Harnessing extracellular vesicles for cell-based therapies

Call: Cell-based therapies and Precision Medicine

Project description

Summary

Background: The ubiquitous nature of extracellular vesicles (EVs) and their diverse biological functions have incited a growing scientific interest in basic and translational research of EV biology and therapeutics. EVs are sub-micron cellular structures with significant involvement in the development and diagnosis of diseases across multiple disciplines Circulating EVs have been detected in the extracellular space and body fluids and hailed as biomarkers for cardiovascular, urological, hepatic and hematological diseases, as well as cancer and central nervous system disorders. EVs are capable of transferring proteins, lipids and various RNAs, and therefore their functional role in regulating cellular signaling and metabolism can be harnessed and used for cell-based therapies.

EVs have the advantage over whole cell-based therapies as they are easier to characterize, possible to control, are GMP-compliant, amendable to changes of cargo and have the possibility to target specific organs or diseases. This SF board application is aimed to isolate, characterize, and develop the therapeutic use of EVs for cell-based therapies at the University Hospital of Bern. Thus, the application of EVs has a much lower hurdle for a potential development for therapies than direct cell therapy. The regulatory hurdles are much less to overcome for the concrete development of a clinical application.

Aim: The proposal aims to establish standard operating procedures for the isolation, purification, characterization, and analysis of EVs from various sources for diagnostic and therapeutic purposes. With this proposal, we plan to create an EV Core Facility at the Medical Faculty and Inselspital, Bern University Hospital. The goal is to make the medical site of the University of Bern and Inselspital a center of excellence for new cell-based therapies and diagnostics and to bring this technology into clinical application.

Project overview: Based on our expertise and the introduction of these novel methods, the following projects will be conducted to target the aims of the proposal:

Project 1: Develop the infrastructure and standard operating procedures for the isolation, purification, characterization of morphological, and EV- and donor cell-specific membrane markers, and analysis of EVs using multiple complementary techniques. The project will also address tissue and patient-related cross-validation of the similarities and differences in their composition caused by disease state, gender, and age-related differences.

Project 2: Establish pipelines for the functional characterization of the molecular composition of EVs from different tissue types and disease models using multi-omics approaches. The project will focus on identifying surface proteins for targeting strategies and developing procedures to characterize RNA, protein, and lipid profiles. The analysis will include comparisons between EVs from healthy and diseased donors, gender and age differences, and different cellular origins. The project will use Deep Learning algorithms to identify novel biomarkers for disease progression and personalized medicine strategies.

Project 3: Advance the diagnostic applications of EVs in multiple disease models by analyzing the disease state-specific characteristics of the EVs' cargo, membrane, and surface through Deep Learning and validating the diagnostic features in a more extensive set of samples. EVs are being explored as potential biomarkers due to their advantages as minimally invasive, stable, and specific diagnostic tools and their molecular composition, especially their non-coding RNA cargo, that makes them ideal candidates for precision medicine.

Project 4: Expand the therapeutic applications of natural and artificial / hybrid EVs. Stem cell-derived EVs have shown potential in improving the conditions of various diseases affecting different organs and recent developments in bioengineering have allowed the refinement of EV-based therapeutics. Hybrid vesicles created by engineering EVs have several advantages, such as biocompatibility, biodegradability, enhanced stability, targeting capabilities and the ability to cross biological barriers, making them a promising candidate for targeted drug delivery applications. We aim to develop the methodology for producing native, synthetic, and modified EVs and assess their stability, delivery, and efficacy for therapeutic applications in in vitro and animal models.

Significance: EVs are highly promising candidates for use in regenerative medicine and therapeutic interventions due to their unique and versatile properties. The therapeutic potential of EVs is significant, and they offer a wide range of potential applications in various fields of medicine. These applications include disease diagnosis, drug delivery, tissue regeneration and immune modulation. The versatility of EVs as therapeutic agents holds great promise, and further research will likely uncover even more potential applications for these remarkable particles. The current project is unique with cross-disciplinary focus that brings insights and expertise from different disciplines to establish and standardize the EVs isolation, characterization and potential use for diagnosis and therapeutic purpose.

Clinical Relevance: EVs hold great promise as diagnostics and therapeutic modalities due to their endogeneous characteristics. For diagnostics purposes, EVs offer the advantages of longer-term stability and high sensitivity and their mirroring of their parental cell in terms of composition, plus the technical capability to extract low levels of signal, makes them an intriguing proposition for use in liquid biopsies. They therefore have the potential to shift healthcare from the present reactive state to a more proactive system. Furthermore, extracellular vesicle-based therapeutics hold great clinical promise, specifically when a combination of native and engineered aspects are utilized. Native EVs hold innate therapeutic potential as they are biocompatible, stable, and due to their specific targeting, facilitate therapeutic use. Moreover, with engineered EVs allowing modified content, increased production, and targeting for improved therapeutic outcome the commercialization and clinical development of EVs can easily be achieved.

Strategy and outlook: The applicants are strategically comprised of local experts in EV biology with access to human samples for a direct translational significance. We have an interdisciplinary team consisting of researchers with clinical support from six Departments. We have already established a strong interactive network in research and teaching in stem cell and regenerative medicine network in Bern and across Switzerland. We are leveraging our collective years of pre-clinical experimental research and individual topical expertise across multiple organ systems to centralize local knowledge of EV biology to accelerate translational utility across multiple disciplines aiming for EV therapeutics.

With the kick-off funding provided by the SF Board call we intend to become a lead center of EV expertise in Switzerland and continue to generate funding through SNF collaborative project (former Sinergia) and Innosuisse. The proposed project will be the first of its kind in Switzerland to develop and combine state-of-the-art technologies for EV purification and in-depth phenotypic and functional characterization to support the translation of future EV-based diagnostics and therapies.

Background

Extracellular vesicles as mediators in cell-cell communication

Extracellular vesicles (EVs) are membrane-bound nanoparticles released from all cells that carry lipids, proteins and nucleic acids [1] and are increasingly recognized for their great importance in the development and diagnosis of a wide range of diseases.

Initially, EV secretion has been believed to serve the disposal of unnecessary proteins [1,2]. However, a quarter of a century ago, the first studies indicated that sEV have a role in intercellular communication [3,4]. This observation was confirmed by subsequent studies, which demonstrated that EVs serve as vehicles for intercellular communication by transferring proteins, lipids, DNA, mRNA, and non-coding RNA, which remained functional when interacting with recipient cells [5-8].

EVs can mediate the transfer of proteins and RNA, including regulatory non-coding RNA, between neighboring and distant cells following their uptake. The uptake can be both specific and non-specific [9,10]. Although the processes involved in the sEV uptake and cargo delivery into the cytoplasm of recipient cells are not yet fully characterized, distinct mechanisms have been described, such as phagocytosis, macropinocytosis, clathrin/caveolin-mediated endocytosis, lipid raft-mediated uptake or direct membrane fusion [3,11]. However, EVs may also remain bound to the target cell's plasma membrane without delivering their content, transmitting information through cell signaling or antigen presentation [10]. The binding of EVs to target cells or EV uptake can result in localized changes in gene expression [4-6]. Generated by nearly all cells and in all organisms, they are believed to constitute a novel, yet poorly understood means of intercellular communication, and play a significant role in a variety of physiological and pathological processes.

Extracellular vesicles in physiology and disease

The ubiquitous nature of EVs and their diverse biological functions have incited growing scientific interest in different fields of basic and biomedical research. The key functions of EVs have been described in many physiological processes, such as immunomodulation [12] and intercellular signaling in the brain [13], and pathological conditions, including tumour progression [14], metastasis [15], as well as cardiovascular, neurologic, and infectious diseases [16]. For example, circulating miRNAs have been detected in the extracellular spaces and body fluids [7] and hailed as biomarkers for cardiovascular and hematological diseases, cancer and CNS disorders [8-10]. Conversely, EV shedding and uptake are important processes during plasma membrane repair and toxin defence [11-13]. There is strong evidence that RNA cargo of EVs can reach the recipient cell and alter its gene expression [1]. This prompted research into applications of EVs for delivery of RNA, proteins and even drugs in a novel type of therapeutics. The advantages of EVs lie in low immunogenicity and toxicity [14], ability to cross the cell and tissue barriers and therefore better delivery efficiency in vivo [15].

EVs have evolved into substantially promising candidates as disease biomarkers [17], therapeutics [18], and drug delivery carriers [19].

Challenges associated with consistent and reliable EV isolation and purification

One of the major problems associated with the isolation and purification of EVs from complex matrices like body fluids is the paucity of quantitative techniques, because their size is below the reach of conventional detection methods such as flow cytometry. Electron microscopy or classical methods of analysis such as dynamic light scattering are often inapplicable to be applied to clinical samples. Several EV-related projects are currently running at the University of Bern use Nanoparticle Tracking Analysis (NTA) to characterize nanoparticles from 10 nm - 2000 nm in solution. NTA is an established method for the direct and real-time visualization and analysis of nanoparticle size distribution in liquids, based on a laser-illuminated microscopic technique [16]. NTA-based NanoSight NS300, which is currently used for EV characterization studies was purchased with an SNF R'Equip grant has supported more than ten publications in the last seven years [17-27].

Establishment of an extracellular vesicles core facility

Aim

The overarching goal of the present proposal is to make the medical site of the University of Bern and Inselspital, Bern University Hospital, **a center of excellence for new cell-based therapies and diagnostics** and to **bring this technology into clinical application**.

Relevance

Extracellular vesicles are an emerging field, and clinical trials using EVs as biomarkers or therapeutics gained strong momentum in the last few years. The clinical trials database ClinicalTrials.gov currently lists 406 studies involving extracellular vesicles (excluding suspended or withdrawn studies) [20]. To ensure the successful clinical translation of extracellular vesicle-based diagnostics and therapies, it is critical to prioritize the rigor and standardization of various aspects of the process, including isolation, purification, characterization, and engineering [8]. Standardizing these procedures can help minimize variability between different batches of vesicles, improve the reproducibility of results, and facilitate comparison between different studies. Moreover, standardization is necessary to ensure regulatory compliance and meet clinical use safety and efficacy requirements. Additionally, the development of standardized protocols will allow for comparing results obtained from different research groups and facilitate the integration of findings into a cohesive body of knowledge. Finally, rigorous characterization and engineering of extracellular vesicles can help to optimize their therapeutic potential, improve their targeting and delivery to specific cell types or tissues, and increase their stability and shelf-life, thereby improving their overall clinical utility.

General strategy

To fulfill our goals, we plan to generate a hub for EV research, diagnostics, and therapies at the Medical Faculty of the University of Bern and Inselspital, Bern University Hospital. The EV Core Facility will establish standard operating procedures (SOPs) for the isolation, purification, characterization, and analysis of EVs from various sources - such as body fluids or cell culture supernatants - for diagnostic and therapeutic purposes, taking into account organ-, disease-, age- and sex-specific differences. This will facilitate and promote the development of therapies targeting disease-relevant cell types to deliver therapeutic molecules. The expertise in extracellular vesicles in the applicants' consortium is complemented by qualified staff providing technical support for EV isolation, purification, characterization, and analysis. Existing EV-specific equipment in the applicants' research groups will be complemented by new, state-of-the-art technologies. The new facility will be open to all members of the Medical Faculty and the Inselspital and other research groups at the University of Bern.

Novelty

At the present time there are only a handful of academic or hospital extracellular vesicle core facilities in Europe (University of Helsinki, Finland [21]; Philipps Universität Marburg, Marburg, Germany [22]; Institut Curie, Paris, France [23]; Paracelsus Medizinische Universität, Salzburg, Austria [24]; University of Galway, Ireland [25]) and worldwide (La Trobe University, Melbourne, VIC, Australia [26]; School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA [27]; Rhode Island Hospital, Providence, RI, USA [28]; Children's Hospital, Los Angeles, CA, USA [29]; Vanderbilt School of Medicine, Nashville, TN, USA, [30]). The proposed project would be the **first of its kind in Switzerland** to develop and combine state-of-the-art technologies for EV purification and in-depth phenotypic and functional characterization to support the translation of future EV-based diagnostics and therapies.

Interdisciplinarity

The development of extracellular vesicle-based technologies requires a multidisciplinary approach, integrating expertise from various fields, including **biology, biochemistry, biophysics, material science, engineering, clinical medicine, and data science**. Understanding the EVs' role in cell biology is critical to developing effective diagnostic and therapeutic applications. The molecular composition of extracellular vesicles, including proteins, lipids, and nucleic acids, requires expertise in biochemistry to develop and optimize the isolation and purification methods for clinical applications. As EVs are heterogeneous in size, shape, and surface properties, knowledge of biophysics is essential for understanding the interaction of these vesicles with biological systems and the development of new analytical tools to characterize their physical properties. Expertise in material science is necessary to develop innovative, engineered, or hybrid vesicles and to exploit the full potential of nanoparticles, lipids, and biomaterials. To bring EV-based therapeutics to clinical application, developing scalable, reproducible, and cost-effective extracellular vesicle-based technologies requires the integration of various engineering disciplines, including biomanufacturing. The analysis and interpretation of large datasets generated by the characterization and functional assays of extracellular vesicles require data science and bioinformatics expertise to identify meaningful patterns and correlations. The interdisciplinarity of the platform ensures that extracellular vesicle-based technologies are developed with a comprehensive understanding of their biological, physical, chemical, and pharmacological properties and are optimized for diagnostic and therapeutic applications.

Our ultimate goal is that **EV-based technologies reach clinical applications and improve patient outcomes**. Translating this into the clinic cannot happen without the close interaction of scientists, biomanufacturing specialists, and clinicians. The applicants of the present proposal include biologists, engineers, physicians, and pharmacologists. All applicants are principal investigators of research groups included in the **Bern Stem Cell and Regenerative Medicine (SCRM) platform** and serve the SCRM as the co-leaders and steering committee members. As such, we are closely collaborating on advancing cell-based therapies at Inselspital, Bern University Hospital, and the Faculty of Medicine and the Faculty of Science, University of Bern.

Research Plan

Project 1 Develop a unified methodology for purification and characterization of EVs from different tissues and body fluids, as defined by applicants' area of expertise

We will solve one of the major disadvantages of working with EVs, which is that there is no uniformity in the field. Heterogeneity often arises from inconsistencies in purification strategies and lack of standard operating procedures (SOPs) for purification and handling [31]. In this first aim, we will build the infrastructure within the FACS core facility of the DBMR for EVs isolation, purification, labeling, morphological, and EV- and donor cell-specific membrane marker molecule characterization. We will develop pipelines and protocols for the participants of the program that will be available for all project participants, Inselspital and DBMR members, and external users.

A particular focus will be on **characterizing EVs in gender- and age-related medicine**.

We will address tissue and patient-related cross-validation of the similarities and differences in their composition, which might be caused by disease state, gender, and age-related differences. Access to multiple sources at the Inselspital, whose patients are genotypically and phenotypically profiled, provides the co-applicants with an unprecedented pool of rigorously controlled sources of primary cells and with a vast number of known reportable preanalytical parameters, both of which are prerequisites of utmost importance to achieve meaningful clinical findings in the EV field.

