UNIVERSITY OF LJUBLJANA | MF Faculty of Medicine

SiN-EV SLOVENIAN NETWORK FOR EXTRACELLULAR VESICLES

SiN-EV MEETING 2026

BOOK OF ABSTRACTS

Ljubljana, 02. 06. 2026

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Book of Abstracts

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OPENING ADDRESS

Dear Colleagues,

We are pleased to welcome you to the SiN-EV Meeting 2026, taking place on the 2nd of June 2026 at the Faculty of Medicine, University of Ljubljana. This year, we are especially proud to celebrate the 10th anniversary of the Slovenian Network of EVs (SiN-EV).

Extracellular vesicles (EVs) represent a heterogeneous population of membrane-bound vesicles released by cells both *in vitro* and *in vivo*. They carry proteins, lipids, nucleic acids, and metabolites that reflect the composition and physiological state of their cell of origin. EVs have growing potential as biomarkers in humans, as they accumulate in body fluids at high concentrations and carry unique molecular signatures. Their key role in mediating many pathological processes makes them a promising therapeutic target, while EVs are also being studied as therapeutics and drug carriers.

This year's SiN-EV Meeting will begin with plenary lectures highlighting recent advances in single-vesicle detection using super-resolution microscopy and flow cytometry, and how these approaches can be applied to fundamental research and biomarker discovery. The program will then continue with two thematic sections: Fundamental EV Biology and Translational Applications of EVs, both showcasing diverse ongoing research across the EV field. The meeting will also include a poster session designed to offer participants the opportunity to present their work informally and engage in open discussions with colleagues.

We hope that the meeting's stimulating scientific atmosphere will inspire new collaborations, spark innovative ideas, and support the continued growth of EV research within the Slovenian community and beyond.

On behalf of the organizing committee, we warmly welcome you to this year's meeting dedicated to the rapidly expanding field of EVs.

Assoc. Prof. Metka Lenassi, PhD
Chair of the organizing committee

Mirijam Kozorog, PhD
Member of the organizing committee

ORGANISATION

SiN-EV Meeting 2026 is organised by

Organising committee

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Katja Goričar
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PROGRAM

Date: *Tuesday, 2nd of June 2026*

Location: *University of Ljubljana, Faculty of Medicine, Small lecture room, Korytkova 2, Ljubljana*

09.00 – 09.05	**Opening address** (Prof. Ksenija Geršak, dr. med., Dean, UL MF, SI)
Session 1	**Plenary talks *(Chairs: Metka Lenassi, Katja Goričan)***
09.05 – 09.40	Tijana Jovanović-Talisman, *Beckman Research Institute of City of Hope, USA* **Democratizing single EV microscopy**
09.40 – 10.15	Rienk Nieuwland, *Amsterdam University Medical Center, NLD* **Detection of extracellular vesicles by flow cytometry: from single to multicenter studies**
10.15 – 10.30	Sandrine Olivier, Malvern Panalytical (sponsor talk) **Analytical solutions for EVs**
10.30 – 11.10	*Coffee break + poster session*
Session 2	**Fundamental EV Biology *(Chairs: Teja Lavrin, Tina Plavec)***
11.10 – 11.30	Veronika Kralj Iglič, UL ZF, SI **Coacervation – a mechanism of extracellular particle formation**
11.30 – 11.45	Nina Kostevšek, IJS, SI **Erythrocyte membrane–derived vesicles as biomimetic platforms for safe and efficient siRNA delivery**
11.45 – 12.00	Samuel Žvanut, UL MF, SI **Characterization of extracellular vesicles carrying HIV-1 Nef: insights into inflammatory cargo**
12.00 – 12.15	Toni Petan, IJS, SI **Lipid droplet metabolism shapes the lipid landscape to control organelle integrity and ferroptosis sensitivity**
12.15 – 12.30	Pia Pužar Dominkuš, UL MF, SI **Gastrin modulates sEV miRNA cargo and promotes sEV-mediated proliferation in gastric cancer cells**
12.30 – 12.50	Carlos Jesus, President of SNEV **Introducing SNEV – Student Network on EVs**
12.50 – 13.05	*Group photo & SiN-EV 10th anniversary celebration*
13.05 – 14.00	*Lunch break & poster session*

