. However, it remains unclear how Wnt activation affects EV secretion and cargo, and which mechanisms are mediated through EV-bound versus non-EV-bound Wnt ligands. Using CRISPR/Cas9, we generated two cell lines from the CRC line HCT116: APCtrunc with a clinically relevant truncation of APC, causing constitutive Wnt pathway activation, and EVIKO, a knock-out of the Wnt cargo receptor EVI/wntless, rendering the pathway inactive. We analyzed Wnt ligand secretion from APCtrunc, EVIKO and wild-type (WT) cells by Western Blots after Wnt ligand pulldown. EV fractions were enriched from the HCT116 lines and healthy colon fibroblasts with differential centrifugation. EV isolation was validated using electron microscopy and Western Blots. The effect of Wnt activation on vesicle quantity and size was investigated using nanoparticle tracking analysis (NTA). Analyses of the protein cargo by mass spectrometry are ongoing. We established and characterized the HCT116 APCtrunc and HCT116 EVIKO cell lines, observing a differential secretion of selected Wnt ligands depending on the Wnt signaling background. Two distinct vesicle size fractions were reproducibly enriched from different cell culture supernatants. These fractions exhibited vesicle morphology and classical EV markers. Cells with different Wnt mutational backgrounds showed changes in the number of particles secreted per cell. Ongoing investigations evaluate differences in the EV cargo derived from HCT116 WT, HCT116 APCtrunc, and HCT116 EVIKO cells. In future studies we will assess the influence of isolated EV and non-EV fractions on cell migration and tumor invasiveness. By investigating the effects of oncogenic Wnt mutations on EV biogenesis and composition, we will improve the understanding of oncogenic signal distribution via EVs in CRC.

### 1.28 Investigation of Radiation-Induced Extracellular Vesicles (EVs) in Head and Neck Cancer

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Radiotherapy has been shown to promote the release of extracellular vesicles (EVs). Here, we aim to investigate the role of radiation-induced EVs in squamous cell carcinoma of the head and neck (HNSCC) using both cancer cell lines and EVs derived from patients which have been treated with radiotherapy. We aim to understand the interaction of radiation-induced EVs with the tumor microenvironment as well as their potential as biomarkers in HNSCC. We have thoroughly established methods for EV isolation and characterization from both cell line supernatants and patient plasma. We are investigating EV cargo with proteomic profiling as well as NGS. We have established live imaging co-culture systems to understand EV uptake, communication and function in various cell types. Our preliminary results confirm that EV release is triggered by radiation and that the cargo of radiation-induced EVs may promote cell invasion and migration. Moreover, we could show that radiation-induced EVs are uptaken more by HNSCC cells which have not been exposed to radiation. We have also identified components of EV cargo which differ in radiation treated cancer patients and compared to healthy controls. Taken together, our findings contribute to better understanding the role of radiation-induced EVs in HNSCC. Our results will be further confirmed and developed to support this hypothesis.

## 1.29 Proteomic analysis of endothelial cell secretomes exposed to hypoxic head and neck cancer cell-derived exosomes

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Head and neck squamous cell carcinomas (HNSCCs), with high mortality and morbidity rates, are associated by enhanced lymphatic and organ metastasis, immune escape, despite effective treatment strategies in diagnostic/therapeutic techniques.In recent studies, it has been shown that changes detected in cancer tissues are mediated by exosomes, which are one of the cellular communication tools between tumor cells and the microenvironment, especially by increased secretion from tumor cells. However, the content of exosomes released under hypoxic conditions in many cancer types, including HNSCCs, and their mechanisms of action on TME (Tumor microenvironment) are not yet fully understood. Therefore, we aimed to examine the changing proteome content the of endothelial cell's secretomes exposed to exosomes released from these cells as a result of exposure of HNSCCs to hypoxic conditions, and to investigate the properties of proteins. Changes in the protein profile of the secretome of human umbilical vein endothelial cells (HUVECs) treated with hypoxia-induced tumor-derived exosomes (hiTDExs) were evaluated using a cytokine antibody array. hiTDExs were significantly changed the levels of cytokines in the endothelial cell secretome. As a result of the cytokine antibody array, at least 10 different cytokines were identified with significantly altered expression in the secretomes of endothelial cells treated with hiTDExs. The contributions of CCL24, EGFR, GRO a/b/g, TNF-RI, and TNF-RII, whose expressions changed significantly in the endothelial cell secretome, in the reorganization of the HNSCC microenvironment need to be characterized through further

functional assays. It has become an urgent necessity to understand the pathogenesis and progression of HNSCC in the molecular level, to develop new and effective treatment strategies, to characterize the cytokines responsible for the aggressive HNSCC phenotype, especially microenvironment components and their interrelationships, and to better understand the molecular mechanisms underlying the disease.

### 1.30 Binding of circulating melanoma cell-derived extracellular vesicles to ultra-large von Willebrand factor induces cancer-associated thrombosis

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Extracellular vesicles (EVs) from tumor cells are being explored as cancer biomarkers due to their varied contents, including proteins and genetic material. Tissue factor-positive (TF)-EVs, linked to tumor progression and cancer-associated thrombosis (CAT), are associated with elevated von Willebrand factor (vWF) levels. This study investigates if melanoma cell-derived TF-EVs promote CAT by interacting with ultra-large vWF (ULvWF). Heparin-coated Fluorescent Polystyrene Particles were used to simulate binding process of cells or EVs to ULvWF. Computer modelling were used to validate the experimental results. Binding of melanoma cell-derived EVs or melanoma cells to ULvWF and subsequent thrombosis formation was investigated by microfluidic experiments. Microfluidic channels coated with human umbilical vein endothelial cells or vWF were perfused with whole human blood supplemented with purified and fluorescently labeled EVs. EVs were characterized by nanoparticle tracking analysis, flow cytometry, super resolution microscopy STED and electron microscopy. The expression of TF on different melanoma cells and melanoma cell-derived EVs was studied by fluorescence microscopy. We found that melanoma cell-derived EVs, but not intact cells, can bind to ULvWF. Smaller EVs had a higher probability of interacting with ULvWF, while the binding rates of larger EVs were very low. ADAMTS13, the cleavage enzyme of vWF, significantly reduced the binding of EVs. EVs bind to ULvWF through interactions involving HS and the vWF A1 domain. Furthermore, binding of TF-EVs to ULvWF activated platelets and induced the formation of microthrombi. Although intact melanoma cells could not bind to ULvWF directly, melanoma cells were trapped in microthrombi. In conclusion, this study demonstrates that melanoma cell-derived EVs induce CAT by binding to ULvWF. While TF-EVs may serve as a promising biomarker, blocking ULvWF formation or EV binding may prevent metastasis.