The International Society for Extracellular Vesicles – through several position papers and regularly updated guidelines – tirelessly calls for standardization in handling, isolation, and analysis methods in EV research. In agreement with the Minimal Information for Studies of Extracellular Vesicles (MISEV) 2018 guidelines [8], the factors that will be considered for standardization of the analyzed biosample will be donor age, biological sex, severity of the disease, diet, and pharmacological treatment. The characterization of the isolated and purified EVs will be carried out by means

of multiple complementary techniques to validate the separation protocols and exclude the absence of artifacts in identifying possible modulators of regeneration pathways. Specifically, the quantification of EVs will be manifold and based on established techniques in the Luciani group [32,33]: particle number via nanoparticle tracking analysis (ZetaView, Particle Metrix); total protein amount (micro-bicinchonic acid, BCA, assay); total lipids (UHPLC-CAD, Thermo Fisher, method developed in the Luciani group [34]); protein identification of EV markers by flow cytometry and/or NTA (Category 1a MISEV: tetraspanins); protein profiling (Proteomics, PMSCF Core facility, University of Bern); EV-RNA content (Next Generation Sequencing (NGS) Platform, Core facility University of Bern). Single vesicle analysis will be conducted via cryogenic electron microscopy (cryo-TEM, Microscopy Imaging Center UniBern).

Primary endpoint Project 1:

- Infrastructure and SOPs for the isolation, purification, and analysis of EVs derived from different tissue.

Individual contributions to Project 1:

Prof. Andreina Schoeberlein

Small EVs derived from mesenchymal stromal cells (MSC) will be prepared after the isolation of MSC from umbilical cords (hUC-MSC) sampled from donors with uncomplicated pregnancies undergoing elective cesarean sections at term, following informed written consent [35]. Supernatants for sEV isolation will be collected from MSC cultured for 36 h in serum-free medium under normoxic (ambient O_2 tension) conditions [35]. Small EV will be characterized and quantified according to Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines [8], including protein expression, electron microscopy, and nanoparticle tracking analysis, as well as additional methods defined in the SOPs established within this project. Small EV derived from human foreskin fibroblasts (HFF; ATCC) will serve as a non-stem cell control.

Prof Deborah Stroka and Prof Paola Luciani

We propose to (i) harvest EVs from primary HSCs from Non-Alcoholic Steatohepatitis (NASH) patients at different stages of their disease. Hepatic stellate cells (HSC) will be isolated from resected or explanted liver tissue obtained from the Department of Visceral Surgery and Medicine of the Inselspital, University Hospital Bern and (ii) profile them with a focus on protein and mRNA content (Project 2). The purified EVs will be incubated with primary human hepatocytes from NASH patients at different stages of their disease in order to understand the biological effect of the cross-talk driven by a purified population of the HSC secretome (Project 3). The information gained through the EV profiling will be correlated with the biological effect observed on NASH hepatocytes and will help in identifying crucial players in the reversion of NASH (final outcome of the project).

Combined anticipated outcomes and benefits of Project 1:

Project 1 will develop a unified methodology for purification and characterization of EVs from various tissues and body fluids. The establishment of an infrastructure and SOPs for the isolation, purification, and analysis of EVs will be a significant achievement that will provide a unified and novel framework for researchers at the Medical Faculty/Inselspital. This will facilitate cross-validation of similarities and differences in EV composition between tissues and patients that may be caused by disease state, gender, and age-related differences. Project 1 will provide an unprecedented pool of rigorously controlled EVs from multiple sources and lead to meaningful clinical outcomes in EVs.

Project 2 Functional characterization of EVs from different tissue types and disease models

We will combine our 'omics' expertise to characterize EVs cargo and shell proteins. We will identify surface proteins for potential targeting strategies. For characterizing RNA, protein, and lipid profiles, we will develop pipelines and make them available for the co-applicants and future investigators. We will focus on EVs characterization and cargo by addressing healthy compared to disease (malignant, inflammatory, genetic dysfunctions) material, origin of the

producing cells, nature of the disease, gender, and age differences across multiple organs of interest. By utilizing deep learning algorithms, we will analyze multi-omic data from EVs isolated from multiple organs of interest, incorporating patient data such as gender and age. Our approach has the potential to identify novel biomarkers associated with disease progression and response to treatment, ultimately leading to the development of personalized medicine strategies for improved patient outcomes.

Primary endpoints Project 2:

- Generation of a comprehensive understanding of the molecular composition and of extracellular vesicles (EVs) using multi-omics approaches and development of pipelines for characterizing RNA, protein, and lipid profiles of EVs, contributing to the standardization of EV research and a better understanding of the fundamental biology of EVs, including their biogenesis, trafficking, and uptake mechanisms.
- Development of personalized medicine strategies for improved patient outcomes, by the identification of novel biomarkers associated with disease progression and response to treatment and the identification of specific EV markers for the development of targeted therapies.

Approach Project 2.1 Characterization of the cargo of isolated extracellular vesicles (EVs) using multidimensional omics approach:

- **Proteomics:** Proteomic analysis will be used to identify the proteins present in EVs. This technique involves separating and analyzing the proteins using techniques such as gel electrophoresis, liquid chromatography, and mass spectrometry.
- **Transcriptomics:** Genomic analysis will be used to identify the RNA content of EVs, including both messenger RNA (mRNA) and non-coding RNA such as microRNA (miRNA) and long non-coding RNA (lncRNA). This technique involves isolating and sequencing the RNA using techniques such as RNA-Seq.
- **Lipidomics:** Lipidomic analysis will be used to identify the lipid content of EVs, including phospholipids, sphingolipids, and cholesterol. This technique involves separating and analyzing the lipids using techniques such as liquid chromatography and mass spectrometry.
- **Metabolomics:** Metabolomic analysis will be used to identify the small molecules present in EVs, including amino acids, sugars, and organic acids. This technique involves separating and analyzing the metabolites using techniques such as gas chromatography and mass spectrometry.
- **Glycomics:** Glycomic analysis will be used to identify the glywill content of EVs. This technique involves separating and analyzing the glywills using techniques such as liquid chromatography and mass spectrometry.

Approach Project 2.2 Characterization of membrane of extracellular vesicles (EVs) using multidimensional omics approach:

- **Proteomics:** Proteomics will be used to identify the proteins present in the EV membrane using mass spectrometry.
- **Lipidomics:** Lipidomics will be used to identify the lipid composition of the EV membrane using mass spectrometry.
- **Glycomics:** Glycomics can be used to identify the glycans present on the surface of EVs using mass spectrometry.

Approach Project 2.3 Characterization of surface charge, physical, and biochemical properties

Defining EV subpopulations based on their size, density, surface markers, lipid composition, and surface charge can provide valuable insights into their biogenesis, trafficking, and uptake mechanisms. Understanding these properties can help to tailor EV-based diagnostic and therapeutic strategies for different diseases, and to optimize their efficacy and safety profiles.

For example, identifying specific surface markers on EVs can inform the development of targeted therapies, as they can be used to selectively deliver therapeutic cargo to specific cells or tissues. Moreover, knowledge of the size and density of EV subpopulations can help to optimize their purification and isolation protocols, and to improve their yield and purity. Characterization of EV surface charge and lipid composition can also inform the development of drug delivery vehicles, as they can be tailored to enhance their stability, specificity, and uptake by target cells or tissues. Therefore we plan to perform the followings:

- **Separation of EV subpopulations based on size:** The size of EVs can play a significant role in their uptake by cells. For example, smaller EVs may have an advantage in crossing the blood-brain barrier, while larger EVs may have a higher capacity for cargo loading. Understanding the size of EVs can help researchers optimize their delivery to specific target cells and tissues. FPLC will be used to separate EVs based on their size, which will provide information about the different subpopulations of EVs present in a sample. Different subpopulations of EVs may have different functions and biogenesis pathways, and their relative abundance may change in different diseases. Therefore, identifying and characterizing different subpopulations of EVs based on size can provide valuable insights into their biological functions and clinical relevance.

- **Separation of EV subpopulations based on density:** The density of EVs can also influence their uptake by cells. For example, exosomes, which have a higher density than other EV subpopulations, may have different mechanisms of uptake by cells. By understanding the density of EVs, researchers can design strategies to enhance their uptake by target cells, potentially improving their efficacy as drug delivery vehicles. FPLC will be used to separate EVs based on their density, which can separate different subpopulations of EVs. For example, exosomes have a higher density than larger microvesicles and apoptotic bodies. Separation of EVs based on density can help to isolate and identify specific subpopulations of EVs, which can provide insights into their biological functions and potential clinical applications.

- **Characterization of EV surface markers:** FPLC can fractionate EVs based on their surface markers by utilizing columns with immobilized ligands that have an affinity for specific surface markers. For example, a column with immobilized antibodies that recognize a specific surface marker can be used to capture and separate EVs that express that marker. The EVs can then be eluted from the column using different buffers or changing the conditions to release the bound EVs. The fractions collected will then be analyzed for their biological and functional properties.

- **Characterization of EV lipid composition**: FPLC will be coupled with other analytical techniques to identify and quantify the lipids present in EVs. This can provide valuable information about the lipid composition of different EV subpopulations and their potential biological functions. For example, identifying specific lipids that are enriched in EV subpopulations derived from diseased tissues or cells can provide insights into the underlying pathophysiology of the disease and potential therapeutic targets.

- **Characterization of EV surface charge:** FPLC can also separate EVs based on their surface charge, which is determined by the presence of specific surface markers. The surface charge of EVs can influence their interactions with other cells and tissues, and can be used to isolate and identify specific subpopulations of EVs. For example, cationic EVs may have different biological functions than anionic EVs, and their relative abundance may change in different diseases. Therefore, separating EVs based on their surface charge can provide valuable insights into their biological functions and potential clinical applications. Additionally, identifying specific surface markers on EVs can inform the development of diagnostic and therapeutic strategies, as mentioned in the previous explanation.

FPLC has several advantages over FACS sorting for EV characterization. Firstly, FPLC can process larger volumes of samples, allowing for higher yields of EVs. In contrast, FACS sorting can only process smaller volumes of samples,

limiting the amount of EVs that can be obtained.Secondly, FPLC is gentler on EVs than FACS sorting, which can subject EVs to potentially damaging forces such as high-pressure flow and shearing. This can result in altered or damaged EVs, which may affect downstream analyses.Thirdly, FPLC allows for the collection of fractions containing different subpopulations of EVs, which can be further analyzed and characterized. In contrast, FACS sorting typically only allows for the collection of a single population of EVs based on surface markers, limiting the ability to study the heterogeneity of EV populations.

Approach Project 2.4 Deep Learning for Personalized Medicine: Identifying Disease Biomarkers through Integration of Patient and EV Omic Data

By combining patient data, such as medical history and symptoms, with omic data from the isolated EVs of those patients, machine learning algorithms will be used to identify specific biomarkers associated with disease progression or treatment response. This can lead to the development of personalized medicine that is tailored to the individual patient's needs. For example, using deep learning to analyze EVs isolated from cancer patients, we can identify specific mutations or protein expressions that are driving cancer and develop drugs that specifically target those mutations or proteins, leading to improved treatment outcomes.

- **Prediction of EV cargo based on RNA-seq data:** We will develop a deep learning algorithm to predict the cargo carried by EVs based on RNA sequencing data from the parent cells. This could involve training a model to predict which genes are most likely to be packaged into EVs based on their expression levels in the parent cells. Possible deep learning algorithms that could be used for this project include convolutional neural networks (CNNs) or recurrent neural networks (RNNs).
- **Classification of EVs based on their biophysical properties:** We will develop a deep learning algorithm to classify EVs based on their biophysical properties, such as size, shape, and surface markers. This could involve training a model to recognize patterns in imaging or flow cytometry data that indicate the presence of specific EV subtypes. Possible deep learning algorithms that could be used for this project include convolutional neural networks (CNNs), recurrent neural networks (RNNs), or deep belief networks (DBNs).
- **Prediction of EV function based on proteomics data:** We plan to develop a deep learning algorithm to predict the function of EVs based on proteomics data. This could involve training a model to recognize patterns in protein expression data that are associated with specific EV functions, such as immune modulation or intercellular communication. Possible deep learning algorithms that could be used for this project include feedforward neural networks (FNNs), deep belief networks (DBNs), or long short-term memory networks (LSTMs).

Approach Project 2.5 Development and Validation of In-Vitro Functional Assays for EV Uptake and Activity in Alignment with Applicant's Research Focus

Extracellular vesicles (EVs) play a crucial role in intercellular communication and are emerging as potential therapeutic agents for various diseases. However, understanding the mechanisms of EV uptake and activity is still a major challenge. In this proposal, we aim to develop and validate two novel in-vitro assays for evaluating EV uptake and activity that align with our research focus.

Investigating EV Uptake and Activity through Reporter Gene Assays : Reporter gene assays are a promising tool for understanding the uptake and activity of EVs. By genetically modifying recipient cells to express a reporter gene in response to EV uptake or activity, we can measure the amount of EVs taken up by cells and their activity in a high-throughput manner. This method will provide quantitative data that can be used to investigate the mechanisms of EV uptake and activity, as well as identify new biomarkers associated with disease progression or treatment response.

Developing a Microfabricated Device for High-Throughput Evaluation of EV Uptake and Activity: We propose to develop a novel high-throughput in-vitro assay for evaluating EV uptake and activity. Our innovative approach will be based on the use of microfabricated devices that mimic the in-vivo environment and provide a platform for studying EV uptake and activity at a single-cell level. The device will be designed to facilitate the formation of stable cell-EV

interactions, and will allow for real-time monitoring of EV uptake and activity. The proposed method will be validated using EVs derived from different cell types and in various disease models, including cancer and inflammation.

Contributions to Project 2:

Project 2 will be a joint effort of all applicants, together with the existing core facilities, including the Flow Cytometry and Cell Sorting Facility (FCCS), Core Facility Proteomics & Mass Spectrometry, Microscopy Imaging Center, Live Cell Imaging (LCI), Interfaculty Bioinformatics Unit, or the Next Generation Sequencing (NGS) Platform.

Combined anticipated outcomes and benefits of Project 2

The proposed project has the potential to generate several outcomes and benefits. By characterizing the cargo and membrane of extracellular vesicles (EVs) using multi-omics approaches, the project will provide a comprehensive understanding of the molecular composition of EVs, which can help to identify novel biomarkers associated with disease progression and response to treatment. The use of deep learning algorithms to analyze the multi-omic data from EVs isolated from multiple organs of interest incorporating patient data such as gender and age can lead to the development of personalized medicine strategies for improved patient outcomes. The identification of specific surface markers on EVs can inform the development of targeted therapies, as they can be used to selectively deliver therapeutic cargo to specific cells or tissues. Furthermore, knowledge of the size, density, surface markers, lipid composition, and surface charge of EV subpopulations can help to tailor EV-based diagnostic and therapeutic strategies for different diseases, optimizing their efficacy and safety profiles.