Session 3	Translational Applications of EVs *(Chairs: Mirijam Kozorog, Nika Breznik)*
14.00 – 14.15	Carlos Jesus, University of Coimbra, PRT **Extracellular vesicles attached to a responsive transporter boosts cardiac targeting and therapeutic effect**
14.15 – 14.30	Eva Drmota, UKC Ljubljana, SI **Analysis of small extracellular vesicles in severe preeclampsia**
14.30 – 14.45	Maja Vodušek, UKC Ljubljana, SI **Urinary extracellular vesicle protein profiling enables identification and stratification of kidney allograft injury**
14.45 – 15.00	Boštjan Korenjak, UL ZF, SI **Determination of extracellular vesicles in canine whole blood, plasma and serum**
15.00 – 15.15	Tina Pavlin, OI, SI **Diagnostic potential of extracellular vesicles and miRNAs in advanced renal cell carcinoma**
15.15	**Closing address:** Metka Lenassi, UL MF, SI

ABSTRACTS

Plenary talks abstracts

Democratizing single EV microscopy

Nan Jiang[1], Benjamin Purnell[1], Mihajlo Radmilovic[1], Balint Beres[1,2], Mahir Patel[3], Rupangi Vasavada[4], Victoria Seewaldt[5], Lisa Feldman[6], Gagandeep Singh[7], Yue Dong[3], Tijana Jovanovic-Talisman[1]

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Introduction: Nanoscopic particles called extracellular vesicles (EVs) are abundant in biofluids and carry molecular signatures reflective of their cells of origin. Because EVs rapidly report on cellular health states, they represent promising disease biomarkers. However, EV populations are heterogeneous in size, molecular cargo, and tissue origin, making it challenging to assess EVs from subpopulations associated with the disease.

Methods: By combining affinity-based EV isolation with super-resolution fluorescence microscopy, we developed three single-EV imaging platforms that enable sensitive, multiparametric characterization of biologically relevant EV subpopulations directly from low-volume biofluid samples.

Results: Our Single Exracellular VEsicle Nanoscopy (SEVEN) platform can robustly assess individual EVs, but requires specialized equipment and fluorescent dyes. To broaden accessibility, we developed the SEVEN-Universal Protocol (SEVEN-UP) and SEVEN-Multiparametric Analysis Expansion (SEVEN-MAX) platforms, which are compatible with a wide range of microscopes and fluorophores. Integration of a user-friendly Python application leveraging GPU acceleration and machine-learning–based analysis enabled rapid data processing and enhanced overall performance.

Conclusions: SEVEN, SEVEN-UP, and SEVEN-MAX platforms robustly quantify EVs across their full biological size range with excellent cargo sensitivity. Importantly, they can enable quantification of rare, disease-enriched EV subpopulations, supporting their potential utility in biomarker discovery and precision diagnostics.

Acknowledgements: HESI Thrive, Layne Foundation, CUBRI, Circle1500, Concern Foundation, and Neuroendocrine Tumor Research Foundation.

Detection of extracellular vesicles by flow cytometry: from single to multicenter studies

Rienk Nieuwland[1]

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Introduction: Body fluids contain clinically relevant information, and for example cell concentrations are amongst the most commonly measured "*biomarkers*". The concentration of a biomarker is information that can help clinician to make a diagnosis. Question is, how does a clinician know when a measured concentration is "normal" or "abnormal"? To help clinicians, biomarkers are measured in healthy individuals and expressed as "*reference ranges*" (a.k.a. *reference interval* or *normal range*). This means that 95% of the measured biomarker values fall within this range, and are considered "normal". Importantly, reference ranges are globally standardized and independent from analysers used.