### 1.31 Characterization and function of extracellular vesicles in Merkel Cell Cancer progression

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The function of extracellular vesicles (EVs) in the pathogenesis of Merkel cell carcinoma (MCC) and concerning modifications and function in the tumor microenvironment (TME) remains elusive. Individual publications mention miRNA-375 as EV cargo, which is transferred via EVs to fibroblasts and possibly activates the TME. Previous work in our group showed that in MCC tumors which are virally induced by MCPyV, the viral oncoprotein sT is crucial for tumor progression by altering surface marker expression and thereby TME interaction. We aimed to conduct a comprehensive description and analysis of the protein and nucleic acid cargos of EVs derived from patient-derived virus –positive MCC cell lines expressing or not expressing sT to elucidate the potential role of MCC EVs in MCC progression and TME. For this analysis, EVs from MCC untreated cells and MCC cells in which sT was downregulated by shRNA expression were isolated via ultracentrifugation. Nanoparticle Tracking Analysis, Imaging Flow Cytometry and Cryo-EM was used for phenotypical analysis. High-throughput sequencing and mass spectrometry was employed to determine the RNA and protein content and composition of MCC EVs. High-throughput sequencing of RNAs together with proteome analysis suggest that EV cargos do not simply reflect the parental cell composition. Notably, most highly expressed proteins, transcripts and miRNAs from the parental cells were not detected in EVs. Proteome analysis shows an enrichment of nuclear-derived proteins, particularly in EVs derived from cells expressing sT protein. Pathway analysis of predicted mRNA targets of the EV-miRNA cargos revealed an enrichment of target genes with predicted functions in cancer-related pathways. Based on this comprehensive characterization of MCC EVs, which hints towards a potential role of the EVs in TME regulation, current experiments address the qualitative differences of EVs in MCC progression and their functional role, especially in the activation of TME resident macrophages and immune evasion.

**OCTOBER 2nd**

## EV methods

### 2.1 In vitro models for induction of MitoEV release from Jurkat cells

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Extracellular vesicles carrying mitochondrial content (MitoEVs) have recently gained much attention as more studies have shown that MitoEVs can deliver mitochondrial components to recipient cells and affect their function. As this is an emerging field, the models for in vitro MitoEV studies are still missing. Here, we present two approaches for the induction of MitoEV production in Jurkat cells. The first model uses ionizing radiation, which induces mitochondrial DNA damage, the second one inhibits lysosomal function and thereby disrupts mitophagy. We characterized mitochondrial cargo within large and small extracellular vesicles released from the cells. Our results showed that mitochondrial DNA and protein can be detected in both large and small EVs after Jurkat cell irradiation and lysosomal inhibition. Using proteinase and DNase protection assays we demonstrated that mitochondrial cargo is attributable to EVs. Nonetheless, we also detected a large proportion of cell-free mitochondria and mtDNA outside of EVs. These data showcase two fast and relatively straightforward approaches for producing MitoEVs in Jurkat cells, together with several assays for MitoEV detection. This protocol can be applied to detect MitoEVs produced by different cell types in vitro. Additionally, generated MitoEVs can be used in a number of downstream applications, including genetic and pharmacological manipulations.

### 2.2 Efficient isolation and characterization of exosomes and insights into their role in prostate cancer progression and treatment response

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Prostate cancer (PCa) is the most prevalent malignant tumor among men. Despite early detection, diagnosis and treatment, PCa remains the second frequent cause of cancer related deaths. With their cargo depending on their parental cell, presenting a potential message to other cells, extracellular vesicles (EVs) present var-

ious opportunities for their application. A primary goal of this study is finding an efficient, time-saving exosome isolation method, as the gold standard method of ultracentrifugation is not suitable for small volumes, time-consuming and poses risks of damaging exosomes. As an additional aim, the potential role of exosomes in prognosis of PCa is analyzed. Hormone-sensitive, human PCa as LNCaP and hormone-resistant ones including LNCaP-sublines were utilized. EVs were isolated by ultracentrifugation, precipitation, and a combined concentration-precipitation method. EVs were analysed by nanoparticle tracking analysis. Exosome characterization was conducted using Western blotting to assess CD9 and CD63 expression. PKH67-staining was utilized to observe exosome uptake. LC-MS/MS was utilized to examine the proteomics of EVs in comparison to their parent cells. EVs isolated from patient-gained tissue slice culture were measured for verification. Our findings suggest that the combined concentration-precipitation method yields the most effective isolation of EVs with the highest concentration. Exosomes were characterized by detecting exosomal markers. Confocal microscopy revealed significant cellular uptake of PKH67-labelled exosome. Proteomic profiles of EVs and their parental cells show notable differences. Significant variations in proteomic profiles were observed between EVs isolated from LNCaP cells and their resistant sublines. Several pathways are enriched exclusively in EVs. These data were further validated using EVs isolated from tumor slice cultures established from patients. Our study highlights the efficacy of our new established isolation method. Furthermore, our findings suggest unique cargo and functional differences between EVs and their parent cells, under scoring their potential roles in prostate cancer progression.

## 2.3 Isolation and Biological Activity of EVs: The Purification Methods Matter

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Different methods to isolate extracellular vesicles (EVs) are discussed in the cross-cutting area between contamination, specificity and yield (MISEV 2024). Here we aim to provide a comprehensive and systematic comparison of purification methods in terms of EV protein cargo loading and a biological function. EVs were isolated from either peritoneal fluid present in ovarian cancer patients (ascites) and ovarian cancer cell (ES-2) supernatant using ultracentrifugation-based (UC) methods or tangential flow filtration-based (TFF) methods followed by ultrafiltration or size exclusion chromatography. EV samples are comprehensively characterized regarding particle number and particle size by via nano flow cytometry (nFC), Western blot analysis, electron microscopy and mass spectrometry-based proteomic approaches (MS). As expected, the EV samples from different purification methods showed distinct features regarding particle number and protein concentration and these data were correlated to MS results. The ratio of known EV marker to the total protein amount and number of peptides detected in MS were indicative for the specificity of the isolation method used. We report that density gradient and TFF combined with size exclusion chromatography produce purer EV samples, whereas differential ultracentrifugation and TFF followed by ultrafiltration result in higher yields with greater protein variability. This study highlights the impact of purification methods on EV characteristics and biological activity. Using NFkB activation in reporter cells, we link purification methods to biological function, underscoring the need for standardized EV isolation protocols to enhance reproducibility.

## 2.4 Novel insights into the isolation of extracellular vesicles by anion exchange chromatography

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Extracellular vesicles (EVs) are membrane-enclosed particles secreted by most cell types. They have gained interest as biomarkers and in drug delivery applications. Currently, the potential of EVs is hampered by a lack of suitable methods for downstream pharmaceutical applications and a lack of standardization. HEK293 derived EVs were isolated using a strong anion exchange resin after nucleic acid digestion and concentration steps. EVs were eluted with a linear sodium chloride gradient up to 1.2M. Chromatographic fractions were analyzed comprehensively using various methods. Using strong anion-exchange resin chromatography (AEX), the majority of the EVs were recovered and the elution fractions showed a reduced protein content throughout the elution. With increasing salt concentration, the mean particle size increased and the protein content decreased. Tetraspanin (TP) composition and colocalization remained consistent, while absolute CD81 content peaked in early elution and decreased throughout, indicating TP-negative vesicle populations in later elution fractions. AEX is a promising tool that could address the current challenges of EVs in therapeutic applications by providing a standardized and scalable process. The results of this work contribute to a better understanding of the underlying mechanism and the interaction of the resin with an efficient and optimized downstream process for EVs can be optimized and established based on these findings.