The development of pipelines for characterizing RNA, protein, and lipid profiles of EVs will be made available to co-applicants and future investigators, contributing to the standardization of EV research. Additionally, the project's results will have implications beyond the scope of the research, contributing to a better understanding of the fundamental biology of EVs, including their biogenesis, trafficking, and uptake mechanisms. The multidisciplinary nature of the project, which involves collaboration between experts in different fields, will also facilitate knowledge transfer and scientific exchange. Finally, the project has the potential to pave the way for the development of new diagnostic and therapeutic approaches for various diseases, contributing to advancements in healthcare.

Project 3 Advance the diagnostic applications of EVs in disease

Extracellular vesicles have several advantages as early diagnostic biomarkers for human disease: (i) EVs can be isolated from various biofluids such as blood, urine, saliva, and cerebrospinal fluid, making their collection minimally invasive. (ii) EVs are stable in biofluids, even under harsh conditions, and can be stored for extended periods without degradation. (iii) EVs can be isolated from specific cell types and disease states, making them potential biomarkers for diseases originating from those cell types. (iv) EVs can contain a wide range of biomolecules, including proteins, lipids, and nucleic acids, providing a comprehensive picture of disease states [17].

EVs and their respective cargo molecules have already been explored as potential biomarkers in a wide range of medical fields. The specificity of the EVs' molecular composition, especially their non-coding RNA cargo, makes them ideal candidates for their use in precision medicine [36]. In oncology, EVs have shown promise as biomarkers for early detection and diagnosis of various cancers and for monitoring treatment response and disease progression [37]. Specifically, in cancers or other diseases of the urinary tract [38,39] or the prostate [40], EVs collected from the urine or seminal fluid bear a great potential as part of a liquid biopsy [41-44]. EV-based biomarkers have been studied in neurodegenerative diseases [45-47], such as Alzheimer's [48] and Parkinson's disease [49], as well as for traumatic brain injury [50], stroke [51], and perinatal brain injury [52]. Biomarkers for cardiovascular diseases, such as myocardial infarction [53,54] and heart failure [55], have also been investigated based on EVs. Autoimmune diseases, such as lupus [56] and multiple sclerosis [57-58], as well as infectious diseases, such as HIV-related neuroinflmmation [59,60] and hepatitis [61][62], might also profit from EV biomarkers in the future. Liver diseases of different etiologies were also investigated for the suitability of EVs as biomarkers [62]. Another exciting field with unmet needs for biomarkers is the various reproductive health conditions, such as preeclampsia, fetal growth restriction, and preterm

birth [63]. Fetal EVs can be detected in the mother's peripheral blood and can provide conclusions about the health of the fetus [64].

Primary endpoints Project 3:

- The information collected in Project 2, namely the disease state-specific characteristics of the EVs' cargo (Project 2.1), membrane (Project 2.2), and surface (Project 2.3), are then analyzed through Deep Learning (Project 2.4) and used for the diagnostics of diseases in the applicants' fields of medical expertise.
- The diagnostic features of EVs are validated in a more extensive set of samples, taking differences in the patients' ages and genders into account.
- The technology will be made available to researchers and clinicians in various disciplines to develop EV-based diagnostic tools for diseases of interest to them.

Individual contributions to Project 3:

Prof. Andreina Schoeberlein and Dr. Amanda Brosius Lutz, MD, PhD: Detection of reactive astrocyte-derived EVs as biomarkers for fetal and neonatal brain injury

This part of the project was conceived by and will be planned and carried out under the guidance of Dr. Amanda Brosius Lutz, MD, PhD, in the Laboratory for Prenatal Medicine, Department for Feto-maternal Medicine.

Our research is based on the hypothesis that reactive astrocyte polarization in the fetal and neonatal brain can be detected remotely by analyzing astrocyte-derived EVs in maternal and neonatal circulation. Inadequate detection of perinatal brain injury in utero and during the neonatal period remains an obstacle to making best-informed delivery and treatment decisions. Therefore, our aim is to test the diagnostic potential of peripherally detected fetal/perinatal astrocyte-derived EVs as a biomarker of astrocyte reactivity. We will use an astrocyte-specific EV transgenic floxed CD63-GFP reporter mouse (strain #:036865, The Jackson Laboratory, Bar Harbor, ME, USA), which allows the restriction of GFP-labeled EV production to a cell type of interest when crossed to the appropriate cell type-specific Cre Recombinase expressing mouse. Cre recombinase activity will be induced, and mouse astrocytes purified from CD63-GFP; Aldh1L1-CreERT2 mice. This astrocyte reporter line has been shown to be highly specific for astrocytes and to label a very high percentage of astrocytes in the brain, including perinatally [65].

Reactive, C3+ astrocytes (C3rAs) will be generated in vitro by the purification of resting astrocytes from neonatal rat brains through immunopanning, followed by the stimulation by C1q, TNFα, and Il-1 [66]. The enrichment of astrocyte-derived exosomes will be accomplished using antibodies to astrocyte marker Glutamate Aspartate Transporter (GLAST) as previously reported [67]. The purity and quality of EVs will be assessed through the methods established in Project 2. Purity and quality of exosome fractions will be assessed through quantification of expression of small The EV protein content will be compared between resting and reactive astrocyte states in order to identify candidate markers significantly up- or downregulated in the C3rA state. Permission to conduct the animal studies required for this aim has been obtained.

We hypothesize formation of reactive astrocytes in utero in pregnancies affected by intrauterine growth retardation (IUGR) leads to changes in fetal astrocyte-derived EVs' protein cargo or surface markers. We will analyze 1-2ml neonatal blood collected from the umbilical arteries at the time of delivery from neonates [68] affected by severe IUGR (birthweight < 3rd percentile) with subsequently confirmed WMI (MRI-based diagnosis) and healthy gestational age-matched controls with normal birthweights 34-40 weeks

gestation. Pregnancies affected by IUGR due to etiologies other than placental insufficiency will be excluded. EVs will be enriched and analyzed as proposed in Projects 1 and 2. We will test whether changes in the protein content of astrocyte-derived EVs can be detected in IUGR-affected infants with WMI compared to healthy matched control infants. We anticipate the identification of one or more proteins that is significantly more highly expressed in astrocyte-derived EVs from neonates affected by placenta insufficiency in comparison with astrocyte-derived EVs from healthy control neonates. After validation using ELISA or other suitable methods, this protein would constitute a candidate peripheral biomarker for astrocyte reactivity.

Using matched samples of maternal peripheral blood collected just prior to delivery, umbilical cord blood collected from the umbilical artery at the time of delivery, and maternal blood collected 6 weeks postpartum, we will test whether fetal astrocyte-derived EVs can be detected and differentiated from maternal astrocyte-derived EVs in maternal peripheral blood. Astrocyte-derived EVs will be purified and then enriched using anti-GLAST antibodies, and analyzed for their protein content (see Project 2). Pregnancies complicated by major maternal co-morbidities and fetal birth defects will be excluded. The aim of these experiments is to identify a marker of fetal astrocyte-derived EVs that can be used to unequivocally distinguish maternal and fetal astrocyte-derived exosomes from one another so that (in combination with an astrocyte marker such as GLAST) fetal astrocyte-derived exosomes can be sorted away from maternal astrocyte-derived exosomes in maternal peripheral blood samples and then carried forward to further analysis of reactivity markers. An ideal marker would not be expressed at all in maternal astrocyte-derived exosomes and highly expressed in fetal astrocyte-derived EVs, or the opposite. We would expect the detection of this marker in neonatal blood and the blood of pregnant women prior to delivery, but not in the blood of postpartum women 6 weeks after giving birth. The next step would be to use validated markers from this aim and the signature reactivity protein(s) identified in to test whether changes in astrocyte reactivity can be detected in fetal astrocyte-derived exosomes isolated from maternal serum in pregnancies affected by IUGR. If successful, this experiment would support the feasibility of using fetal astrocyte-derived exosomes as biomarkers of fetal brain health during pregnancy.

Prof. Katia Monastyrskaya: Investigating the molecular mechanisms of lower urinary tract diseases (LUTDs)

While investigating the molecular mechanisms of lower urinary tract diseases (LUTDs), particularly focus on the role of miRNAs in the bladder organ remodeling during obstructive and neurogenic bladder dysfunction [38,69,70]. The group studies the switch from bladder hypertrophy to fibrosis and acontractility. We hypothesized that regulatory miRNA molecules may serve as reliable biomarkers of the progression of bladder fibrosis. The EV analysis is a valuable tool to discover urinary biomarkers, and can be applied to LUTDs. We are currently creating a database of EVs, originating from different organs and tissues, using single cell RNA sequencing data and state-of–the art bioinformatic approaches. Experimental validation of our molecular classifiers will involve isolation of EVs from selected cells and tissue types and specific immunolabelling with antibodies against marker membrane and intracellular proteins.

Own work on EVs as biomarkers for lower urinary tract diseases (LUTDs)

The Functional Urology Group within the Urology Research Lab at DBMR, University of Bern, has a long-standing interest in the pathophysiological processes of bladder dysfunction. We investigate the regulatory mechanisms underlying the gene expression changes observed in the bladder diseases, and have pioneered microRNA (miRNA) research in bladder pain syndrome [38]. In a follow-up study of the role of miRNAs in urothelial permeability [70], we identified miR-199a-5p as an important regulator of intercellular junctions. Interestingly, the function of miR-199a-5p in the bladder is not limited to the urothelium. We showed that miR-199a-5p was highly expressed in bladder smooth muscle [70], where it plays a critical role in the WNT2-mediated regulation of proliferative and differentiation processes [69]. Recently, we completed quantitative transcriptome and miRNA profiling of different urodynamically-defined phenotypes of BPO in humans using comprehensive Next-generation sequencing (NGS)-derived gene expression data [43]. We identified 3 mRNAs (NRXN3, BMP7, and UPK1A) and 3 miRNAs (miR-103a-3p, miR-10a-5p, and miR-199a-3p) characteristic of each urodynamic phenotype. We showed that TNF-alpha was the top upstream regulator of signaling, potentially contributing to organ remodeling [43]. Our follow-up study addressed the role of TNF-alpha in the disease [71], implicating its role in the loss of contractility in patients with advanced

obstruction-induced LUTD. Using comprehensive NGS technology and pathway analysis, we performed paired miRNA and mRNA expression profiling in bladder biopsies from BPS patients with non-Hunner BPS phenotype, and showed gene expression changes consistent with cell cycle regulation, chemotaxis of immune cells, muscle development, muscle contraction, remodeling of extracellular matrix and peripheral nervous system organization and development [42].

Circulating miRNAs have been proposed as biomarkers [36,72,73]. Urine is a useful source of circulating RNAs as it is easy to collect non-invasively in large amounts. Circulating RNAs can be packaged in secreted urinary extracellular vesicles (uEVs) and thus protected from degradation. Various stimuli can induce shedding of microvesicles, including mechanical stress in fibroblasts [74], PKC activation [75], and the increase of intracellular Ca^{2+}. To this end, we characterized the mechanisms of microvesicle shedding following exposure of cells to the bacterial toxin Streptolysin O (SLO) and showed that Ca^{2+} and phospholipid binding proteins annexins were involved in the processes of membrane repair [76,77]. Recurrent urinary tract infections are caused by uropathogenic *Escherichia coli* (UPEC) and may be relevant in chronic bladder dysfunction [39]. UPEC produce a bacterial pore-forming toxin alpha-hemolysin [78], and our study in the urothelium established the role of microvesicle shedding in the urothelial cell survival during a toxin attack [79].

Major difficulties in application of uEVs for a clinical application are the high variability and low reproducibility of uEV isolation methods. In order to address the question of urinary biomarkers for BPO, we first established protocols for miRNA isolation from urine. In our recently published study [44], we used five different methods to isolate uEVs and compared the size distribution, morphology, yield, presence of exosomal protein markers and RNA content of uEVs. We developed an optimized ultracentrifugation and size exclusion chromatography approach for highly reproducible isolation for 50-150 nm uEVs, corresponding to the exosomes, from 50 ml of urine (Fig. 1). UC-SEC purified uEVs are free from contaminating soluble urinary proteins and DTT, allowing the downstream analysis of their cargo as well as functional assays. We profiled the miRNA content of uEVs and total urine from the same samples with the NanoString platform and validated the data using qPCR [44].

Urine is a good source of circulating miRNAs, but sex- and age-matched controls are important for urinary metabolite comparison. Recently using urinary exosome profiling, we investigated the number and composition of urinary exosomes in different age groups [41]. In two groups of healthy subjects (average age 32 and 57 years old, respectively) the total protein and RNA content was very similar between age groups, but the number of secreted uEVs and expression of several miRNAs were higher in the young healthy male volunteers. Timing of urine collection was not important for these parameters. We also evaluated the suitability of urinary miRNAs for non-invasive diagnosis of BPO. A three urinary miRNA signature (miR-10a-5p, miR-301b-3p and miR-363-3p) could discriminate between controls and patients with LUTD (BPO and neurogenic origin). This panel of representative miRNAs can be further explored to develop a non-invasive diagnostic test for BOO. The age-related discrepancy in the urinary miRNA content observed in this study points to the importance of selecting appropriate, age-matched controls [41].

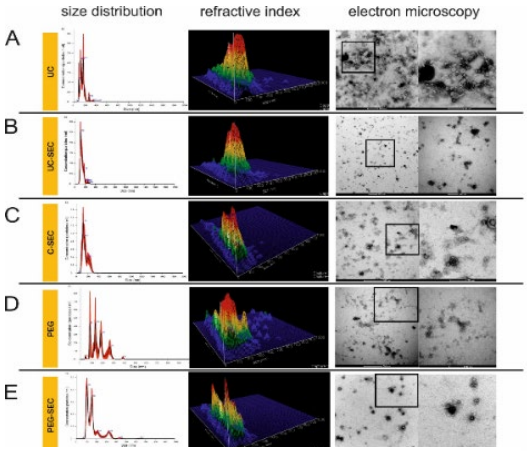

Figure 1 NTA and EM analysis of uEVs. UC = ultracentrifugation, UC SEC = ultracentrifugation followed by size exclusion chromatography, C SEC = concentration followed by size exclusion chromatography, PEG = polyethylene glycol precipitation, PEG-SEC = polyethylene glycol followed by size exclusion chromatography.

Specific aims

Fibrosis is a common end-result of pressure overload-induced bladder hypertrophy during BPO and leads to bladder decompensation and failure. We showed that miRNAs mediate molecular changes in the bladder wall and urinary miRNAs profile is influenced by LUTD. We hypothesise that the composition of uEVs and their cargo reflects the biological processes affecting the bladder and leading to its fibrotic remodelling during BPO.

We aim to answer the following questions:

1) Does cellular stress during LUTD (functional obstruction or BPO) affect the release and contents (protein, RNA) of urinary EVs?
2) Can we delineate the cellular origin of the EVs detected in urine?
3) Which factors affect the loading of miRNAs into EVs?