Research question: Platelets are the smallest cell (diameter 2-4 µm) present in any human body fluid. Still, the platelet reference range is firmly established, but concentration measurements of platelet-derived EVs (PEVs) and other blood cells are not. Why not?

Methods and results: I will try to explain the challenges encountered when we and others tried to establish reference ranges for blood cell-derived EVs, and how by a complex interplay between improvements of pre-analytics, knowledge exchange (e.g. Blood EV task force of ISEV), intersociety collaboration (ISEV-ISAC-ISTH), development of calibration requirements and procedures (e.g. development of stable EV test samples and suitable reference materials, software), performing interlaboratory comparison studies (to validate calibration), and by promoting transparent reporting (MiBlood-EV, MiFlowCyt-EV) and supporting education (compendium, Summerschool), all have contributed to improving comparability of measuring EVs by flow cytometry.

Conclusions: Truly understanding, optimizing and exploring flow cytometry as a reliable and robust instrument capable of measuring concentrations of EVs likely improves comparability of results between laboratories and enables establishment of clinically-relevant reference ranges.

Acknowledgements: All collaborators, including members from ISEV, ISAC, ISTH and researchers from my lab who contributed to the incredible progress together we made during the last decade.

Short talks abstracts

Coacervation - a mechanism of extracellular particle formation

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Frequently outlined mechanisms of extracellular particle (EP) formation include fragmentation in apoptosis (apoptotic bodies), vesiculation of the membrane (microvesicles), and formation in internal compartments with release (exosomes). However, there may be other mechanisms, such as fragmentation due to mechanical forces and coacervation. The question is which mechanism is relevant for a particular sample - to present representative EPs. Isolation and concentration procedures yield EPs in samples in space and time, however, the information on their sources and mechanisms is indirect. Imaging, modelling and assessment of composition are combined to reveal the mechanism of their formation. Membrane - enclosed extracellular vesicles have no internal structure and their shape is determined by the minimization of the membrane free energy. The shape of EVs is in general flaccid, as the membrane is easily bent, but not compressed or stretched. If spherical EV is subjected to volume increase, the membrane bursts, so the probability to find a population of sphere-like EVs is small. We have observed sphere-like EVs in diluted plasma, conditioned media of microalgae, and isolates from plant culture. Based on these observations and also on other experimental data, it is indicated that the shape of these particles is not determined by the membrane properties. We suggest that they are formed by a segregation mechanism called coacervation. In the lecture, an overview of the mechanisms of EP formation will be given and supported by the theoretical considerations.

Acknowledgements: ARIS P3-0388, J3-60063 and European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie Staff Exchange project "FarmEVs," grant agreement No. 101131175.

Erythrocyte Membrane–Derived Vesicles as Biomimetic Platforms for Safe and Efficient siRNA Delivery

Nina Kostevšek[1,2], Giulia Della Pelle[1,2], Tim Božič[3], Jernej Šribar[1], Boštjan Markelc[3]

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Introduction: Erythrocytes are the most abundant circulatory cells and can be isolated in large quantities, making them an attractive, low-cost source for therapeutic delivery systems. In this study, we developed a neutral siRNA delivery system using red blood cell-derived membrane vesicles (EMVs). We selected siRNA for its small size and established clinical relevance. As a proof-of-concept, we loaded anti-TdTomato siRNA into EMVs and demonstrated their safety, efficiency, and affordability in vitro and in vivo using a melanoma model (1).

Methods: We optimized EMV purification and storage protocols. Vesicle morphology was characterized by TEM and cryo-TEM. siRNA-loaded EMVs were assessed for nuclease resistance, release kinetics, and RNA interference activity *in vitro*. For *in vivo* evaluation, Cy5-labeled siRNA-EMVs were further stained with Vybrant-DiO and injected into tumor-bearing mice (1.5 mg/kg; n=19). Mice were sacrificed at 2, 24, and 48 hours post-injection. Blood and major organs were collected for confocal imaging and RNA quantification (RT-PCR).