## 2.5 Optimization of salivary and blood-derived EV enrichment for the detection of the Parkinson´s disease-related biomarker aSyn

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Aggregates of aSyn in the CNS are pathological hallmarks of Parkinson´s disease (PD), and findings indicate that pathological forms of the protein are also present in extracellular vesicles (EVs) in peripheral tissues. Thus, EVs deserve particular interest in PD research as they can serve as a gateway to a better understanding of the disease mechanism and inter-organ spreading, and as potential sources of early disease biomarkers. The aim of this work is to establish an effective aSyn seed amplification assay (SAA), which enables detection of minimal amounts of pathological aSyn forms in EVs derived from peripheral body-fluids. For this, we compare various EV-enrichment methods and identify factors which influence aSyn-SAA. We

isolated EVs from blood and saliva with methods based on polymer-precipitation, membrane-affinity, ultracentrifugation, and size-exclusion chromatography, and characterized the obtained samples for size and protein markers via transmission electron microscopy, nanoparticle tracking analysis, and western blots. For the optimization of pathological aSyn detection we assessed the influence of monomer and seed concentrations, and reagents commonly used during sample preparation on the sensitivity and reliability of aSyn-SAA. We show that different EV-enrichment techniques result in significant differences in yield, integrity, and purity of vesicle preparations. We demonstrate that under optimal conditions aSyn-SAA enables the detection of picograms of pathological aSyn conformers. However, the assay sensitivity and reproducibility greatly depend on the optimal monomer concentration and seed quality, and is influenced by the presence of reagents commonly used for sample preparation. The detection of pathological aSyn seed in EVs obtained from peripheral tissue via minimally invasive route is a promising approach for PD diagnosis. However, due to the required very high sensitivity of the assay, the EV-enrichment method used for aSyn seed isolation has to be chosen carefully, and the seed amplification assays need to be performed with great scrutiny.

## 2.6 Comparison of EV enrichment methods from human plasma samples for mass spectrometry analysis

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For early cancer detection, minimally invasive biomarkers with high specificity and sensitivity are needed. Extracellular vesicles (EV) from plasma could serve this purpose, but their enrichment is technically challenging, due to interference from highly abundant plasma proteins with analytical applications such as mass spectrometry (MS). In this study, plasma from three healthy donors was used to compare a self-designed EV enrichment method based on density cushion centrifugation (DCC) combined with size exclusion chromatography (SEC) and concentration (method 1), with the exoRNeasy midi kit from Qiagen (method 2), and with unprocessed plasma. Nanoparticle tracking analysis resulted in median particle diameters ranging from 148.1 to 173.4 nm for samples generated with method 1, and from 171.3 to 189.2 nm for method 2, while diameters for unprocessed plasma were 100.2 to 130.6 nm. The EV tetraspanins CD9, CD63 and CD81 were detected by flow cytometry in samples from method 1 and unprocessed plasma, while in method 2 only CD9 could be detected. MS analysis revealed that EV markers, such as CD81, Flotillin-1, Syntenin-1, and Programmed Cell Death 6-Interacting Protein, could not be detected in unprocessed plasma and were only sporadically found in samples generated with method 2. Using method 1, all markers were present in all samples. The levels of highly abundant plasma proteins, such as albumin and serotransferrin, tended to be lower in samples generated with method 1 and 2 compared to unprocessed plasma. In summary, the combination of DCC, SEC and concentration proved to be a promising approach for EV enrichment from plasma. In future research, this method could be applied to discover EV-based biomarker proteins from plasma of patients with cancer.

## 2.7 Methodological Overview of Isolation and Characterization of Patient-derived Serum and Fecal EVs

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The research field of extracellular vesicles (EVs) is becoming increasingly popular, but the isolation of EVs derived from patients samples still faces some challenges, due to, for example, the high complexity and diversity of particles and microorganisms in stool samples. Here, we provide a methodological overview of isolation and characterization of patient-derived serum and fecal EVs, to summarize general-used protocols for patient-derived EVs preparation. We employed ultracentrifugation for fecal EVs isolation, while there are commercial isolation kits available for serum EVs. Then transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), Western Blot (WB) and metabolomic analysis were included to determine biophysical properties and for quality control. Furthermore, TLR4-reporter-assay and LOL assay were used for functional analysis. A high number of EVs could be isolated by ultracentrifugation, meanwhile the results of main parameters including concentration, diameter and shape showed high quality of isolated EVs. Functional analysis demonstrate that EVs contain high level of LPS and NF-κB induction. Ultracentrifugation and isolation kits are well-established protocols for isolating patient-derived EVs. Techniques such as TEM, NTA, and metabolomic analysis aid in the characterization of EVs. Patient-derived serum and fecal EVs could act as regulators of the immune response.

## 2.8 Thoracic Aortic Diseases: Identification of Diagnostic Biomarkers Using Bottom- Up Proteomic Analysis of Extracellular Vesicles and Parallel Reaction Monitoring of Plasma Samples

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Thoracic aortic aneurysms (TAA) are common causes of death in western industrial nations. Diagnosis and risk assessment in patients with TAA remains challenging. Therefore, we aimed to identify possible circu-

lating blood biomarkers for the diagnosis and prognostic assessment of thoracic aortic diseases by analyzing extracellular vesicles as well as human plasma samples by using mass spectrometry-based proteomics. A total of 80 patients were divided into an aneurysm group (n=40) and a control group (n=40), and plasma samples were taken from each patient. To identify a potential biomarker for the diagnosis of TAA, plasma samples were analyzed using parallel reaction monitoring and extracellular vesicles extracted from plasma were analyzed using a bottom-up proteomics approach. Overall, 1077 proteins were successfully identified in extracellular vesicle samples, including several well-known EV markers such as CD47, CD63, CD73, CD81 and FLOT1. In total, student's t-tests revealed 14 proteins to be significantly upregulated ($p$-value $< 0.05$, fold change > 1.5) in patient samples (i.e. Myeloperoxidase: $p=0.03$, fold change=2.0; Enolase 2: $p=0.01$, fold change=3.8) while 14 proteins were significantly upregulated in control samples. At the same time, PRM measurements show that the C-reactive Protein is significantly upregulated in patient samples. A range of potential TAA biomarkers were found among the significantly regulated proteins. For instance, Myeloperoxidase and Enolase 2 were both found to be associated with aortic disease in prior analyses and may therefore be valuable biomarkers for TAA. The identified biomarker candidates need to be validated in larger, external TAA cohorts before they can be used clinically for the diagnosis and prognostic assessment of TAA.

## 2.9 Extracellular vesicles (EV) Purification by Anion-exchange Membrane Chromatography reveals EV Subpopulation Separation

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Heterogeneous bulk isolates of EV are used in (pre-)clinical studies, limiting their therapeutic potential. Current methods for separating EV subpopulations are not scalable or lack purity and yield. Large-scale purification processes are needed to address the interest in EVs as drug carriers. Ion-exchange chromatography (IEX) is a well-established method in the biochemical and pharmaceutical industries. Specific charges on the surface of EVs and their interaction with the functional groups of the IEX carrier can be used to separate subpopulations. In particular, membrane technology as a tool offers the advantage of high flow rates so that EV purification of cell culture supernatants can be performed in short process times without prior concentration. We established a scalable membrane-based EV separation and purification procedure. For a detailed insight, all chromatographic fractions were observed by several EV analysis techniques. Particle concentration and size distribution, surface protein and membrane labeling were analyzed by nano flow cytometry, protein content, dsDNA and HCP levels were determined, and fluorescent microscopy was performed. Using an anion-exchange membrane, two populations can be eluted using a salt gradient. Both populations appear to differ in particle size and the data indicate an enrichment of MemGlow™- and CD81-positive particles in the 2nd elution peak. HCP and dsDNA levels can be reduced in both elution peaks compared to the cell culture supernatant. Furthermore, our studies show high batch-to-batch variability of cell culture supernatants and that preprocessing greatly affects chromatography. Our scalable, membrane-based process effectively separates EV subpopulations and reduces contaminants such as proteins and

dsDNA. This method improves the purification of EVs and enhances their potential as therapeutic drug carriers.