By altering the expression levels of miRNAs, affecting the key signalling pathways activated in fibrosis, it would be possible to prevent bladder acontractility, and assist full restoration of the bladder function following the relief of obstruction.

Methods

We plan to quantify and characterize urine-derived exosomes, isolated methods established in Project 1, and correlate the amounts of cargo RNA to the particle number to optimize the purification procedures.

To answer the first question about the impact of stress on EV composition and numbers, we will culture primary human bladder smooth muscle (SMC) and urothelial (UE) cells and subject them to a variety of stressors, corresponding to those occurring during BPO (hypoxia, oxidative stress, exposure to TNF-alpha and other cytokines, exposure to LPS and HMGB1). Our preliminary data show that hypoxia conditioning for 48 hrs significantly increased the number of EVs released from the SMC. Now we will perform proteomics on the EVs collected under different conditions and assess the changes in the protein composition of the vesicles. Based on the approaches established previously, we will select the strongest candidates as classifiers for each condition and cell type, and find appropriate specific antibodies, labeling these proteins. We will label the EVs from each batch with a combination of exosome-specific (anti-CD63, -CD9 or CD81) and classifier protein antibodies to confirm their expression on the EVs released by the stressed bladder cells in vitro. We will then apply the same combinations of antibodies to the purified urinary EVs from the urine of healthy controls and patients with BPO to confirm the presence of proteins, indicative of the type of cellular stress encountered during obstruction.

To answer the second question about the cellular origin of urinary EVs, we will collect urine samples directly from the kidney of the catheterized urolithiasis patients, and compare them to the urinary samples collected from the bladder of the same person. Labeling the uEVs with bladder-specific vs. kidney-specific marker antibodies and counting the relative amounts of EVs of each type in different samples will help elucidate the contribution of EVs originating from the bladder in the total urinary EVs. This will be followed by capturing this population and analyzing its intravesical cargo (other proteins, RNAs, etc.) to delineate the impact of the bladder to the urinary miRNAs and better understand the relevance of the changes in the urinary miRNA profiles observed in patients with LUTD. Additionally, by applying the computational tools established by us (see Project 2.4) to the single-cell RNA sequencing datasets of the normal and diseased bladder, we will define the classifiers of different cell types, potentially contributing to the bladder exosomes. We will label the EVs, isolated from urine, and from the bladder cell suspension and count the number of double-labeled vesicles per population the technology established in this project.

To study the loading of miRNAs into the urinary EVs, we will first label them with fluorescent RNA-specific dyes (RNA-SYTO and RiboGreen) in combination with antibody labeling (anti-CD9 and anti-CD81, as these are most prominently expressed in uEVs based on our previous results [41]). We will assess the percentage of EVs carrying RNA. Following uEVs isolation, we will compare the amount of EV-packaged RNA to the total urinary RNA and non-packaged RNA in the same sample and assess whether LUTD influences this ratio by increasing the RNA packaging into the EVs. In a series of in-vitro experiments using immortalized UE cell lines and primary SMCs transfected with fluorescently-labeled pre-miRNA miR-199a-5p, we will study the uptake and release of the miRNAs in the EVs. Using computational tools, we will screen the available databases of urinary RNA for the specific sequences, coding for cellular release and retention (EXOmotifs, CGGGAG) [80]. We will modify the relevant sequences of miR-199a-5p and study its packaging and release.

Combined anticipated outcomes and benefits of Project 3:

EVs can contain a wide range of biomolecules, including proteins, nucleic acids, and lipids, providing a comprehensive picture of disease states. One of the expected benefits of using EVs as biomarkers is their non-invasive collection and stable nature, facilitating their storage. Project 3 will develop EV-based diagnostic tools for diseases by analyzing the cell type and disease state-specific characteristics of the EVs' cargo, membrane, and surface using Deep Learning, validating the diagnostic features of EVs in a more extensive set of samples, and making the technology available to researchers and clinicians in various disciplines.. This will allow for the detection of biomarkers for diseases of interest, making early diagnosis and informed treatment decisions possible.

Project 4 Therapeutic applications of natural and artificial / hybrid EVs

Project 4.1 Native EVs for therapeutic applications

EVs, or extracellular vesicles, have gained recognition as a promising option for therapeutic purposes due to their small size on a nanoscale level, stability, ability to interact with cells, and compatibility with living organisms. Research has demonstrated the therapeutic potential of EVs, with clinical trials in phase I/II testing their ability to regenerate tissue, function as vaccines, provide immunotherapy, and act as delivery agents. On-going preclinical and therapeutic studies have shown that EVs hold great promise for repairing and regenerating tissues to treat a variety of illnesses [81].

Extracellular vesicles (EVs) derived from stem cells have shown potential in improving the conditions of various diseases affecting different organs, including the liver (fibrosis, hepatitis, inflammation), brain (stroke), heart (myocardial infarction, contractility), kidney (renal ischemia, stenosis), and the immune system [18,82]. These EVs have the ability to regulate the immune system by either enhancing or inhibiting the immune response, depending on the immune context and the source of the initial cells. This suggests that EVs have the potential to be used in immunotherapy [14]. Their therapeutic effects are due to their complex bioactive properties, enabling efficient cell communication and overcoming biological barriers.

The benefits of EVs stem from their cargo, which includes proteins, nucleic acids, and lipids, that work synergistically to provide their multiple therapeutic effects. These molecular components influence their clinical potential by regulating protein expression and surface receptor-mediated interactions with recipient cells, including fusion and uptake. Understanding EVs' biochemical and biophysical heterogeneity, variable composition, pharmacokinetic behavior, and functional diversity is necessary to develop them as effective next-generation therapeutics [81,82].

To achieve this goal, technological advances are required to monitor EVs’ molecular and structural levels reproducibly. This includes hybrid or engineered EVs or nanovesicles [84], which are an alternative to native EVs, to address issues such as production, scalability, purity, and cost-effectiveness. Recent developments in bioengineering have allowed the refinement of EV-based therapeutics, both native and mimetic, by modifying their cargo, target capacity, and pharmacokinetic properties. Therefore, EV-based therapies show great potential for the development of effective treatments for a wide range of pathologies.

Project 4.2 Hybrid vesicles for cell specific targeting

Drug delivery systems play a crucial role in modern medicine by enhancing the efficacy and safety of therapeutic agents. One approach to achieving targeted drug delivery is through the use of vesicular carriers, such as liposomes and polymeric nanoparticles [85]. Liposomes are spherical lipid-based vesicles that can encapsulate hydrophilic and hydrophobic drugs, providing protection against degradation and rapid clearance by the immune system. Polymeric nanoparticles, on the other hand, are typically made from biodegradable and biocompatible polymers, which can be functionalized to target specific cell types through ligand-receptor interactions [86]. Despite their promising potential, both liposomes and polymeric nanoparticles have limitations, such as poor stability, limited drug loading capacity, and insufficient targeting capabilities. Extracellular vesicles (EVs), naturally occurring membrane-bound vesicles that are released by cells into the extracellular space, have emerged as promising carriers for targeted drug delivery due to their natural ability to transport bioactive molecules between cells [19]. EVs have been shown to play important roles

in cell-to-cell communication, delivering bioactive molecules such as proteins, nucleic acids, and lipids to recipient cells. EVs are highly versatile and can be isolated from a wide range of cell types, making them attractive candidates for drug delivery applications [87].

By engineering hybrid vesicles using EVs as a starting material, it is possible to create a platform for cell-specific drug delivery with enhanced stability and targeting capabilities [88,89]. By functionalizing the surface of EV-derived hybrid vesicles with specific ligands that will recognize receptors on the surface of target cells, thus drug delivery can be localized to the site of action, minimizing off-target effects and reducing systemic toxicity. Furthermore, EV-derived hybrid vesicles offer several advantages over other drug delivery systems. EVs are biocompatible and biodegradable, reducing the risk of toxicity and immune reactions. They also can cross biological barriers, such as the blood-brain barrier, enabling drug delivery to previously inaccessible sites [89].

Primary endpoints Project 4:

- Development of the methodology for producing several types of native, synthetic and modified EVs, including the addition of specific proteins on the surface to facilitate targeting to the defined cell types or loading the EVs with the cargo of choice (siRNA, miRNA, specific soluble proteins, drugs, etc.).
- Methods to assess the EVs' stability, delivery, and efficacy.
- Therapeutic applications in animal models to study tissue biodistribution of the targeted EVs and their cargo.

Individual contributions to Project 4:

Project 4.1 Native EVs for therapeutic applications

Prof. Andreina Schoeberlein: Therapy of perinatal brain injury with umbilical cord tissue-derived EVs

Mesenchymal stromal cells (MSC) derived from perinatal tissues are recognized as promising tools in regenerative medicine through their immunomodulatory, anti-microbial and anti-inflammatory capacities. Perinatal tissues have gained increasing interest as sources of stem cells, due to their availability, minimally invasive collection, and low immunogenicity. The administration of MSC-EVs was shown to promote neuroregeneration in a variety of disease models. Their cargo, specifically micro RNAs (miRNAs), have gained increasing interest for their regulatory functions in brain development and neurological disorders.

White matter disease leads to severe neonatal morbidity and mortality and to long-term neurological deficits. Etiologies mainly include hypoxia-ischemia due to reduced cerebral blood flow and maternal/fetal infection and inflammation. In animal models of neurodegenerative disorders, the administrations of MSC-EVs contributed to neural repair and functional recovery.

The therapeutic potential of sEV derived from MSC is widely recognized [90]. Amongst others, umbilical cord-derived MSC sEV ameliorated oxidative stress and apoptosis, attenuated inflammatory immune reactions and promoted cell proliferation and angiogenesis. Besides our own publications [35,91,92], there are only few reports of the application of MSC-derived sEV in models of perinatal brain injury [93-97], although sEV have been proposed as a promising neuroregenerative therapy [7]. We have successfully administered the EVs intranasally in rodent models of perterm birth-related white matter injury, where we showed that they attenuated neuroinflammation and promoted myelination, leading also to improved functional outcomes. More recently, we have been investigating the role of the EVs' miRNA cargo and assessed their functionality using luciferase assays to prove that the EVs' miRNAs specifically downregulated the expression of apoptosis genes TP53 and TAOK1. In addition, we knocked-down DROSHA in the cells secreting the EVs. DROSHA is a key protein of the canonical pathway of miRNA processing, and its knock-down results in a sharp drop in mature miRNAs. Indeed, the EVs produced by DROSHA knock-down cells were not effective in down-regulating genes previously identified by us to be involved in the EVs' anti-inflammatory and pro-myelinating effects [35], in contrast to EVs produced by native cells (Tscherrig V et al. submitted to Stem Cell Rev

Rep, 28.04.2023). As a next step, DROSHA knock-down EVs will be intranasally administered to neonatal rat pups. Our hypothesis is that these EVs are less effective in attenuating neuoinflammation and in promoting myelination, in contrast to native EVs (Figure 2).

In Project 4.1, we plan to assess the biodistribition and kinetics of the EVs administered intranasally in our rat animal model of preterm birth-related WMI using IVIS® Spectrum CT. To achieve this goal, we will label the EVs with dyes (DiR, TurboFP635) to allow for their detection in the computer tomography. Alternatively, we will transfect the umbilical cord mesenchymal stromal cells producing the EVs with a CD63-eGFP vector, resulting in the insertion of a green fluorescent protein tag in CD63, one of the tetraspanins present in EV membranes (Figure 2).

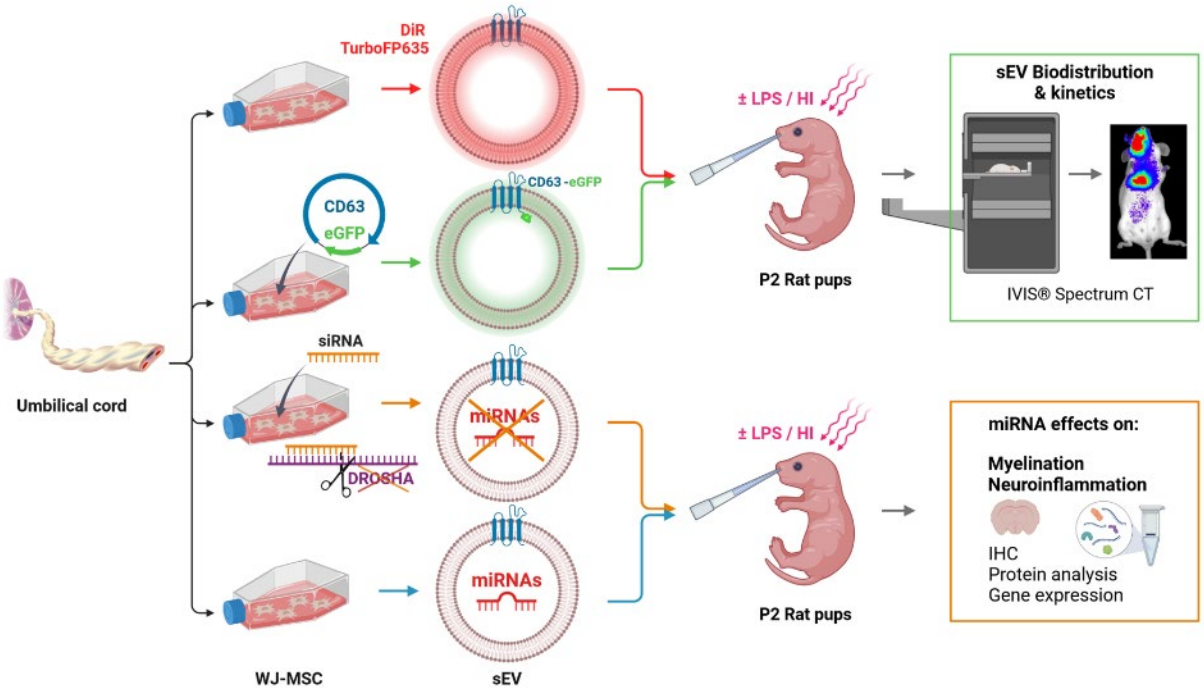

Figure 2 Experimental layout to assess the biodistribition and kinetics of EVs administered intranasally in our postnatal day 2 (P2) rat model of preterm birth-related WMI using IVIS® Spectrum CT (upper half of the panel). EVs secreted from DROSHA knock-down umbilical cord MSC, containing only minimal amounts of miRNA, are expected to be less effective in attenuating WMI after intranasal administration to P2 rat pups, as compared to native EVs (lower half of the panel).

Prof. Benjamin Gantenbein: Regeneration of bone and joints

Low back pain is a significant problem given demographic and ageing population dynamics affecting the quality of life, placing a substantial burden on healthcare resources. Degeneration of the intervertebral disc (IVD) results in a loss of proteoglycans and water content with a consequential decrease in disc height. It has been recognised as a significant “discogenic” pain issue [98,99]. Identifying a suitable cell source to enhance regeneration is a pivotal challenge for clinical translation [100]. Recently, a particular native progenitor cell population has been identified within the niche of the IVD, which are positive for Angiopoietin-1 receptor (so called Tie2). It has been demonstrated that these so-called nucleus pulposus progenitor cells (NPPC) are relatively rare (~2-10% of all IVD cells) in the human intervertebral disc and diminish in number with increasing age (>40 yrs) [101,102]. Nevertheless, these cells may represent a very important source for future cell therapy applications. A current SNF-bridge project has been started together with the Lucerne University of Applied Sciences and Arts and there is a special focus on these Tie2+ cells. EVs shall be studied and characterized in more detail in future projects concerning these progenitor cells to possibly facilitate the development of clinical therapy [102,104].