Results: siRNA was successfully encapsulated within EMVs, as confirmed by freeze-fracture TEM and STED confocal microscopy. The vesicles protected siRNA from RNAse A degradation and achieved ~80% gene silencing at just 0.3 nM in vitro—outperforming HEK293 and Neuro2a-derived EVs. Efficacy was consistent across various cell lines (CT26, B16F10, NHLF), suggesting a copy number–dependent mechanism. In vivo, siRNA-EMVs achieved ~60% silencing of TdTomato expression in melanoma-bearing mice and remained detectable in circulation 48 hours post-injection.
Conclusions: We developed a scalable, low-cost, and effective siRNA delivery platform based on erythrocyte membranes. Future directions include tumor-targeted delivery and the co-loading of imaging agents for theranostic applications.

Acknowledgements: The study was supported by the Slovenian Research Agency ARIS (program numbers P2-0084, P3-0003, project numbers PR-1290, J4-50150 and J4-70159).

References:

(1) Giulia Della Pelle, et al. Red blood cell membrane vesicles for siRNA delivery: A biocompatible carrier with passive tumor targeting and prolonged plasma residency. *International journal of nanomedicine*, 2025, 20, 3269-3301. DOI: 10.2147/IJN.S504644.

Characterization of extracellular vesicles carrying HIV-1 Nef: insights into inflammatory cargo

Samuel Žvanut[1], Teja Lavrin[1], Marija Holcar[1], Valentina Levak[2,3], Aleksandra Usenik[4,5], Magda Tušek Žnidarič[2], Dušan Turk[4,5], Metka Lenassi[1]

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Introduction: Neuroinflammation sustained by microglial HIV-1 reservoirs is increasingly recognized as a contributing factor to HIV-1-associated neurocognitive disorders (HAND), which persist despite effective antiretroviral therapy. Extracellular vesicles (EVs) released from microglia expressing the HIV-1 protein Nef may contribute to neuroinflammatory processes through their associated inflammatory cargo. Here, we developed an integrated experimental toolbox to characterize extracellular vesicles released from Nef.GFP-expressing microglia (Nef-EVs), with a particular focus on their inflammatory profile.

Methods: To this end, we established a model of microglial HIV reservoir by integrating Nef.GFP construct into immortalized microglia by lentiviral transduction. We developed an in house nanobody-based ELISA (Nef nanoELISA) to quantify Nef release into cell culture media. Further, we subjected the media to differential ultracentrifugation (dUC) and iodixanol density gradient centrifugation (dUC-DG) for EV enrichment. EV-enriched samples were further characterized for particle concentration and size using NTA and nano-flow cytometry (nFC), Nef concentration (Nef nanoELISA) and proportion of GFP+ particles (nFC). To determine the type of Nef-EV association, the samples were analyzed by Nef nanoELISA and transmission electron microscopy (TEM) in the absence and presence of detergent. Lastly, we profiled the inflammatory factors associated with Nef-EVs by semi-quantitative Proteome Profiler Human Cytokine Array Kit. Specifically enriched factors were further analyzed by nFC.

Results: Nef concentration in conditioned media strongly correlated with the proportion of Nef-EGFP–expressing microglia. Following EV enrichment and density gradient separation, Nef localized to discrete iodixanol fractions enriched in EV markers, supporting its association with extracellular vesicles. Detergent permeabilization increased Nef detection by 77.13% in conditioned medium and by 88.94% in EV-enriched preparations, indicating a predominantly membrane-protected topology of extracellular Nef, further confirmed by immunogold TEM. Nano-flow cytometry identified distinct GFP-positive EV populations released from Nef.GFP-expressing microglia. Semi-quantitative inflammatory profiling revealed altered levels of MIF, Serpin E1, CXCL12, and ICAM-1 in Nef-EVs compared with control EVs, with ICAM-1 enrichment additionally confirmed at the single-vesicle level by nano-flow cytometry.