## 2.10 A New Class Of Universal Reference EVs

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Extracellular vesicles hold great promise as circulating biomarkers for diagnostic purposes ("liquid biopsy") and, thus, many research groups world-wide investigate various aspects of EVs, their biology and medical significance. However, scientific results from different laboratories (and experimental setups) are often difficult to compare and interpretation of diagnostic tests may be problematic mainly because reliable reference EVs are not available. Here, we introduce a new class of reference EVs to be used as an internal standard for quantitative assessment of EV preparation and improvement of overall data quality. HEK293-cells were transduced to express horseradish peroxidase (HRP) on their surface. Cell-culture-derived HRP-positive EVs (collected from serum-free conditioned medium) were evaluated for their performance in spike/recovery experiments in a complex matrix using different EV preparation methods (e.g. SEC, EV-precipitation, Density gradient centrifugation). Easy and fast quantification of HRP-positive EVs is possible via TMB-based colorimetric detection. Our HRP-positive EVs exhibit common EV-characteristics (particle size distribution, density, EV-markers). The HRP-activity which was shown to be associated with EV-particles is stable after spike-in and recovery from tested complex biological matrices. In individual EV-preparations (n=4; SEC-based or precipitation-based preparation methods), the recovery of HRP-positive EVs (% relative to input) was calculated and used for normalization of the particle-concentration as measured by NTA. Compared to the absolute results of the particle-concentration, the variability (coefficient of variation) between individual EV-preparations was reduced from 22% to 16% (precipitation-based method) and 16% to 3% (SEC-based method) using the normalized data. Our HRP-positive reference EVs feature robust and sensitive detection via an enzymatic reaction and, thus, can support harmonization and standardization in the EV-field. Their general suitability for data-normalization has been shown in this study and, in the future, could be expanded to clinical applications as a mean of quality control and for normalization of biological specimen.

## 2.11 V-Disk: Automated Multimodal EV Purification from 1 ml of Plasma

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The clinical application of extracellular vesicle (EV) research, such as in liquid biopsies, is limited by the lack of reproducible, automated, and affordable EV purification methods. An ideal method would standardize the entire workflow from blood collection and plasma separation to EV purification for reliable and comparable results. Our microfluidic V-Disk cartridge aims to achieve this. The current V-Disk processes 1 ml of pretreated plasma, and the next version will automate plasma separation from 3 ml of blood, completing the purification workflow. Using disposable centrifugal microfluidic cartridges manufactured by injection molding, we automate a multimodal EV purification process consisting of cation exchange chromatography (CEX), two filtration steps to remove particles >800 nm and <20 nm and concentrate the EVs, and a final treatment with a mixed-mode chromatography (MMC) resin to remove remaining protein contaminants. After establishing the layout and manufacturing processes of the V-Disk, we conducted an initial study comparing it with size-exclusion chromatography (SEC), using samples from melanoma patients and one healthy control. ELISA results of EV surface markers reveal that the V-Disk isolates exhibit a higher EV yield than those obtained via SEC. Measuring proteins, HDLs and VLDLs we demonstrate that the V-Disk is capable of removing impurities from plasma samples to achieve purity comparable to that of SEC. Nanoparticle-tracking analysis reveal that the size distribution of isolated particles is around 100 nm. Starting from CEX-treated plasma, the current version of the V-Disk already demonstrates significant potential, competing with SEC in terms of EV purity and yield. In the future, the V-Disk will offer a fully automated, standardized label free isolation of EVs from whole blood in a highly reproducible manner in a small point-of-care compatible device.

## 2.12 Comparative Analysis of Tube Coatings and Size Exclusion Chromatography Columns for Optimal Extracellular Vesicle Isolation

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Efficient isolation of extracellular vesicles (EVs) is crucial for various scientific questions. Size exclusion chromatography (SEC) has emerged as an effective methodology to deplete soluble proteins. This study comparatively analyzes the performance of three SEC columns. Additionally, it examines the impact of tube coatings for ultracentrifugation and storage. The aim is to optimize EV yield and purity, providing a protocol for the EV community to perform impactful studies. Initially, the impact of ultracentrifugation tube coatings with BSA, Triton, Ethanol, and Tween was tested. After separating large and small EVs originating from cell culture supernatant and plasma through ultracentrifugation, EV eluates were equally split onto the three tested SEC, namely IZON qEVsingle, manually manufactured SEC (mmSEC), and SmartSEC Single. Finally, storage tubes were coated with Ethanol, ProteinLoBind, and PBS-HAT before use. EV quantity and quality was assessed by protein measurements, nanoparticle tracking analysis, electron microscopy and mass spectrometry. Coating of ultracentrifugation tubes led to differences in particle size and number as well as non-EV contaminations with Ethanol showing superior performance. SEC choice did not show significant difference in EV yield or purity. While SmartSEC showed highest yields and protein complexity, IZON and mmSEC

showed slightly higher EV enrichment. In general, all investigated columns were effective in eliminating contaminants. Storage tube coating showed minor impact on yield, integrity and purity with the exception of PBS-HAT which led to higher numbers of intact EVs coupled with a substantial protein contamination. In summary, SmartSEC is advantageous for high throughput and high yield isolations, while mmSEC and IZON can provide a higher purity. Tube coating can influence yield and purity during isolation and storage. Our analysis underscores the importance of analytical considerations depending on the research objectives and sample availability, as choice of SEC column and tube coating can significantly impact yield and purity.

## 2.13 The Extracellular Vesicle Adipocyte Proteome is Severely Affected by the Isolation Method

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20 years after the discovery of leptin, adipose tissue is recognized as a true endocrine organ. In addition to its homeostatic role, its impact has also been highlighted in other contexts, like aging or tumor progression, involving not only adipokines but also extracellular vesicles (EVs). Remarkably, adipose tissue has been described as a major contributor to circulating EVs. That finding, however, relies on scarce evidence from a few adipocyte-specific markers. In this work, we describe several adipocyte-specific markers enriched on EVs and investigate whether their pattern is influenced by aging and tumor status in plasma samples. Label-free mass-spectrometry analyses have been performed on EV preparations from in vitro differentiated adipocytes (3T3-L1) as well as plasma-derived EVs. Different isolation procedures were carried out including differential ultracentrifugation (dUC), tangential-flow filtration (TFF), and size-exclusion chromatography (SEC). High-throughput proteomics confirmed that EVs derived from in vitro differentiated adipocytes were significantly enriched in adiponectin (Adipoq), leptin (Lep), and fatty-acid binding protein 4 (Fabp4), among others. Interestingly, the relative abundance of these molecules as well as a number of EV-related markers were affected by the isolation method. These observations were further confirmed for plasma-derived EVs, where the isolation method had a stronger discriminatory effect than sample grouping, which included ovarian cancer patients and age-matched healthy donors. Remarkably, adipocyte-related markers like ADIPOQ displayed different abundance patterns depending on the isolation method of circulating EVs. The functional implications of adipocyte-derived factors like adiponectin or leptin have been repeatedly described, albeit controversially. Our data show the relative abundance of these molecules on EVs to be altered by the isolation method, which may explain some of the contradictory results from the literature. Our results provide new evidence on the necessity of well-described and highly standardized methods, either when working with biofluids or with in vitro-derived samples.