Here, on this proposal of a shared EV platform we intend to characterise the EVs of these Tie2+ progenitors cells and to test if these EVs have regenerative potential similarly to EVs isolated from primary bone-marrow derived mesenchymal stromal cells (MSCs).

We will isolate the EVs and characterise these in a first step, in a second step we will investigate their regenerative potential in an established pre-clinical organ culture model of the IVD [105]. Previous research has been proposed in this direction in the field of IVD research [106-108]. However, current knowledge whether Tie2+ derived EVs contain even more potent components to activate endogenous IVD cells is currently unknown.

Here, *ex-vivo* tissue and organ culture models of the IVD will be investigated and the importance of EVs for cell therapy shall be explored.

In vivo validation models: Exploration of the regenerative effects in an in-vivo rat-tail animal study. Adult Wistar rats (aged around 10-12 weeks, male and females alike) shall be used. Anesthesia and post-analgesic care will be used as previously described [109]. Rat tail disc at the level of caudal (Cd)-2-Cd3 will be degenerated by a puncture needle model [110,111]. After needle puncture with 25G needle and fixed depth during anesthesia, IVDs will be monitored using MRI and digital X-ray (Compai Truevue system 100) to monitor disc height. 2 weeks post-op Tie2+ cells, EVs or biomaterial only will be injected with a 25G needle into the degenerated discs. 1M expanded human Tie2+ cells will be monitored using red fluorescence membrane dye (PKH26, Sigma-Aldrich or DIL form Thermo-fisher) and using histology in paraffin sections to track survival of Tie2+ cells.

Project 4.2 Hybrid vesicles for cell-specific targeting

PD Dr. Amiq Gazdhar and Prof. Paola Luciani: Generation of hybrid vesicles for cell-specific targeting

In the current proposal we aim at developing hybrid EVs that can target specific lung cells (alveolar epithelial cells, fibroblasts and macrophages) in vivo as a promising cell specific drug delivery cargo offering enhanced stability, targeting capabilities, and biocompatibility for chronic lung disease like Idiopathic pulmonary fibrosis (IPF) . IPF is an age related progressive interstitial lung disease of unknown etiology and very high mortality, with no curative treatment available [112,113]. Recent research indicates that multiple complex mechanisms involving different cell types and mediators trigger the onset and progression of this disease [114,115]. Secreted mediators of Induced pluripotent stem cells (iPSCs), the iPSCs-conditioned medium (CM) have been tested in various preclinical settings with promising results, we have successfully demonstrated antifibrotic effect of iPSCs-CM in preclinical settings [116]. We applied the multiomics approach and performed interactome analysis and identified few key components present in the iPSCs-CM that effect different cell types [117-118]. Therefore, we will load cell specific hybrid vesicles with cell specific key components and test them in the in vivo rodent model of lung injury and fibrosis.

Own work on cell-specific targeting using EVs

In an ongoing project we have developed EVs derived hybrid vesicles for targeting hepatocytes in vivo.

Isolation of Extracellular vesicles (EVs) from mouse hepatocyte: Mouse hepatocyte (Hepa1-6) were labelled using the live cell dye (DiO) cultured to confluence in a serum-free media and the supernatant was collected, standard protocol of EVs isolation and characterization was performed as described before (Figure 3). The cell specificity was tested by in vitro test (Figure 4). The EVs were injected intravenous (i.v.) into the tail vein of the mice and the mice were sacrificed 48 h later, the liver was harvested and sectioned to evaluate the cell specificity of the EVs (Figure 5).

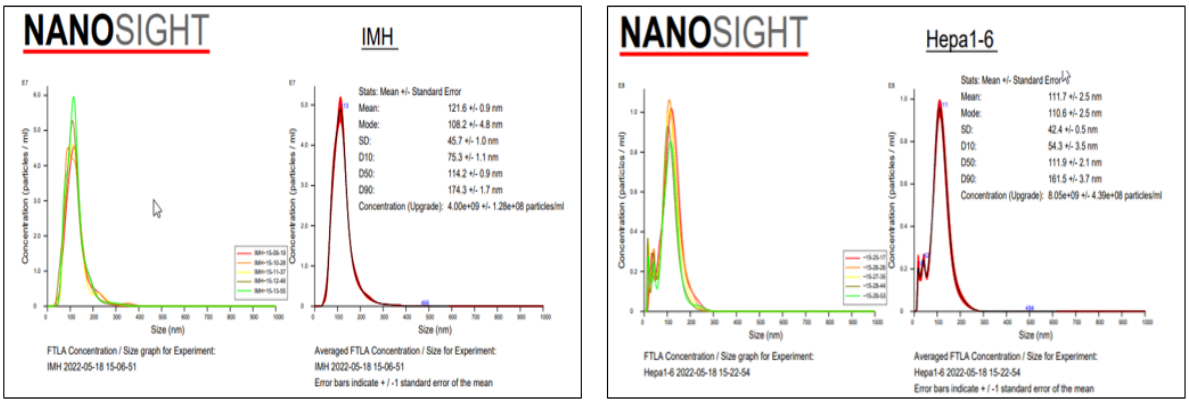

Figure 3 Extracellular vesicles (EVs) were isolated from human and mouse hepatocyte cell lines. Nanosight analysis was performed for characterization. Size and concentrations of EVs coming from Human hepatocytes (IMH cell line) and mouse hepatocytes (Hepa1-6).

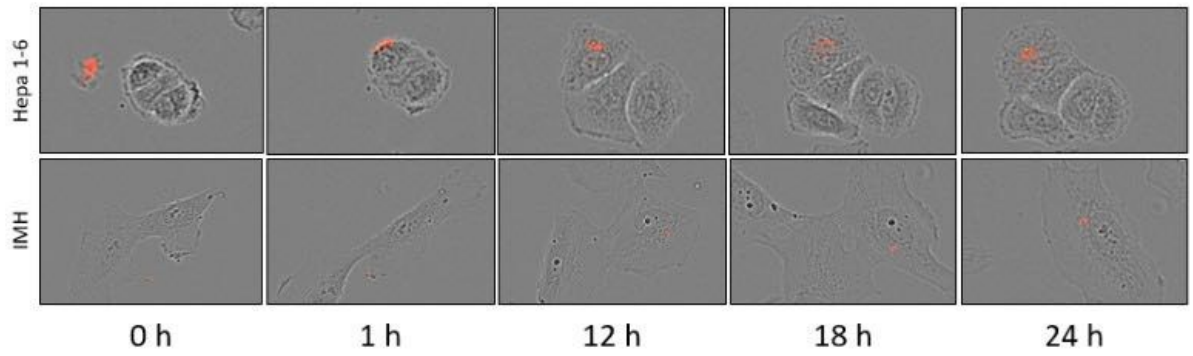

Figure 4 EVs where isolated and different cells were treated with the EVs. Human hepatocytes (IMH), mouse hepatocytes (Hepa1-6), and human lung alveolar epithelial cells (A549) were treated with either EVs isolated from IMH or Hepa1-6. Microscopic images taken after 24 h from EV isolation revealed cell-specific uptake of the EVs, Hepa1-6 cells only internalized the EVs from Hepa1-6 cells and IMH cells only internalized the EVs from IMH cells. Interestingly, no EV internalization was observed in A549 cells indicating the cell specific uptake of EVs thus proving our hypothesis. The images shown here are representative of > 100 images analysed to demonstrate the specific EV-cell interaction.

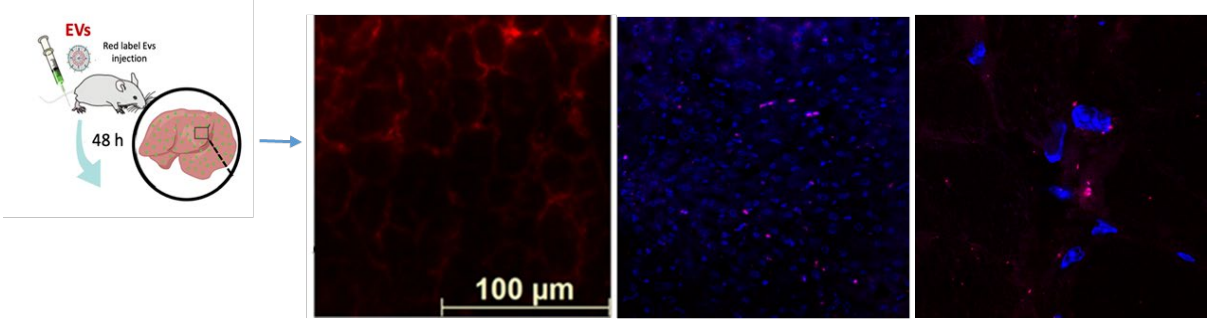

Figure 5 Mouse hepatocytes (Hepa1-6) cells were labelled with PKH26 red fluorescent cell linker and EVs isolated and injected into the mice tail vein. Mice were sacrificed after 48 hours and liver were observed under microscope. The red labelled EVs were specifically seen in hepatocyte only. (EVs were not seen in any other organ, data not shown).

Specific aims of Project 4.2

In the current proposal, we aim:

- To develop lung cell-specific hybrid vesicles that can target alveolar epithelial cells or fibroblasts or macrophages.
- To test the specificity in healthy and bleomycin-injured rodent lung
- To load cell-specific hybrid EVs with lead compounds to target specific cell types in bleomycin-injured rodent lung.

Methods

Lung cell-specific liposome/virosome

The different primary cells will be labeled with different colors and membrane proteins from mouse lung cells (alveolar epithelial cells, fibroblasts and macrophages) will be purified from primary mouse cells by isolating the cell membrane fraction and purifying the membrane proteins by a detergent process, or by isolating the EVs from the cells as described below. At the end of the process, purified membrane proteins will be solubilized in a non-ionic detergent and subsequently inserted into the virosome or liposome lipid membrane. Virosomes will be prepared from inactivated and purified influenza virus (A/Brisbane/59/2007 (H1N1), Seqirus, Australia). The final liquid hepatocyte-specific virosomes will be terminally filtered through a 0.2 μm sterile filter. Quality controls will be conducted with appropriate methods for determining protein concentration and particle size and homogeneity. Lung cell-specific liposome formulations will be manufactured with the same process but in the absence of influenza hemagglutinin. Alternatively, the Nano-Assemblr Ignite® microfluidic mixer (Precision Nanosystems, Canada, available in the laboratories of Prof. Dr. Paola Luciani at the DCBP, University of Bern) will be used to prepare protein- and drug-containing liposomes by a microfluidic process. After dilution and dialysis, quality controls will be applied to all formulations.

Hybrid EVs

Primary mouse lung cells (alveolar epithelial cells, fibroblasts, and macrophages) will be grown to 90% confluency in 150 mm tissue culture-treated dishes using cell-specific media. Cells will be washed with PBS twice and cultured in KO-DMEM without serum and will be grown for 24 h. Culture supernatant will be collected and centrifuged at 2,000 × *g* for 20 min to remove cells and cellular debris. Subsequently, the supernatant will be extracted and ultracentrifuged at 70,000 × *g* for 20 min. The supernatant will be again collected and further ultracentrifuged at 120,000 × *g* for 90 min to pellet EVs. Pellets will be re-suspended in PBS and characterized by nanoparticle tracking analysis using Zeta view or stored at −80°C until further use. For characterization of EVs, western blot (detection of CD9, CD63, CD81) and electron microscopy will be performed. Hybrid EVs will be produced by incubating naïve EVs with liposomes. The plasmid–liposome complex will be added to EVs and incubated at 37 °C for 12 h. The nucleic acid-loaded lipid nanoparticles (LNPs) will be prepared by using the NanoAssemblr Ignite. The preparation method will be optimized starting from the several recently reported ones [120]. Plasmid DNA (0.1 mg/mL in acetate buffer at pH 4) in aqueous buffer will be mixed with lipids (specifically, the ionizable lipid 1,2-distearoyl-3-dimethylammonium-propane (16:0 DAP), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG), and cholesterol as in [118] (10 mg/mL total lipid concentration) in ethanol solution at a flow rate ratio (FRR) of 3:1 (aqueous:ethanol) at room temperature through NxGen micromixers at a total flow rate (TFR) of 12 mL/min. Ethanol present in the resulting mixture will be removed by dialysis using a 1000 kDa Float-A-Lyzer G2 dialysis device (Spectrum Labs) at 4 °C overnight against PBS pH 7.4. The removal of organic solvents will be confirmed by gas chromatography (GC), a service available at the Department of Chemistry, Biochemistry and Pharmaceutical Sciences at the University of Bern. After ethanol removal, the LNPs will be characterized for their plasmid DNA content using a Quant-iT™ PicoGreen™ dsDBA Assay Kit (Thermo Fisher Scientific). Size, PDI and zeta potential of the LNPs will be assessed via a LiteSizer500 (Anton Paar, available in Prof. Luciani's labs). The morphology of the particles will be determined via cryo-TEM (service available at the MIC Center of UniBern). In view of the subsequent cellular assays, the resistance of LNPs to serum will be evaluated by incubating them for 24 h with PBS with or without fetal bovine serum (10% or 50%) at 37 °C, followed by treatment with or without Triton X-100 (0.5%). LNPs will be then resolved on agarose gel (2%) and the bands analyzed

semiquantitatively via ImageJ. The same procedure will be followed to prepare hybrid LNPs, having care of using in-line dilution NxGen cartridges. These cartridges profit from a third inlet that will be used to load the previously isolated exosomes. The resulting hybrid LNPs will be characterized as described above.

In vivo evaluation of specificity in healthy and diseased lung

Adult male (C57BL/6) mice will be used for the study, after approval of the protocol by the local Cantonal ethics committee. The different cell-specific hybrid EVs will be instilled in the trachea and the animals will be sacrificed 48 hours later and lungs will be harvested and cryosections will be performed. The sections will be evaluated under fluorescent microscopy.

Loading cell-specific hybrid EVs with lead compounds identified by proteomic analysis of induced pluripotent stem cells

We have performed mu**l**tiomics, interactome analysis to identify the key components that regulate different pathways in different cells. We will experimentally validate the interactome analysis. The interactome analysis identified key mediators present in iPSCs-CM such as: ACAA2 and 1, FASN, ATP5F1 A&B, and COX5A and 4Il that regulate fibroblasts; AAP and ELVL1 that regulate macrophages; VCP, HSPA4, ACTN4 that regulate alveolar epithelial cells. The identified cell-specific key mediators will be purchased as proteins and the cell-specific hybrid EVs will be loaded following the standard protocol as described below.