Conclusions: We provide a detailed characterization of Nef-containing EVs from microglia, establishing a foundation for future studies on their potential role in neuroinflammation and HAND.

Acknowledgements: The study was supported by research grant P1-0170 and young researcher grant funded by Slovenian Research and Innovation Agency.

Lipid droplet metabolism shapes the lipid landscape to control organelle integrity and ferroptosis sensitivity

Leja Perne[1,2], Špela Koren[1,2], Eva Jarc Jovičić[1], Ana Kump[1,2], Kristyna Brejchova[3], Michele Wölk[4], Ondrej Kuda[3], Maria Fedorova[4], Toni Petan[1]

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Introduction: Lipid droplets are cytosolic fat storage organelles with a central role in lipid metabolism and cellular stress responses. They are formed within the endoplasmic reticulum membrane, integrating intracellular and extracellular lipid sources to synthesize inert neutral lipids that are stored within their protective core. Lipid droplets are continuously formed and degraded, dynamically exchanging lipids and proteins through interactions with other organelles. In cancer, lipid droplets are emerging as key organelles contributing to metabolic and oxidative stress tolerance, as well as therapy resistance. However, how lipid droplets balance lipid distribution among essential cellular processes, such as membrane biogenesis, organelle homeostasis and energy production, remains unclear.

Methods: To explore lipid droplet function across metabolic states and stress conditions in human cancer cells, we have generated cellular models reflecting distinct nutrient and redox imbalances. Using genetic and pharmacological approaches, we modulate lipid droplet metabolism together with autophagy, mitochondrial metabolism and membrane oxidation to disentangle key pathways responsible for lipid flux control under stress. By combining advanced lipidomics, live-cell confocal imaging, and functional assays, we investigate lipid droplet-mediated lipidome remodeling, organelle dynamics, and cell death pathways.

Results: Our findings reveal that lipid droplets support membrane and mitochondrial homeostasis and restrain ferroptosis, a form of iron- and lipid peroxidation-dependent programmed cell death with growing relevance for cancer therapy. We uncover how lipid droplet biogenesis and breakdown mechanisms shape ferroptosis susceptibility by regulating the intracellular trafficking of oxidation-prone polyunsaturated fatty acids. Importantly, we demonstrate that the balance between survival and death in aggressive cancer cells shifts depending on extracellular lipid availability and lipid droplet function, creating unexpected windows of vulnerability and resistance.

Conclusions: Our work highlights lipid droplets as key regulators of the lipid landscape that underlies membrane and organelle homeostasis during stress and determines cancer cell sensitivity to ferroptotic cell death.

Gastrin modulates sEV miRNA cargo and promotes sEV-mediated proliferation in gastric cancer cells

Pia Pužar Dominkuš[1,5], Vesna Bricman[2], Alja Zottel[3,5], Nataša Resnik[4], Petra Hudler[5]

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Introduction: Gastrin is a gastrointestinal hormone that regulates gastric acid secretion and maintains gastric mucosal homeostasis. Elevated plasma gastrin levels are associated with increased gastric cancer risk and tumour progression. Gastrin may also alter small extracellular vesicle (sEV) cargo, promoting carcinogenesis. We aimed to investigate the effects of gastrin on sEV miRNA cargo and function in gastric cancer cells.

Methods: We treated MKN45 gastric cancer cell line with gastrin, isolated sEVs by ultracentrifugation, and characterised them by western blotting, transmission electron microscopy, and nanoparticle tracking analysis. We isolated total RNA from treated and untreated cells for miRNA sequencing and validated candidate miRNAs in sEVs by qPCR. We conducted *in silico* expression, survival, Gene Ontology and pathway enrichment analysis of differentially expressed miRNAs, using Kaplan-Meier plotter, UALCAN (TCGA stomach adenocarcinoma cohort), and STRING tools. Functional effects of gastrin-induced sEVs were assessed in MKN45 and normal epithelial MCF10A cell lines using cell proliferation and migration assays.