## 2.14 Optimizing the CFDA-SE Labeling Method for Extracellular Vesicles Derived from Clinical Samples

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Efficient labeling of extracellular vesicles (EVs) is crucial for their characterization and tracking. The cell-permeable dye carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), which turns into its fluorescent variant CFSE intravesical, is commonly used for fluorescent labeling of EVs in flow cytometry. Although several methods for the CFDA-SE labeling of EVs have been described, there is significant variability in labeling efficiency and reproducibility. This study aimed to optimize the CFDA-SE labeling conditions for EVs derived from diverse clinical samples. EVs were isolated from pooled human urine, plasma, and serum samples by sequential centrifugation. Key parameters, including EV concentration, CFDA-SE concentration, incubation temperature, and use of one-step protocols, were systematically evaluated to optimize labeling efficiency. Flow cytometry was used to quantify the efficacy of labeling. Clear EV signals were achieved using one-step protocols. Longer staining times and lower CFDA-SE dilutions resulted in higher EV counts. Incubation at different temperatures (4°C, room temperature, and 37°C) influenced EV counts, with room temperature providing the most stable results. A 20-minute incubation at room temperature produced efficient labeling, resulting in a strong signal. Optimized CFDA-SE labeling conditions provide stable and efficient quantification of EVs derived from clinical samples. This protocol refinement improves the reliability and efficiency of flow cytometry in EV quantification.

## 2.15 Towards analysis of exogenous siRNA loading homogeneity of bovine milk extracellular vesicles

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In a prior study, we established a siRNA loading strategy yielding an average of 150 siRNA copies per milk extracellular vesicle (mEV), but we assume this siRNA distribution is uneven [1]. A homogenous siRNA loading is crucial for reliable siRNA dosing. To distinguish between loaded and non-loaded vesicles, we systematically investigated different analytical methods. After enrichment, the mEV pellet was loaded with fluorescently labeled siRNA bound to calcium phosphate nanoparticles (CaP-NP) using dual centrifugation [1]. As control, mEV mimicking liposomes (EVmL) were manufactured and loaded with CaP-NP or just with siRNA. Analytical methods - for fluorescence detection on single vesicles: nanoparticle tracking analysis (NTA), flow cytometry and confocal microscopy. For total loading efficiency: Fluorescence spectroscopy with a plate reader. NTA analysis showed big differences in fluorescence between with CaP-NP loaded mEVs and EVmL loaded without CaP-NP. We detected much more siRNA-loaded EVmL than mEVs. The fraction of fluorescent mEVs and EVmL, presumably carrying CaP-NP showed higher particles size than average. With flow cytometry we confirmed a small mEV population with large particles, loaded with fluorescent siRNA.

We also stained CaP-NP with Calcein to circumvent low fluorescence intensity when using siRNA. Interestingly when measuring total siRNA loading in a plate reader after lysis, we detected much higher fluorescence in mEV and EVmL than expected by the small fraction of positive vesicles. Our findings show that direct measurement of siRNA loading inside single mEVs is possible but may be limited by a minimal fluorescence yield per vesicles, depending on the instrument employed. The finding that fluorescence was mainly found in large vesicles my result from the concept to encapsulate CaP-NP in mEVs or just by the minimum fluorescence intensity of a vesicle. Finally, there is a need for higher sensitivity instruments for assessing mEV loading in future research.

### 2.16 Evaluation of a Novel Magnetic Bead-Based Method for Isolation of Intact EVs or EV-RNA

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Intact extracellular vesicles (EVs) and EV-derived RNA were isolated from different sources. The EVs were then quantified and characterized by different methods and compared to alternative methods to assess usefulness in various fields and applications. EVs were isolated using a novel bead-based workflow from human peritoneal fluid (ascites) and ES-2 cell culture supernatant. Size and concentration were determined using nanoparticle tracking analysis and nano-flow cytometry, with further characterization of EV markers CD9 and CD63 via nano-flow cytometry. Additionally, protein quantification was performed using the BCA assay. EV-RNA was isolated from human plasma and characterized using qPCR and dPCR. The recovery of EVs using the new bead-based assay is comparable to isolation via TFF-SEC. RNA recovery and specificity of EV and non-EV miRNA separation are comparable with an already commercially available spin column-based method, but with a significantly shorter workflow. The integration of magnetic beads and a streamlined workflow enhances efficiency, enabling the processing of larger numbers of samples in parallel without compromising performance. This novel method also paves the way for future automation, making it an ideal choice for high-throughput applications.

### 2.17 Small RNA-sequencing (miND®) Enables Discovery of EV-specific MicroRNA Signatures to Support EV Therapeutics Development

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Extracellular vesicles (EVs) are promising drug products or drug delivery tools. Information about the biodistribution of native or engineered EVs is important to validate drug delivery and to take decisions on target organs and indications. We hypothesize that the identification of EV-specific or -enriched microRNA

(miRNA) cargo can provide valuable insights into the biodistribution and uptake of EVs in target cells. We performed a comprehensive NGS-based analysis of human Wharton's Jelly (WJ) MSC-EV RNA cargo as well of lung, liver, bone, and blood (mouse) as a panel of common target tissues (Figure 1). We utilized the miRNA Next-Generation-Sequencing Discovery Assay (miND®), which uses spike-ins to enable absolute quantitation of small RNAs including miRNAs. We went on to validate the utility of miRNA as tracers of EV biodistribution by spiking defined amounts of WJ-MSC EVs (100 to 109) into homogenized mouse tissues corresponding to 1/6 to 1/3 of total organ mass. We identified a cluster of miRNAs with significant enrichment in WJ-MSC derived EVs in comparison to the selected target tissues. We developed a miRNA RT-qPCR assay for targeted analysis of 5 WJMSC-EV-specific miRNAs, and added four control miRNA assays consisting of a liver (miR-122-5p), lung (miR-150-5p), bone (miR-140-3p), and blood-enriched (miR-451a) miRNA. Using the miRNA signature of WJ-MSCs we found that 105 WJ-MSCs are sufficient to obtain a significant increase in signals for the EV signature compared to control miRNA levels. These results demonstrate that information on EV miRNA composition may be utilized to develop biomarker signatures for tracing the distribution and accumulation of EVs in-vivo. A small RNA-sequencing workflow that enables absolute quantification of miRNAs is a prerequisite to perform such analyses.

## EV Therapeutics

### 2.18 Preclinical Studies on Safety and Biodistribution in mice and guinea pigs of Vesicle-enriched Secretome Fraction after Cochlear Implantation Trauma

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After cochlear implantation, immune response can impair hearing performance. So far, there are no approved drugs available. A novel drug candidate exerting immunomodulatory or neuroprotective effects is the vesicle-enriched secretome fraction (VSF) derived from human umbilical cord mesenchymal stromal cells. Previously, efficacy was shown in vitro in spiral ganglion neurons and in vivo by attenuation of threshold shifts and protection of hair cells after trauma. To prepare a clinical trial, safety and biodistribution of VSF in implantation trauma animal models was investigated. Safety was tested by single intracochlear application of different doses of VSF or control. Guinea pigs were applied with VSF for 4 weeks (n=18) and 6 month (n=11). Hearing was assessed in all animals. Toxicity (GLP-compliant) was investigated in mice (n=90) with implantation trauma. Biodistribution of DiD-labelled extracellular vesicles (EV) was assessed initially in guinea pigs (n=3) and extended in mice (n=25). VSF improved hearing after implantation trauma

in the guinea pig cochlear implant group when compared to the control. Six months after VSF application, ABR thresholds remained on a similar level compared to the control and no adverse effects were revealed within electrophysiological and histological investigations. Within toxicity study, general behavior and locomotor activity of animals revealed no abnormalities and no substance related clinical symptoms were observed. Regardless the species, DiD-labelled EV were traced in a time dependent manner and were detected at the applied cochleae (hair cells, supporting cells and spiral ganglion neurons) and in the kidney indicating a physiological degradation. Single application of VSF did not raise any specific safety concern in any of the described animal models. VSF is able to enter inner ear specific cells enabling a possible direct modulation of homeostasis and maintain gross structural integrity. Thus, VSF seems to be a safe new drug to be evaluated in a clinical trial.