EVs will be loaded in 50 µl recombinant protein and incubated for 15–45 min with gentle agitation on a HulaMixer™ at room temperature. After centrifugation for 30 s at 14,000 × g, the supernatant will be collected leaving the resin with the bound protein that was not associated with the EVs [121,122].

In vivo validation in mice lung injury model

Male C57BL/6jRJ mice will be anesthetized, and instilled intratracheally with bleomycin (Cat. No: 2634B5012, Baxter, USA) (1.52 U/mice) in a volume of 50µL of saline to both lungs, for equal distribution a bolus of air will also be instilled.

To evaluate the effect of hybrid EVs, seven days after bleomycin administration animals will receive cell specific hybrid EVs through intratracheal administration. The animals will be euthanized as described above seven days after treatment and lungs will be harvested for further investigation. Anesthesia and euthanasia will be carried out strictly following approved, institutional SOPs. Histology, total collagen content and various cytokines will be measured following standard procedure as described before.

Prof. Deborah Stroka: New therapeutic approach for autoimmune cholangiopathies

Using EVs to treat autoimmune cholangiopathies (AC). Patients with AC such as primary sclerosing cholangitis and primary biliary cholangitis suffer from burdensome and life-threatening symptoms, including pruritus, metabolic dysfunction, cholestasis, and fibrosis. Current treatments for AC are insufficient and patients with advanced AC often require liver transplantation. The role of EVs in autoimmune diseases is increasingly recognized. In AC, EVs carry immunogens (e.g., PDC-E2) and pro-inflammatory factors, which activate antigen-presenting cells priming a disease-inducing autoimmune response. Our working hypothesis is clearance of cholangiocyte-dervied EVs will decrease autoimmune cholangiopathy. Our aim is to develop GalNAc-based lysosome-targeting chimeras (LYTAC) binders that recognizes surface molecules on diseased cholangiocyte-derived EVs. The binding of the GalNAc with the asialoglycoprotein receptor will trigger the endocytosis of LYTAC binder with the cholangiocyte EVs by hepatocytes and induce their lysosomal degradation. Based on expertise within our group and direct access to human material, we will isolate and characterize EVs from patients with AC (Aim1). Next we will target cholangiocyte EVs surface protein(s) (e.g. Muc1/4) using our established LYTAC technology and evaluate the feasibility of EV clearance in vitro, using primary human hepatocyte cultures. With mouse models of autoimmune cholangiopathy induced by AC patient derived EVs, we will evaluate the efficacy of our new therapeutic approach. We will monitor autoimmune injury in the liver and, quantifiy the effect of disease severity with our expertise in mass cytometry (MC) and imaging MC (www.imc.unibe.ch).

Combined anticipated outcomes and benefits of Project 4:

The anticipated outcomes and benefits of Project 4 are the development of different types of native, synthetic, and modified EVs, that will provide researchers and clinicians with a range of options for addressing specific disease targets. The methods to assess EVs' delivery, and efficacy will enable researchers to monitor and optimize the therapeutic potential of these EVs. This will ensure that EV-based therapies are safe and effective for human use. The biodistribution of EVs and their cargo will be studied in animal models to determine the optimal dosing and delivery routes for clinical trials. The EVs can be engineered with specific proteins on their surface to facilitate targeting to defined cell types or loaded with various cargoes to improve therapeutic efficacy. The use of EVs can offer a personalized approach to treatment, tailoring the EVs' cargo to the specific needs of each patient.

Precision medicine is a rapidly advancing field that aims to tailor medical treatments to an individual's genetic makeup, environment, and lifestyle. One key aspect of precision medicine is the development of targeted drug delivery systems that can specifically deliver drugs to the affected cells within the tissue. Use of cell-specific hybrid vesicles allows for the targeted delivery of drugs, minimizing damage to healthy cells and improving treatment outcomes. The specificity of cell-specific hybrid EVs enables them to target-specific cell types, which can be especially beneficial in the treatment of complex diseases. Furthermore, the use of cell-specific hybrid EVs for drug delivery has several advantages over conventional drug delivery systems. For instance, they can protect drugs from degradation, increase their stability, and improve their bioavailability, all of which can result in better treatment outcomes. Finally, this project will help develop the much needed targeted delivery and pave the way for novel clinical applications for the treatment of chronic disease, thus addressing the unmet clinical need. Developing new and improved EV-based therapies has the potential to transform the way we approach patient care, offering personalized and effective treatments with minimal adverse effects.

Promotion of young researchers and diversity

The proposed projects are a solid basis for several academic studies that are suitable for the full range of different thesis projects ranging from Master and MD theses for medical students to MD-PhD projects both in the Graduate School for Cellular and Biomedical Sciences (GCB) and the Graduate School for Health Sciences (GHS). The data of the projects have the potential to be analysed by clinician-scientists leveraging the bio-informatic skills that will be required for this future generation of clinical researchers.

Young researchers who are already involved in the design of the project and the preliminary findings are:

- Vera Tscherrig, MSc (supervised by Andreina Schoeberlein and Marianne Joerger-Messerli), with her PhD project “MicroRNAs in Wharton's jelly-derived small extracellular vesicles (sEV) and their potential role in neuroregeneration”.
- Marel Steinfort, MSc (supervised by Amanda Brosius Lutz and Andreina Schoeberlein), with the PhD project “Astrocyte reactivity in perinatal white matter injury: heterogeneity, role in myelination defects and potential as a biomarker”.

Outlook

Scientific impact

The EV Core Facility will have a significant scientific impact by enabling research on EVs, which are increasingly recognized as potential therapeutics and biomarkers for various diseases. By providing services such as isolation, characterization, and analysis of EVs, the EV Core Facility will help standardize and improve the quality of EV research, which is still a relatively new field. In particular, the project will have the following scientific impact:

- Advancing our understanding of EV biology: by providing high quality EVs and analytical tools, the EV Core Facility will help provide new insights into the biology of these vesicles.

- Improving biomarker discovery: EVs are being explored as potential biomarkers for a wide range of diseases, and the EV Core Facility can contribute to this effort by providing standardized methods for EV isolation and analysis. This can help identify reliable EV biomarkers that can be used for diagnostic or prognostic purposes.
- Supporting drug development and targeted drug delivery: EVs are also being explored as potential therapeutic agents, and the EV Core Facility can help advance this research by providing researchers with high-quality EVs for use in preclinical studies. This can help identify promising therapeutic targets and accelerate the development of EV-based therapies.

Overall, the EV Core Facility will have a significant scientific impact by advancing our understanding of EV biology, improving biomarker discovery, supporting drug development, and developing novel therapeutics.

Clinical impact

The extracellular vesicle core facility can have a significant clinical impact by providing researchers and clinicians with standardized and quality-controlled EV isolation, characterization, and analysis services. The core facility can act as a centralized resource that offers a range of EV-related services, including isolation of EVs from different biofluids, characterization of EVs' physical and biochemical properties, and analysis of EV contents such as proteins, nucleic acids, and lipids. By providing standardized, quality-controlled EV services, the core facility can help ensure that EV research is reproducible, reliable, and clinically relevant. This will be an indispensable prerequisite for the future provision of Good Manufacturing Practice (GMP)-certified therapies and diagnostic tools and their approval by regulatory authorities.

Moreover, the EV core facility can play a crucial role in facilitating the translation of EV research into clinical applications. By providing high-quality, standardized EV services, the core facility can help accelerate the development of EV-based diagnostic, prognostic, and therapeutic tools. For example, the EV core facility can support the development of EV-based liquid biopsies for cancer diagnosis or the development of EV-based drug delivery systems. The core facility can also provide education and training for researchers and clinicians on the isolation, characterization, and analysis of EVs, further promoting the clinical translation of EV research.

Sustainability

The sustainability of the “platform” will be based on a circular model. Researchers will be able to use the combined expertise and methodology of the platform to generate more funding and resources to further develop the local expertise. We aim to develop a centralized facility and will work closely by establishing standard protocols that will be shared and used to establish EVs for diagnostics and will be open for external collaboration; this will be a unique set up in Switzerland creating an ecosystem with a circular loop model to offer EV isolation and downstream characterization. The operational sustainability is a fundamental part of the project by having clear policies and procedures in place, implementing best practices for EV sample processing and analysis, and continually evaluating and improving its performance. While the project will begin on a small scale, incorporating expertise and equipment already available in the applicants' research groups and augmented by new technological platforms and dedicated technical personnel, it has the potential to expand as the need for EV research and clinical application increases and more funding becomes available. Upon establishing the EVs for therapeutics as described in Project 4, we will consolidate the use of EVs for targeted therapy and will open new avenues for internal and external collaboration to explore more cell-specific precision medicine approaches. We are confident that the we will be able to create a self-sustainable conducive ecosystem with strong collaboration and teaching involving young talents. The already established collaborations will lead to innovation and giving the platform a strong intellectual property portfolio and further creating and strengthening a more sustainable and resilient future.

Contributions to the proposal

To fulfil the aims of the projects, we request for additional personnel to run the facility on a daily basis and assist with EV isolation, purification and characterization. The main workload to carry out all the project-specific tasts, however, will be absorbed in the involved research groups with the current manpower.

Main Applicant

Prof. Dr. Andreina Schoeberlein, University Women's Hospital, Obstetrics and Feto-maternal Medicine, has a long-standing experience in the field of stem cell-based experimental therapies. The main applicant is the lead of the Bern Stem Cell and Regenerative Medicine Platform (SCRM) and has central roles in the European and international cell therapy research communities by serving as the President-Elect of the International Placenta Stem Cell Society (IPLASS) and as the leader of the "Pre-clinical studies" working group of the COST Action CA17116 "International Network for Translating Research on Perinatal Derivatives into Therapeutic Approaches (SPRINT)". Her research focus in the last 20+ years was to develop treatments for diseases affecting the fetus and the neonate. The Perinatal Medicine lab has access to umbilical cord tissue to isolate stem & stromal cells used in therapeutic approaches for premature white matter injury. The group has extensively studied the mechanisms of action of umbilical cord mesenchymal stromal cells (UC-MSC) and of the extracellular vesicles secreted by UC-MSC using in vitro functional assays and in vivo disease models. More recently, we analysed the functionality of the miRNA cargo of the UC-MSC-EVs.

Co-applicants

PD Dr. Amiq Gazdhar

Prof. Dr. Katia Monastyrskaya-Staeuber is the head of the Functional Urology Research Group, DBMR, Medical Faculty. Her group's research focuses on investigating the molecular mechanisms of lower urinary tract diseases (LUTDs), particularly the role of miRNAs in the bladder organ remodelling during obstructive and neurogenic bladder dysfunction [38,69,70]. The group studies the switch from bladder hypertrophy to fibrosis and acontractility. They hypothesized that regulatory miRNA molecules may serve as reliable biomarkers of the progression of bladder fibrosis. The EV analysis is a valuable tool to discover urinary biomarkers, and can be applied to LUTDs. The research group is currently creating a database of EVs, originating from different organs and tissues, using single cell RNA sequencing data and state-of–the art bioinformatic approaches. Experimental validation of our molecular classifiers will involve the isolation of EVs from selected cells and tissue types and specific immunolabelling with antibodies against marker membrane and intracellular proteins.

Prof Dr. Deborah Stroka is a group leader in the Department of Visceral Surgery and Medicine works in the field of liver cell biology since >20 years. Her research interest lies in the understanding of how the liver regenerates with a focus on defining mechanisms of hepatoprotection. Recently, she has studied NAFLD/NASH and liver regeneration at a single cell level with RNA seq in mouse and human livers. In addition, she is currently supervising a PhD project, funded by the Bern Center of Precision Medicine, which is characterizing NAFLD and NASH patient material and samples from a preclinical mouse model. Blood and liver samples are analyzed by mass cytometry and imaging mass cytometry for deep phenotyping at a single cell level. Her laboratory in the Department of Visceral Surgery and Medicine has full access to discarded liver tissue from consented patients and is fully proficient in isolating liver cells, including hepatocytes, stellate cells, Kupffer cells and immune cells.

Prof. Dr. Benjamin Gantenbein, PhD, P.I., is group leader of "Orthopaedic Research" at the Department for BioMedical Research, Bone & Joint Research Cluster, University of Bern, to conduct basic and translational research in the field of the spine motional segment and the knee joint. For the current project, he unites the knowledge from two entirely separate fields, i.e. molecular biology and genetics (as he was initially trained as a population geneticist) combined with orthopaedic research of musculoskeletal tissues (2nd education in tissue engineering). He and his team currently investigate regenerative approaches with clinically relevant cells and organ culture models such as stromal

cells, IVD cells and/or biomaterials for more than twelve years [123-128]. He has been awarded in the field of IVD research with awards, one from AOSpine International – the Hansjörg Wyss start-up grant.

Project Partner

Prof. Dr. Paola Luciani. University of Bern, Department of Chemistry, Biochemistry and Pharmaceutical Sciences works in the field of drug delivery since >15 years. Her research interest lies in the development of lipid-based drug carrier and diagnostic tools, with a focus on fibrotic and inflammatory pathologies. Besides having identified bioactive phospholipids with antifibrotic properties [129], which her group is now formulating as solid dosage forms for the treatment of chronic liver disease, she actively investigates the role of extracellular vesicles in liver fibrosis [130] with the aim of identifying biomarkers and modulators in response to the pharmacological treatment [32,33]. Her laboratories of Pharmaceutical Technology in Bern are fully equipped to harvest, purify and characterise EVs according to the latest Minimal Information for Studies of Extracellular Vesicles (MISEV) 2018 guidelines [8].

References

1 Harding, C., Heuser, J. & Stahl, P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol* **97**, 329-339, doi:10.1083/jcb.97.2.329 (1983).

2 Pan, B. T., Teng, K., Wu, C., Adam, M. & Johnstone, R. M. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J Cell Biol* **101**, 942-948, doi:10.1083/jcb.101.3.942 (1985).

3 Raposo, G. *et al.* B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* **183**, 1161-1172, doi:10.1084/jem.183.3.1161 (1996).

4 Wolfers, J. *et al.* Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med* **7**, 297-303, doi:10.1038/85438 (2001).

5 Valadi, H. *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* **9**, 654-659, doi:10.1038/ncb1596 (2007).

6 Yáñez-Mó, M. *et al.* Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* **4**, 27066, doi:10.3402/jev.v4.27066 (2015).

7 Braccioli, L., van Velthoven, C. & Heijnen, C. J. Exosomes: a new weapon to treat the central nervous system. *Mol Neurobiol* **49**, 113-119, doi:10.1007/s12035-013-8504-9 (2014).

8 Théry, C. *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* **7**, 1535750, doi:10.1080/20013078.2018.1535750 (2018).

9 Mathieu, M., Martin-Jaular, L., Lavieu, G. & Thery, C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol* **21**, 9-17, doi:10.1038/s41556-018-0250-9 (2019).

10 van Niel, G., D'Angelo, G. & Raposo, G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* **19**, 213-228, doi:10.1038/nrm.2017.125 (2018).