## 2.19 Indomethacin Increased Astrocytes-derived miR-27b-3p-enriched Anti-inflammatory Exosomes and Reduced the Redox-induced NF-κB/PGE2/TNF-α Response

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Astrocytes, known for their distinctive morphological and functional characteristics, respond to various stimuli through reactive astrogliosis. This study aimed to explore the complex response of astrocytes to redox imbalance, the role of exosomes in this context, and the potential neuroprotective effects of Indomethacin. We induced redox imbalance by treating primary mouse astrocytes with tBHP, and then evaluated cellular and molecular changes using various methods, including real-time PCR, Western blotting, and immunocytochemistry. Additionally, we assessed exosome production and miRNA content during this period, both with and without Indomethacin intervention. Our study found that tBHP-induced redox imbalance led to increased PGE2 synthesis, GFAP and GAP43 expression, and the formation of fibrous astrocytes, accompanied by activation of the NF-κB pathway and an aggravated inflammatory response. Indomethacin treatment reduced fibrous astrocytes and GFAP expression, while increasing protoplasmic astrocytes and AQP-4 expression through modulation of PGE2 synthesis. Astrocyte-derived exosomes under redox imbalance promoted inflammatory signatures in normal astrocytes, but exosomes derived from Indomethacin-treated astrocytes reduced inflammation by delivering miR-27b-3p. This reduction in inflammation was confirmed as exosomes lacking miR-27b-3p did not decrease inflammatory markers. We showed that Indomethacin induced exosome miR-27b-3p content, leading to reduced inflammatory response in astrocytes. Understanding this intricate mechanism of cellular communication influenced by Indomethacin and other NSAIDs requires further investigation, particularly into their epigenetic effects and role in cellular communication via exosomes.

## 2.20 Reprogramming the tumor milieu by T cell-derived extracellular vesicle delivery of miR-155

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Immunoregulatory networks within the tumor microenvironment (TME) limit the infiltration and function of antitumor T cells, compromising the efficacy of cancer immunotherapies. Strategies to reverse this immunosuppressive environment often lack the ability to influence multiple intracellular pathways to alter cell fate and behavior1. microRNAs (miRNAs) are abundant in small extracellular vesicles (sEVs), enabling fine-tuning of regulatory components in immune system homeostasis and immune response2. sEV-mediated delivery of immunostimulatory miRNAs, such as miR-155, by adoptively transferred tumor-specific T cells might represent a safe and effective approach to reprogram the TME. We engineered pmel-1 CD8+ T cells recognizing the tumor antigen gp100 to overexpress (OE) miR-155 or a scrambled miRNA and adoptively transferred them into B16F10 melanoma-bearing C57BL/6 mice. In some experiments, we overexpressed human miR-155 in miR-155-deficient pmel-1 T cells and transferred them into miR-155-deficient mice bearing miR-155-deficient B16F10 melanoma (triple-KO), ensuring the only source of miR-155 would be the engineered one. We evaluated the transfer and uptake of the human miR-155 in sorted cell populations of the TME by qPCR. We characterized the TME using scRNA-seq (including CITE- and TCR-seq) as well as multiparametric flow cytometry. T cells overexpressing miR-155 showed better engraftment and T cell function, resulting in superior antitumor responses in the absence of exogenous cytokine support or lymphodepletion. In the triple-KO experiment, the T cell-derived human miR-155 was detected in various cell types in the TME. In C57BL/6 mice that received miR-155OE pmel-1 T cells, we observed intratumoral expansion of endogenous CD8+ effector memory T cells by scRNA-seq and flow cytometry. We demonstrate the potential of a dual hit approach of adoptive T cell therapy in controlling tumor growth through, cell-intrinsic cytotoxicity and miRNA-mediated reprogramming of the TME. sEVs contained overexpressed miR-155 and may thus contribute to local miR-155 delivery. Boosting sEV biogenesis represents a viable route for further potentiating T cell anti-tumor efficacy.

## EVs in pathology

### 2.21 Communication of Proteus mirabilis with Gut Epithelial Cells via Outer Membrane Vesicles

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There is increasing evidence that extracellular vesicles (EVs) and EV-RNA play an important role in cell-cell communication, which might be related to the progression of several diseases. The objective of this study was to analyze the communication between Proteus mirabilis (P. mirabilis) and gut epithelial cells via outer membrane vesicles (OMVs), and in particular OMV-RNAs, in the context of Parkinson's Disease (PD). Using ultracentrifugation and size-exclusion chromatography, we isolated and purified P. mirabilis OMVs. The internalization of the OMVs into Caco-2 cells has been analyzed using flow cytometry and confocal laser scanning microscopy. Using next generation sequencing, we investigate changes in the transcriptome of Caco-2 cells after incubation with OMVs or transfection with artificial liposomes carrying isolated OMV-RNA. We observed that human Caco-2 cells internalize OMVs from P. mirabilis in a time- and concentration-dependent manner without causing any cytotoxic effects. Moreover, we found that the OMVs carried mainly smallRNAs with a length under 200 nt. Analysis of Caco-2 cell transcriptome revealed minor effects of OMVs related to immune response, which were different from the effects caused by lipopolysaccharide (LPS) only. This study provides first insights into communication of P. mirabilis with human gut cells via OMVs. Our findings showed the involvement of OMVs in triggering transcriptomic changes of immune response related genes, which might be an early event in the development or progression of PD.

## 2.22 Large Extracellular Vesicles (EVs) from Phosphate-stimulated Endothelial Cells Self-Perpetuate Unfolded Protein Response Independently of Cell Survival

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Patients with chronic kidney disease (CKD) have an increased risk for hyperphosphatemia and thereby mediated cardiovascular disease (CVD). Maladaptive unfolded protein response (UPR) of the endoplasmic reticulum and endothelial dysfunction are known milestones in the pathogenesis of CVD. We hypothesize large EVs from high Pi-treated endothelial cells (Pi-EVs) can induce UPR and function as paracrine mediators of endothelial dysfunction in Pi toxicity. Endothelial cells (ECs; EA.hy926) were incubated with 0.9mM or 5mM Pi for 90min to induce vesicle formation. EVs were isolated via differential ultracentrifugation (20.000g). EVs from physiologic conditions (Ct-EVs) and EV-depleted supernatants (SUP) served as controls. ECs were incubated with EVs or SUPs for 24h. Cell survival was assessed through ATP andMTT assays. The activation of the three canonical signaling branches of UPR (protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme type 1 (IRE1α), and activating transcription factor 6 (ATF6)) were analyzed through quantitative real-time PCR. Subsequently, gene expression patterns after incubation with GSK2606414, a PERK inhibitor or vehicle were assessed. UPR markers downstream of PERK and IRE1α, but not ATF6 were elevated in ECs exposed to Pi-EVs after 24h. ATF4, an integrated stress response transcription factor, and XBP1, an IRE1α-dependent splicing product, showed a 2-fold upregulation. Furthermore, CHOP and TRIB3, both proapoptotic factors and PERK pathway effectors, were 3-fold increased. Inhibition of PERK with GSK2606414 partially inhibited the expression of CHOP and TRIB3, while ATF4 was not significantly affected. Viability was reduced after 48h. UPR is distinctly dysregulated in endothelial

cells after exposure to Pi-EVs. A specific expression pattern suggests the PERK pathway as primary but not exclusively mediating UPR branch. These findings reveal a novel aspect of Pi toxicity in endothelial cells, highlight functional implications of Pi-EVs, and provide a potential new therapeutic target for UPR-mediated CVD in hyperphosphatemic CKD.