11 Mulcahy, L. A., Pink, R. C. & Carter, D. R. Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles* **3**, doi:10.3402/jev.v3.24641 (2014).

12 Robbins, P. D. & Morelli, A. E. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* **14**, 195-208, doi:10.1038/nri3622 (2014).

13 Frühbeis, C., Fröhlich, D. & Kramer-Albers, E. M. Emerging roles of exosomes in neuron-glia communication. *Front Physiol* **3**, 119, doi:10.3389/fphys.2012.00119 (2012).

14 Marar, C., Starich, B. & Wirtz, D. Extracellular vesicles in immunomodulation and tumor progression. *Nat Immunol* **22**, 560-570, doi:10.1038/s41590-021-00899-0 (2021).

15 Becker, A. *et al.* Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. *Cancer Cell* **30**, 836-848, doi:10.1016/j.ccell.2016.10.009 (2016).

16 Shah, R., Patel, T. & Freedman, J. E. Circulating Extracellular Vesicles in Human Disease. *N Engl J Med* **379**, 2180-2181, doi:10.1056/NEJMc1813170 (2018).

17 Schou, A. S., Nielsen, J. E., Askeland, A. & Jorgensen, M. M. Extracellular vesicle-associated proteins as potential biomarkers. *Adv Clin Chem* **99**, 1-48, doi:10.1016/bs.acc.2020.02.011 (2020).

18 Wiklander, O. P. B., Brennan, M. A., Lotvall, J., Breakefield, X. O. & El Andaloussi, S. Advances in therapeutic applications of extracellular vesicles. *Sci Transl Med* **11**, doi:10.1126/scitranslmed.aav8521 (2019).

19 Herrmann, I. K., Wood, M. J. A. & Fuhrmann, G. Extracellular vesicles as a next-generation drug delivery platform. *Nat Nanotechnol* **16**, 748-759, doi:10.1038/s41565-021-00931-2 (2021).

20 *ClinicalTrials.gov*, <https://beta.clinicaltrials.gov/search?distance=50&term=Extracellular%20Vesicles%20OR%20Exosomes&aggFilters=status:ter%20not%20rec%20com%20act%20enr%20unk> (

21 *FIMM HiPREP Extracellular Vesicles Core*, University of Helsinki, Finland. <https://www.helsinki.fi/en/infrastructures/fimm-technology-centre/fimm-hiprep-extracellular-vesicles-core> (accessed 28.04.2023).

22 *EV - Core Facility*, Philipps Universität Marburg, Marburg, Germany. <https://www.uni-marburg.de/en/fb20/departments/zti/institute-for-tumor-immunology/ev-core-facility> (accessed 28.04.2023).

23 *Curie CoreTech Extracellular Vesicles facility*, Institut Curie, Paris, France <https://institut-curie.org/curie-coretech-extracellular-vesicles-facility> (accessed 28.04.2023).

24 *Transfer Center for Extracellular Vesicles Theralytic Technologies (EV-TT)*, Paracelsus Medizinische Universität, Salzburg, Austria. <https://evtt.pmu.ac.at/en/> (accessed 28.04.2023).

25 *The REMEDI Extracellular Vesicle programme,* University of Galway, Ireland. <https://www.universityofgalway.ie/remedi/research-themes/extracellularvesicles/#> (accessed 28.04.2023).

26 *Research Centre for Extracellular Vesicles*, La Trobe University, Melbourne, VIC, Australia .<https://www.latrobe.edu.au/research/centres/extracellular-vesicles> (accessed 28.04.2023).

27 *Extracellular Vesicle Core*, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA .<https://www.vet.upenn.edu/research/core-resources-facilities/extracellular-vesicle-core> (accessed 28.04.2023).

28 *Extracellular Vesicle Core*, Rhode Island Hospital, Providence, RI, USA. <https://www.lifespan.org/centers-services/cobre-center-stem-cells-and-aging/extracellular-vesicle-core> (accessed 28.04.2023).

29 *Extracellular Vesicle Core*, Children's Hospital, Los Angeles, CA, USA <https://www.chla.org/research/extracellular-vesicle-core> (accessed 28.04.2023).

30 *The Vanderbilt Center for Extracellular Vesicle Research*, Vanderbilt School of Medicine, Nashville, TN, USA. < https://medschool.vanderbilt.edu/evresearch/about/> (accessed 28.04.2023).

31 Bordanaba-Florit, G., Royo, F., Kruglik, S. G. & Falcón-Pérez, J. M. Using single-vesicle technologies to unravel the heterogeneity of extracellular vesicles. *Nature protocols* **16**, 3163-3185, doi:10.1038/s41596-021-00551-z (2021).

32 Zivko, C., Fuhrmann, K., Fuhrmann, G. & Luciani, P. Tracking matricellular protein SPARC in extracellular vesicles as a non-destructive method to evaluate lipid-based antifibrotic treatments. *Communications biology* **5**, 1155, doi:10.1038/s42003-022-04123-z (2022).

33 Zivko, C., Witt, F., Koeberle, A., Fuhrmann, G. & Luciani, P. Formulating elafibranor and obeticholic acid with phospholipids decreases drug-induced association of SPARC to extracellular vesicles from LX-2 human hepatic stellate cells. *European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V* **182**, 32-40, doi:10.1016/j.ejpb.2022.11.025 (2023).

34 Weber, F., Rahnfeld, L. & Luciani, P. Analytical profiling and stability evaluation of liposomal drug delivery systems: A rapid UHPLC-CAD-based approach for phospholipids in research and quality control. *Talanta* **220**, 121320, doi:10.1016/j.talanta.2020.121320 (2020).

35 Joerger-Messerli, M. S. *et al.* Human Wharton's Jelly Mesenchymal Stromal Cell-Derived Small Extracellular Vesicles Drive Oligodendroglial Maturation by Restraining MAPK/ERK and Notch Signaling Pathways. *Front Cell Dev Biol* **9**, 622539, doi:10.3389/fcell.2021.622539 (2021).

36 Ajit, S. K. Circulating microRNAs as biomarkers, therapeutic targets, and signaling molecules. *Sensors (Basel)* **12**, 3359-3369, doi:10.3390/s120303359 (2012).

37 Tatischeff, I. Extracellular Vesicle-DNA: The Next Liquid Biopsy Biomarker for Early Cancer Diagnosis? *Cancers* **15**, doi:10.3390/cancers15051456 (2023).

38 Sanchez Freire, V. *et al.* MicroRNAs may mediate the down-regulation of neurokinin-1 receptor in chronic bladder pain syndrome. *Am J Pathol* **176**, 288-303, doi:10.2353/ajpath.2010.090552 (2010).

39 Schilling, J. D., Mulvey, M. A. & Hultgren, S. J. Dynamic interactions between host and pathogen during acute urinary tract infections. *Urology* **57**, 56-61 (2001).

40 Jain, G. *et al.* Urinary extracellular vesicles miRNA-A new era of prostate cancer biomarkers. *Frontiers in genetics* **14**, 1065757, doi:10.3389/fgene.2023.1065757 (2023).

41 von Siebenthal, M. *et al.* Urinary miRNA profiles discriminate between obstruction-induced bladder dysfunction and healthy controls. *Sci Rep* **11**, 10204, doi:10.1038/s41598-021-89535-3 (2021).

42 Gheinani, A. H. *et al.* Integrated mRNA-miRNA transcriptome analysis of bladder biopsies from patients with bladder pain syndrome identifies signaling alterations contributing to the disease pathogenesis. *BMC urology* **21**, 172, doi:10.1186/s12894-021-00934-0 (2021).

43 Gheinani, A. H. *et al.* Characterization of miRNA-regulated networks, hubs of signaling, and biomarkers in obstruction-induced bladder dysfunction. *JCI insight* **2**, e89560, doi:10.1172/jci.insight.89560 (2017).

44 Gheinani, A. H. *et al.* Improved isolation strategies to increase the yield and purity of human urinary exosomes for biomarker discovery. *Sci Rep* **8**, 3945, doi:10.1038/s41598-018-22142-x (2018).

45 Younas, N., Fernandez Flores, L. C., Hopfner, F., Höglinger, G. U. & Zerr, I. A new paradigm for diagnosis of neurodegenerative diseases: peripheral exosomes of brain origin. *Translational neurodegeneration* **11**, 28, doi:10.1186/s40035-022-00301-5 (2022).

46 Hornung, S., Dutta, S. & Bitan, G. CNS-Derived Blood Exosomes as a Promising Source of Biomarkers: Opportunities and Challenges. *Front Mol Neurosci* **13**, 38, doi:10.3389/fnmol.2020.00038 (2020).

47 Hagey, D. W. & El Andaloussi, S. The promise and challenges of extracellular vesicles in the diagnosis of neurodegenerative diseases. *Handbook of clinical neurology* **193**, 227-241, doi:10.1016/b978-0-323-85555-6.00014-x (2023).

48 Goetzl, E. J., Schwartz, J. B., Abner, E. L., Jicha, G. A. & Kapogiannis, D. High complement levels in astrocyte-derived exosomes of Alzheimer disease. *Ann Neurol* **83**, 544-552, doi:10.1002/ana.25172 (2018).

49 Nila, I. S. *et al.* Identification of exosomal biomarkers and its optimal isolation and detection method for the diagnosis of Parkinson's disease: A systematic review and meta-analysis. *Ageing research reviews* **82**, 101764, doi:10.1016/j.arr.2022.101764 (2022).

50 Guedes, V. A. *et al.* Extracellular Vesicle Proteins and MicroRNAs as Biomarkers for Traumatic Brain Injury. *Front Neurol* **11**, 663, doi:10.3389/fneur.2020.00663 (2020).

51 Ollen-Bittle, N., Roseborough, A. D., Wang, W., Wu, J. D. & Whitehead, S. N. Mechanisms and Biomarker Potential of Extracellular Vesicles in Stroke. *Biology* **11**, doi:10.3390/biology11081231 (2022).

52 Bersani, I. *et al.* Early predictors of perinatal brain damage: the role of neurobiomarkers. *Clin Chem Lab Med* **58**, 471-486, doi:10.1515/cclm-2019-0725 (2020).

53 Li, J. *et al.* Promising roles of non-exosomal and exosomal non-coding RNAs in the regulatory mechanism and as diagnostic biomarkers in myocardial infarction. *Journal of Zhejiang University. Science. B* **24**, 281-300, doi:10.1631/jzus.B2200459 (2023).

54 Zhao, Y. *et al.* Exosomes Derived from Human Umbilical Cord Mesenchymal Stem Cells Relieve Acute Myocardial Ischemic Injury. *Stem Cells Int* **2015**, 761643, doi:10.1155/2015/761643 (2015).

55 Zhou, R. *et al.* Circulating exosomal microRNAs as emerging non-invasive clinical biomarkers in heart failure: Mega bio-roles of a nano bio-particle. *IUBMB life* **72**, 2546-2562, doi:10.1002/iub.2396 (2020).

56 Perez-Hernandez, J. & Cortes, R. Extracellular Vesicles as Biomarkers of Systemic Lupus Erythematosus. *Disease markers* **2015**, 613536, doi:10.1155/2015/613536 (2015).

57 Rademacher, T. D., Meuth, S. G., Wiendl, H., Johnen, A. & Landmeyer, N. C. Molecular biomarkers and cognitive impairment in multiple sclerosis: State of the field, limitations, and future direction - A systematic review and meta-analysis. *Neuroscience and biobehavioral reviews* **146**, 105035, doi:10.1016/j.neubiorev.2023.105035 (2023).

58 Manu, M. S., Hohjoh, H. & Yamamura, T. Extracellular Vesicles as Pro- and Anti-inflammatory Mediators, Biomarkers and Potential Therapeutic Agents in Multiple Sclerosis. *Aging and disease* **12**, 1451-1461, doi:10.14336/ad.2021.0513 (2021).

59 Mahajan, S. D., Ordain, N. S., Kutscher, H., Karki, S. & Reynolds, J. L. HIV Neuroinflammation: The Role of Exosomes in Cell Signaling, Prognostic and Diagnostic Biomarkers and Drug Delivery. *Front Cell Dev Biol* **9**, 637192, doi:10.3389/fcell.2021.637192 (2021).

60 Pulliam, L., Sun, B., Mustapic, M., Chawla, S. & Kapogiannis, D. Plasma neuronal exosomes serve as biomarkers of cognitive impairment in HIV infection and Alzheimer's disease. *Journal of neurovirology* **25**, 702-709, doi:10.1007/s13365-018-0695-4 (2019).

61 Bandopadhyay, M. & Bharadwaj, M. Exosomal miRNAs in hepatitis B virus related liver disease: a new hope for biomarker. *Gut pathogens* **12**, 23, doi:10.1186/s13099-020-00353-w (2020).

62 Thietart, S. & Rautou, P. E. Extracellular vesicles as biomarkers in liver diseases: A clinician's point of view. *Journal of hepatology* **73**, 1507-1525, doi:10.1016/j.jhep.2020.07.014 (2020).

63 Farrelly, R., Kennedy, M. G., Spencer, R. & Forbes, K. Extracellular vesicles as markers and mediators of pregnancy complications: gestational diabetes, pre-eclampsia, preterm birth and fetal growth restriction. *The Journal of physiology*, doi:10.1113/jp282849 (2023).

64 Goetzl, L., Darbinian, N. & Merabova, N. Noninvasive assessment of fetal central nervous system insult: Potential application to prenatal diagnosis. *Prenat Diagn* **39**, 609-615, doi:10.1002/pd.5474 (2019).

65 Yu, X., Nagai, J. & Khakh, B. S. Improved tools to study astrocytes. *Nat Rev Neurosci* **21**, 121-138, doi:10.1038/s41583-020-0264-8 (2020).

66 Foo, L. C. *et al.* Development of a method for the purification and culture of rodent astrocytes. *Neuron* **71**, 799-811, doi:10.1016/j.neuron.2011.07.022 (2011).

67 Goetzl, E. J. *et al.* Cargo proteins of plasma astrocyte-derived exosomes in Alzheimer's disease. *Faseb j* **30**, 3853-3859, doi:10.1096/fj.201600756R (2016).

68 Goetzl, L. *et al.* Diagnostic Potential of Neural Exosome Cargo as Biomarkers for Acute Brain Injury. *Ann Clin Transl Neurol* **5**, 4-10, doi:10.1002/acn3.499 (2018).

69 Hashemi Gheinani, A., Burkhard, F. C., Rehrauer, H., Aquino Fournier, C. & Monastyrskaya, K. MicroRNA MiR-199a-5p Regulates Smooth Muscle Cell Proliferation and Morphology by Targeting WNT2 Signaling Pathway. *The Journal of biological chemistry* **290**, 7067-7086, doi:10.1074/jbc.M114.618694 (2015).

70 Monastyrskaya, K. *et al.* miR-199a-5p regulates urothelial permeability and may play a role in bladder pain syndrome. *Am J Pathol* **182**, 431-448, doi:10.1016/j.ajpath.2012.10.020 (2013).

71 Koeck, I. *et al.* Tumor Necrosis Factor-alpha Initiates miRNA-mRNA Signaling Cascades in Obstruction-Induced Bladder Dysfunction. *Am J Pathol* **188**, 1847-1864, doi:10.1016/j.ajpath.2018.05.008 (2018).

72 Cortez, M. A., Welsh, J. W. & Calin, G. A. Circulating microRNAs as noninvasive biomarkers in breast cancer. *Recent Results Cancer Res* **195**, 151-161, doi:10.1007/978-3-642-28160-0_13 (2012).

73 Redis, R. S., Calin, S., Yang, Y., You, M. J. & Calin, G. A. Cell-to-cell miRNA transfer: from body homeostasis to therapy. *Pharmacol Ther* **136**, 169-174, doi:10.1016/j.pharmthera.2012.08.003 (2012).

74 Cocucci, E., Racchetti, G. & Meldolesi, J. Shedding microvesicles: artefacts no more. *Trends Cell Biol* **19**, 43-51, doi:10.1016/j.tcb.2008.11.003 (2009).

75 Sidhu, S. S., Mengistab, A. T., Tauscher, A. N., LaVail, J. & Basbaum, C. The microvesicle as a vehicle for EMMPRIN in tumor-stromal interactions. *Oncogene* **23**, 956-963, doi:10.1038/sj.onc.1207070 (2004).

76 Babiychuk, E. B., Monastyrskaya, K., Potez, S. & Draeger, A. Intracellular Ca(2+) operates a switch between repair and lysis of streptolysin O-perforated cells. *Cell Death.Differ.* (2009).

77 Potez, S. *et al.* Tailored protection against plasmalemmal injury by annexins with different Ca2+ sensitivities. *The Journal of biological chemistry* **286**, 17982-17991, doi:10.1074/jbc.M110.187625 (2011).

78 Garcia, T. A., Ventura, C. L., Smith, M. A., Merrell, D. S. & O'Brien, A. D. Cytotoxic Necrotizing Factor 1 and Hemolysin from Uropathogenic Escherichia coli Elicit Different Host Responses in the Murine Bladder. *Infection and immunity*, doi:10.1128/iai.00605-12 (2012).

79 Monastyrskaya, K., Babiychuk, E. B., Draeger, A. & Burkhard, F. C. Down-Regulation of Annexin A1 in the Urothelium Decreases Cell Survival After Bacterial Toxin Exposure. *J Urol*, doi:10.1016/j.juro.2013.01.088 (2013).

80 Garcia-Martin, R. *et al.* MicroRNA sequence codes for small extracellular vesicle release and cellular retention. *Nature* **601**, 446-451, doi:10.1038/s41586-021-04234-3 (2022).

81 Kalluri, R. & LeBleu, V. S. The biology, function, and biomedical applications of exosomes. *Science* **367**, eaau6977, doi:10.1126/science.aau6977 (2020).

82 Claridge, B., Lozano, J., Poh, Q. H. & Greening, D. W. Development of Extracellular Vesicle Therapeutics: Challenges, Considerations, and Opportunities. *Frontiers in Cell and Developmental Biology* **9**, doi:10.3389/fcell.2021.734720 (2021).

83 Buzas, E. I. The roles of extracellular vesicles in the immune system. *Nat Rev Immunol* **23**, 236-250, doi:10.1038/s41577-022-00763-8 (2023).

84 Sterzenbach, U. *et al.* Engineered Exosomes as Vehicles for Biologically Active Proteins. *Mol Ther* **25**, 1269-1278, doi:10.1016/j.ymthe.2017.03.030 (2017).

85 Blanco, E., Shen, H. & Ferrari, M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nat Biotechnol* **33**, 941-951, doi:10.1038/nbt.3330 (2015).

86 Hua, S., de Matos, M. B. C., Metselaar, J. M. & Storm, G. Current Trends and Challenges in the Clinical Translation of Nanoparticulate Nanomedicines: Pathways for Translational Development and Commercialization. *Front Pharmacol* **9**, 790, doi:10.3389/fphar.2018.00790 (2018).

87 Elsharkasy, O. M. *et al.* Extracellular vesicles as drug delivery systems: Why and how? *Adv Drug Deliv Rev* **159**, 332-343, doi:10.1016/j.addr.2020.04.004 (2020).

88 Piffoux, M., Silva, A. K. A., Gazeau, F. & Tareste, D. Generation of Hybrid Extracellular Vesicles by Fusion with Functionalized Liposomes. *Methods Mol Biol* **2473**, 385-396, doi:10.1007/978-1-0716-2209-4_24 (2022).

89 Rodríguez, D. A. & Vader, P. Extracellular Vesicle-Based Hybrid Systems for Advanced Drug Delivery. *Pharmaceutics* **14**, doi:10.3390/pharmaceutics14020267 (2022).

90 Katsuda, T., Kosaka, N., Takeshita, F. & Ochiya, T. The therapeutic potential of mesenchymal stem cell-derived extracellular vesicles. *Proteomics* **13**, 1637-1653, doi:10.1002/pmic.201200373 (2013).

91 Joerger-Messerli, M. S. *et al.* Extracellular Vesicles Derived from Wharton's Jelly Mesenchymal Stem Cells Prevent and Resolve Programmed Cell Death Mediated by Perinatal Hypoxia-Ischemia in Neuronal Cells. *Cell Transplant* **27**, 168-180, doi:10.1177/0963689717738256 (2018).

92 Thomi, G. *et al.* Intranasally Administered Exosomes from Umbilical Cord Stem Cells Have Preventive Neuroprotective Effects and Contribute to Functional Recovery after Perinatal Brain Injury. *Cells* **8**, doi:10.3390/cells8080855 (2019).

93 Ophelders, D. R. *et al.* Mesenchymal Stromal Cell-Derived Extracellular Vesicles Protect the Fetal Brain After Hypoxia-Ischemia. *Stem Cells Transl Med* **5**, 754-763, doi:10.5966/sctm.2015-0197 (2016).

94 Drommelschmidt, K. *et al.* Mesenchymal stem cell-derived extracellular vesicles ameliorate inflammation-induced preterm brain injury. *Brain Behav Immun* **60**, 220-232, doi:10.1016/j.bbi.2016.11.011 (2017).

95 Sisa, C. *et al.* Mesenchymal Stromal Cell Derived Extracellular Vesicles Reduce Hypoxia-Ischaemia Induced Perinatal Brain Injury. *Front Physiol* **10**, 282, doi:10.3389/fphys.2019.00282 (2019).

96 Gussenhoven, R. *et al.* Annexin A1 as Neuroprotective Determinant for Blood-Brain Barrier Integrity in Neonatal Hypoxic-Ischemic Encephalopathy. *J Clin Med* **8**, doi:10.3390/jcm8020137 (2019).

97 Wang, W., Ji, Z., Yuan, C. & Yang, Y. Mechanism of Human Umbilical Cord Mesenchymal Stem Cells Derived-Extracellular Vesicle in Cerebral Ischemia-Reperfusion Injury. *Neurochem Res* **46**, 455-467, doi:10.1007/s11064-020-03179-9 (2021).

98 Disease, G. B. D., Injury, I. & Prevalence, C. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet* **392**, 1789-1858, doi:10.1016/S0140-6736(18)32279-7 (2018).

99 Vos, T. *et al.* Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **380**, 2163-2196, doi:10.1016/S0140-6736(12)61729-2 (2012).

100 Sakai, D. & Grad, S. Advancing the cellular and molecular therapy for intervertebral disc disease. *Adv Drug Deliv Rev* **84**, 159-171, doi:10.1016/j.addr.2014.06.009 (2015).

101 Sakai, D. *et al.* Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the intervertebral disc. *Nat Commun* **3**, 1264, doi:10.1038/ncomms2226 (2012).

102 Sakai, D. *et al.* Successful fishing for nucleus pulposus progenitor cells of the intervertebral disc across species. *JOR Spine* **1**, e1018, doi:10.1002/jsp2.1018 (2018).

103 Hu, Y. C. *et al.* Nanoscale Treatment of Intervertebral Disc Degeneration: Mesenchymal Stem Cell Exosome Transplantation. *Curr Stem Cell Res Ther* **18**, 163-173, doi:10.2174/1574888X17666220422093103 (2023).

104 Tilotta, V. *et al.* Mesenchymal stem cell-derived secretome enhances nucleus pulposus cell metabolism and modulates extracellular matrix gene expression in vitro. *Frontiers in bioengineering and biotechnology* **11**, 1152207, doi:10.3389/fbioe.2023.1152207 (2023).

105 Tilotta, V. *et al.* Mesenchymal Stem Cell-Derived Exosomes: The New Frontier for the Treatment of Intervertebral Disc Degeneration. *Applied Sciences* **11**, 11222, doi:10.3390/app112311222 (2021).

106 Liang, W., Han, B., Hai, Y., Sun, D. & Yin, P. Mechanism of Action of Mesenchymal Stem Cell-Derived Exosomes in the Intervertebral Disc Degeneration Treatment and Bone Repair and Regeneration. *Front Cell Dev Biol* **9**, 833840, doi:10.3389/fcell.2021.833840 (2021).

107 Zhao, X. *et al.* Insights into Exosome in the Intervertebral Disc: Emerging Role for Disc Homeostasis and Normal Function. *Int J Med Sci* **19**, 1695-1705, doi:10.7150/ijms.75285 (2022).

108 Bhujel, B., Shin, H. E., Choi, D. J. & Han, I. Mesenchymal Stem Cell-Derived Exosomes and Intervertebral Disc Regeneration: Review. *Int J Mol Sci* **23**, doi:10.3390/ijms23137306 (2022).

109 Oswald, K. A. C. *et al.* Establishment of a Novel Method for Spinal Discectomy Surgery in Elderly Rats in an In Vivo Spinal Fusion Model. *Methods Protoc* **4**, doi:10.3390/mps4040079 (2021).

110 Hu, M. H. *et al.* Optimization of puncture injury to rat caudal disc for mimicking early degeneration of intervertebral disc. *J Orthop Res* **36**, 202-211, doi:10.1002/jor.23628 (2018).

111 Barcellona, M. N., McDonnell, E. E., Samuel, S. & Buckley, C. T. Rat tail models for the assessment of injectable nucleus pulposus regeneration strategies. *JOR Spine* **5**, e1216, doi:10.1002/jsp2.1216 (2022).

112 Thannickal, V. J. Mechanistic links between aging and lung fibrosis. *Biogerontology* **14**, 609-615, doi:10.1007/s10522-013-9451-6 (2013).

113 Selman, M. & Pardo, A. Revealing the pathogenic and aging-related mechanisms of the enigmatic idiopathic pulmonary fibrosis. an integral model. *American journal of respiratory and critical care medicine* **189**, 1161-1172, doi:10.1164/rccm.201312-2221PP (2014).

114 Mora, A. L., Bueno, M. & Rojas, M. Mitochondria in the spotlight of aging and idiopathic pulmonary fibrosis. *The Journal of clinical investigation* **127**, 405-414, doi:10.1172/jci87440 (2017).

115 Shenderov, K., Collins, S. L., Powell, J. D. & Horton, M. R. Immune dysregulation as a driver of idiopathic pulmonary fibrosis. *The Journal of clinical investigation* **131**, doi:10.1172/jci143226 (2021).

116 Gazdhar, A. *et al.* The secretome of induced pluripotent stem cells reduces lung fibrosis in part by hepatocyte growth factor. *Stem Cell Res Ther* **5**, 123, doi:10.1186/scrt513 (2014).

117 Tamò, L. *et al.* Interactome Analysis of iPSC Secretome and Its Effect on Macrophages In Vitro. *Int J Mol Sci* **22**, doi:10.3390/ijms22020958 (2021).

118 Tamò, L. *et al.* Gene Network Analysis of Interstitial Macrophages After Treatment with Induced Pluripotent Stem Cells Secretome (iPSC-cm) in the Bleomycin Injured Rat Lung. *Stem Cell Rev Rep* **14**, 412-424, doi:10.1007/s12015-017-9790-9 (2018).

119 Zinger, A. *et al.* Humanized Biomimetic Nanovesicles for Neuron Targeting. *Advanced science (Weinheim, Baden-Wurttemberg, Germany)* **8**, e2101437, doi:10.1002/advs.202101437 (2021).

120 Quagliarini, E *et al.* Microfluidic Formulation of DNA-Loaded Multicomponent Lipid Nanoparticles for Gene Delivery. *Pharmaceutics* **13**(8):1292, doi: 10.3390/pharmaceutics13081292 (2021).

121 Kooijmans, S. A. *et al.* Display of GPI-anchored anti-EGFR nanobodies on extracellular vesicles promotes tumour cell targeting. *J Extracell Vesicles* **5**, 31053, doi:10.3402/jev.v5.31053 (2016).

122 Crouch, C. H. *et al.* Optimization of Detergent-Mediated Reconstitution of Influenza A M2 Protein into Proteoliposomes. *Membranes* **8**, doi:10.3390/membranes8040103 (2018).

123 Malonzo, C. *et al.* A papain-induced disc degeneration model for the assessment of thermo-reversible hydrogel-cells therapeutic approach. *J Tissue Eng Regen Med* **9**, E167-176, doi:10.1002/term.1667 (2015).

124 Chan, S. C., Ferguson, S. J., Wuertz, K. & Gantenbein-Ritter, B. Biological response of the intervertebral disc to repetitive short-term cyclic torsion. *Spine (Phila Pa 1976)* **36**, 2021-2030, doi:10.1097/BRS.0b013e318203aea5 (2011).

125 Chan, S. C. *et al.* Region specific response of intervertebral disc cells to complex dynamic loading: an organ culture study using a dynamic torsion-compression bioreactor. *PLoS One* **8**, e72489, doi:10.1371/journal.pone.0072489 (2013).

126 Croft, A. S. *et al.* Repairing Annulus Fibrosus Fissures Using Methacrylated Gellan Gum Combined with Novel Silk. *Materials* **16**, 3173 (2023).

127 Croft, A. S. *et al.* In Situ Cell Signalling of the Hippo-YAP/TAZ Pathway in Reaction to Complex Dynamic Loading in an Intervertebral Disc Organ Culture. *International Journal of Molecular Sciences* **22**, 13641 (2021).

128 Gantenbein, B. *et al.* Organ culture bioreactors--platforms to study human intervertebral disc degeneration and regenerative therapy. *Curr Stem Cell Res Ther* **10**, 339-352, doi:10.2174/1574888x10666150312102948 (2015).

129 Valentino, G., Zivko, C., Weber, F., Brülisauer, L. & Luciani, P. Synergy of Phospholipid-Drug Formulations Significantly Deactivates Profibrogenic Human Hepatic Stellate Cells. *Pharmaceutics* **11**, doi:10.3390/pharmaceutics11120676 (2019).

130 Zivko, C., Fuhrmann, G. & Luciani, P. Liver-derived extracellular vesicles: A cell by cell overview to isolation and characterization practices. *Biochimica et biophysica acta. General subjects* **1865**, 129559, doi:10.1016/j.bbagen.2020.129559 (2021).