### 2.23 The Role of Bacterial Extracellular Vesicles on Immunometabolic and Inflammatory Pathways along the Gut-Brain-Axis

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Neuroinflammation is a hallmark of neurodegenerative diseases, characterized by chronic activation of central nervous system (CNS) resident cells and the release of inflammatory mediators, thereby contributing to neuronal damage and dysfunction. Profound research on the Gut-Brain-Axis has rendered the gut microbiota as a key player in this process, prompting our group to investigate the role of bacterial extracellular vesicles (BEVs) in immunometabolic and inflammatory pathways in CNS resident cells. The isolation of extracellular vesicles (EVs) and the use of biomedical endotoxin assays, such as the TLR4 reporter assay, allow us to identify and quantify BEVs in the sera of multiple sclerosis (MS) patients, inflammatory bowel disease (IBD) patients, and healthy controls. By stimulating human induced pluripotent stem cell (iPSC)- derived brain cells with BEVs isolated from different bacterial strains, we can characterize the immunomodulatory properties of BEVs on CNS resident cell types and shed light on their role in neuroinflammation. Higher numbers of BEVs were detected in the sera of MS patients (both during relapse and remission) compared to healthy controls, indicative of increased translocation of BEVs into systemic circulation, thereby representing a possible route for BEVs to reach the CNS. As the immune sentinels of the brain, microglia moved into focus of our investigations and stimulation of human iPSC-derived microglia revealed an increase in inflammatory gene expression upon BEV challenge. Further, utilization of different bacterial strains with either present or lacking lipopolysaccharide (LPS) uncovered both LPS-dependent and -independent responses. Our findings suggest the translocation of BEVs into the CNS and their effect on CNS resident cells as a potential pathway through which the gut microbiota impacts immunological processes in the brain and might contribute to the pathogenesis of neurological disorders. Finally, understanding the underlying mechanisms of this communication system holds great potential for finding novel therapeutic avenues.

### 2.24 Bacterial Extracellular Vesicles as Mediators of Gut-Brain Communication

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In the context of inflammatory diseases such as inflammatory bowel disease (IBD) and multiple sclerosis (MS), a bidirectional link exists between an inflamed gut, altered microbiota, and neuroinflammation. This study focuses on bacterial extracellular vesicles (BEVs) released from the gut microbiota, exploring their potential role as biological shuttle system. We aim to uncover novel routes of microbiota-brain communication and examine the impact of BEVs on disease-relevant cell types in the brain. In vivo studies utilizing Rosa26tdTomato mice as a reporter model inoculated with engineered bacteria producing cre-recombinase allow us to track BEV translocation and identify target cells. Injection of germ-free mice with BEVs reveals their isolated effects, while in vitro challenges of primary murine glial cells with BEVs derived from various bacterial strains provide insights into their impact on disease-relevant brain cell types in mice. In vivo studies demonstrated that BEVs, but not whole bacteria, translocate across barriers along the gut-brain axis. Focusing on microglia as main target cell type in the brain, in vitro challenges of primary microglia with BEVs derived from various bacterial strains revealed their pro-inflammatory nature by increasing cytokine expression. This was confirmed in vivo using germ-free mice, which showed altered cluster abundances within the brain, along with elevated cytokine levels and activation markers in microglia.
These findings suggest that within an inflammatory environment, gut microbiota-derived BEVs shuttle along the gut-brain axis to enter the brain, where they interact with glial cells contributing to neuroinflammation. Concluding, this study highlights the potential of the role of BEVs in inflammatory diseases of the central nervous system, offering avenues for future research and therapeutic interventions.

## 2.25 Bacterial Extracellular Vesicles Relevance and Signaling in IBD and CRC

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Multiple sources indicated bacterial extracellular vesicles (BEVs) to play a pivotal role in mediating signals within host microbiome communication. A better understanding of cross-species interaction mechanisms is crucial for comprehending gut-related pathologies like inflammatory bowel disease (IBD) and colorectal cancer (CRC), as these conditions are associated not only with changes in gut microbiome composition but also with the aggravation of the pathology by specific bacterial strains in IBD and colibactin-expressing bacteria in CRC. BEVs were purified from pathological and probiotic bacteria, tested for quality and quantity, and selected BEVs were further characterized through proteomic analysis. Bulk RNA sequencing was conducted to identify differentially expressed genes in small intestinal and colon-derived human organoids

upon BEV treatment to elucidate vesicle signaling mechanisms. BEVs differed in proteomic characteristics, and potential proteins contributing to pathogenic effects were identified. Human intestinal organoids treated with BEVs derived from pathological bacteria exhibited stronger responses compared to BEVs of probiotic bacteria. Specifically bacterial toxins like colibactin could be transported by BEVs, as indicated from a treatment comparison with colibactin positive and -negative BEVs. Encapsulation and delivery of bacterial toxin colibactin in BEVs may have different effects due to an altered uptake and signaling mechanism of vesicles and their cargo by host cells. Results indicate BEVs as an important component of signal transmission from bacteria to host. Bacterial toxins might be delivered in BEVs to host cells. Thereby BEVs might be relevant in development or progression of diseases. The negative effects of pathological Klebsiella Pneumoniae BEVs on host cells are essential for further investigation in view of multiple observations identifying Klebsiella pneumoniae as driver of IBD, where vesicles could be an overseen link. Moreover, BEVs could be targeted for new diagnostic and therapeutic strategies of gastroenterological diseases.

### 2.26 Translation aspects of extracellular vesicles in Alzheimer's disease pathology

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Discovering new strategies that enhance natural response mechanisms in Alzheimer's disease (AD) could lead to non-invasive therapies. This study examines the role of extracellular vesicles (EVs) in this context. EVs naturally might carry protective mechanisms (such as the prion protein, PrP) that reduce amyloid toxic species (Aβ) aggregation. Here, we aim to characterize brain-derived EVs (BDEVs) from AD patients and investigate their molecular mechanisms using in vitro neuronal models. BDEVs were isolated from the frontal cortex autopsies of AD patients and age-matched non-demented individuals using a novel, non-enzymatic disaggregation method, followed by differential ultracentrifugation and iodixanol-based density gradient. Characterization involved nanoparticle tracking analysis (NTA), western blotting, and transmission electron microscopy (TEM). Human induced pluripotent stem cell (hiPSC)-derived cortical neuronal cultures were treated with patient-derived EVs and recombinant Aβ. Cell viability and activity were analyzed using LDH, immunofluorescence, and live calcium imaging. The role of PrP on EVs in modulating Aβ toxicity was evaluated by treating N2a and primary neurons with PrP-KO and PrP-Wt EVs from N2a cells together with Aβ protein. NTA and TEM analysis showed no differences in BDEVs during AD progression.

The non-enzymatic approach preserved the integrity of the EV membrane proteins. Western blotting confirmed the presence of AD hallmarks (p-Tau and Aβ) and EV markers. The iPSC-derived neuronal model showed cortical neuron fate. The preliminary results obtained treating iPSC-derived neurons with AD-EVs (±Aβ) are promising for understanding the effect of the AD-EVs in the Aβ-derived toxicity in neuronal cultures. Additionally, N2a and primary neurons treated with PrP-KO and PrP-Wt N2a-derived EVs and Aβ showed relevant results regarding PrP-EV-mediated regulation of AD toxicity. Here, we show that AD-BDEVs can describe the pathological state of the brain region as conventional histology does. On top of that, we validated the expression of other relevant proteins in the disease, such as the PrP, suggesting a relevant role of EVs in AD pathology, mediating the Aβ-derived toxicity.

## 2.27 Extracellular vesicles influence Amyloid plaque formation in Alzheimer's disease through their surface Prion protein molecules

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Ultrastructural electron microscopy of Aβ plaques from Alzheimer's disease (AD) brains reveals EVs associated with plaque core. Our data demonstrate that EVs, particularly those expressing high levels of cellular prion protein (PrPC), accelerate Aβ aggregation in vitro. However, it remains unclear whether the EV surface PrPC molecules mediate the EV-Aβ interaction in the AD brain. This study aims to elucidate AD-related changes in PrPC expression in brain-derived EVs (BDEVs), and investigate if EV association with Aβ plaques is driven by EV surface PrPC molecules. Brain-derived EVs (BDEVs) were isolated from frontal cortex autopsy material of AD and age-matched controls, as well as from amyloid pathology mouse model with wild type background: 5xFADPrnp+/+ and with PrPC knock-out background: 5xFADPrnp-/-. Proteomics composition of BDEVs and their PrPC expression was assessed. We further examined the correlation of expression levels of EV markers and PrPC in Aβ plaques from both systems. Immunohistochemistry and expansion microscopy were employed to visualize the EV markers and PrPC expression in Aβ plaques. We observed a positive correlation between BDEVs associated Aβ amounts and BDEVs-PrPC levels. Likewise, EV markers and PrPC expression were correlated in the Aβ plaques. These findings were also augmented by the Immunohistochemistry. Our data suggests a clear role of EV-expressed PrPC in the association of EVs to the amyloid plaques in AD. Taken together with the mouse model and in vitro data, we conclude that PrPC expression on EVs is crucial for their Aβ sequestration leading to plaque formation in AD.

### 2.28 MAFB Expression in LPS-Induced Exosomes: Revealing the Connection to sepsis-triggered Hepatic Injury

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Sepsis poses a significant global health threat, necessitating extensive exploration of indicators tied to its pathological mechanisms and multi-organ dysfunction. While murine studies have shed light on sepsis, the intricate cellular and molecular landscape in human sepsis remains enigmatic. Exploring the influence of activated monocyte-derived exosomes in sepsis sheds light on a promising pathway for understanding the intricate cellular and molecular mechanisms involved in this condition in humans. In sepsis, exosome-borne mRNA and miRNA orchestrate immune response gene expression in recipient cells. Yet, the specifics of exosome-mediated cell-to-cell communication, especially how mRNA cargoes modulate gene expression in recipient cells, remain poorly understood. This study focuses on the role of activated monocyte-derived exosomes in sepsis, specifically investigating how exosomal mRNA cargoes, particularly MAFB, influence gene expression in liver cells. THP-1 cells were treated with LPS to induce changes in exosomal RNA profiles. Exosomes were isolated and characterized using microscopy and mass spectrometry. RNA was extracted from exosomes and sequenced. The most abundant exosomal mRNAs were subjected to GO analysis for functional annotation analysis and KEGG database analysis to identify the involved enriched pathways. PCR (Polymerase Chain Reaction), RNA sequencing, and Western blotting were involved to analyze changes in gene expression, protein levels, and signaling pathways within the liver cells( HepG2) after exposure to exosomal MAFB. This study pinpoints exosomal MAFB as a potential key regulator linked to liver cell damage during sepsis, along with associated genes (miR155HG, H3F3A, and possibly JARD2) forming a crucial molecular pathway contributing to liver cell injury. Together, these elements indicate a vital molecular pathway that plays a significant role in the emergence of liver cell injury during sepsis. These findings suggest the importance of further research on these components for potential therapeutic interventions in managing acute liver damage in sepsis.

### 2.29 MiR-1825/TSC2/mTOR axis mediates hypoxia-induced exosome-driven angiogenesis in oral squamous cell carcinoma

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Oral squamous cell carcinoma (OSCC) is characterized by enhanced angiogenesis resulting in poor prognosis despite improvements in diagnostic/therapeutic techniques. Here, we aimed at investigating potential

roles of miR-1825 enclosed in OSCC-derived exosomes on angiogenesis under hypoxic conditions. Effects of miR-1825 mimic/inhibitor as well as hypoxia-induced tumor derived exosomes on human umbilical vein endothelial cells (HUVECs) were evaluated using cell viability, migration/invasion, tube formation, and spheroid-based 3D angiogenesis assays. Hypoxic conditions caused significant increase in miR-1825 levels in OSCC cells and hiTDEs (Hypoxia induced tumor derived exosomes). miR-1825 alone and within hiTDEs promoted endothelial cell viability, migration, invasion, and angiogenic potential, which is reversed via inhibition of miR-1825 expression. miR-1825 within hiTDEs altered the angiogenesis potential of HUVEC cells via deregulation of TSC2/mTOR axis. We showed that hypoxia led to OSCC-derived exosome mediated transfer of miR-1825 to HUVECs and enhanced angiogenesis in OSCC in vitro. Collectively, we suggest a new role for miR-1825 during cancer-induced angiogenesis and provide new insights into the angiogenic processes carried out under hypoxic conditions. Our data illustrated that exosome- shuttled miR-1825 derived from hypoxic OSCC cells clearly increased angiogenesis via targeting TSC2/mTOR axis. Therefore, we propose a new axis as a prominent target for HNSCC treatment.

## 1.19 Bacterial Derived Extracellular Vesicles Isolation and Quality Control

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Bacterial-derived Extracellular Vesicles (bEVs) localize not only in the primary site, the gut, but in multiple organs, where they have been identified as novel player in inflammation. The molecular mechanisms behind bEVs involvement in inflammatory processes are being investigated through in vivo and in vitro studies. Consequently, efficient production and isolation of bEVs from many bacterial species is increasingly important for advancing this research. In our laboratory we established, tested and improved the in vitro production and isolation of bEVs from many bacteria species including probiotics (E. coli Nissle) and gut pathobionts (Klebsiella pneumoniae (K. pneumoniae), Proteus mirabilis (P. mirabilis)) derived from isolates of patients as well as bioengineered bacterial strains. bEVs were isolated by Ultracentrifugation or dual-mode Chromatography (DMC), characterization was done through Nanoparticle Tracking Analysis and Electron Microscopy. After ultracentrifugation, nanoparticle tracking analysis detected bEVs in concentrations ranging from 1010 to 1012 particles/mL from 1 liter of culture medium harvested during the log-phase. Transmission electron microscopy (TEM) provided detailed insights into the purity and morphology of the isolated bEVs. bEVs from laboratory strains appeared clean and homogeneous. However, bEVs from wildtype strains such as Escherichia coli and gut pathobionts displayed contamination with other bacterial components, including flagella, requiring an additional purification step. To test additional methods, we performed, Dual-Mode Chromatography (DMC), to enrich EVs. Applying DMC for flagella exclusion and enhancing bEV purity has shown promising results, with improved morphological quality of the vesicles as observed through TEM analyses. In conclusion, while ultracentrifugation and DMC are effective in isolating concentrated and pure bEVs, further optimization of DMC is required to enhance its efficiency for larger sample volumes.

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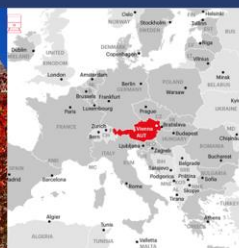

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