Results: Isolated sEVs showed a characteristic cup-shaped morphology and measured 182.4 nm in diameter. They were enriched in the endosomal marker proteins Alix and CD9. Fifty-three miRNAs were differentially expressed in treated compared to untreated cells. Thirteen miRNAs ($log_2FC \geq 2.0$ or ≤ -2.0) were selected for further analysis in sEVs. MiR-199a-3p was under-expressed in gastrin-induced sEVs compared to control (FC=−1.735, p=0.0242) and has been associated with primary tumours (FC=1.51, $p=1.626\times10^{-12}$) and poorer overall survival (HR=1.47, p=0.028) in the TCGA stomach adenocarcinoma cohort. This miRNA regulates target genes involved in cancer, cell proliferation, and migration, according to enrichment analysis. Gastrin-induced sEVs increased the proliferation of naïve gastric cancer cells (FC=1.29, p=0.046) but did not affect cell migration.

Conclusions: Gastrin modulates miRNA expression in gastric cancer cells, decreases miR-199a-3p levels in sEVs, and promotes sEV-mediated cell proliferation.

Acknowledgements: This research was supported by the ARIS programme grant P1-0390.

Extracellular vesicles attached to a responsive transporter boosts cardiac targeting and therapeutic effect

Carlos Jesus[1,2,3,4], Andreia Vilaça[2,3], Arnab Banerjee[2,3], Mohamed Bellahcene[4], Marta Barão[2,3,5], Andrea Cafarelli[6], Liliana Moreira-Costa[7], Pedro Mendes-Ferreira[7], José Sereno[8,9], Leonardo Ricotti[6], Antero Abrunhosa[8,9,10], Costanza Emanueli[4], Hugo Fernandes[2,3,11], Lino Ferreira[1,2,3*]

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Introduction: Extracellular vesicles (EVs), small endogenous, lipid-bilayer membrane compartments involved in intercellular communication and containing bioactive miRNAs and proteins, have been widely used in preclinical studies and, more recently, in clinical trials for the treatment of acute myocardial infarction (AMI). Although targeting strategies have been developed to enhance myocardial accumulation following intravenous administration, limitations remain regarding cardiac retention and delivery to ischemic cardiomyocytes.

Methods: Here, we developed a platform designed to increase EV retention in the heart after intravenous injection by coupling EVs to a transporter via a specific linker and subsequently releasing them at the myocardium through the application of a cardiac-focused external stimulus.

Results: This study showed a remarkable increase in the EV cardiac accumulation in both healthy and AMI mice models (9- and 4.5-fold, respectively) using this technology. Further, a single dose treatment of this technology led to significant improvements in cardiac function in an AMI mice model. Lastly, miR-199a-3p was loaded into EVs using a post-isolation EV engineering strategy and was conjugated to the responsive transporter, which led to a 2-fold increase of miRNA delivery to the heart of pig model upon cardiac treatment with the external stimulus.

Conclusions: The tool developed within this work represents a significant advancement in the field of cardiac nanomedicine and EV delivery and therapeutic fields, offering a highly effective and versatile technology for the treatment of the ischemic heart upon infarction, with the capacity to be easily adapted to other diseases.

Acknowledgements: The authors would like to acknowledge the funding by the FCT PhD Studentship to Carlos Jesus (SFRH/BD/144092/2019); travelling fellowship from the Company of Biologists to Carlos Jesus (DMMTF2208814); MIA-Portugal (European Union's Horizon 2020 No 857524) (Hugo Fernandes); Programa Operacional Competividade e Internacionalização (POCI) na sua componente FEDER e pelo orçamento da Fundação para a Ciência e a Tecnologia na sua componente OE (Project 2022.03308.PTDC; 2022.07615.PTDC; 2022.02803.PTDC); EC projects REBORN (Ref. 101091852); PRR project HfPT - Health from Portugal (Ref: 02/C05-i01.01/2022.PC644937233-00000047) and "Project RESET_BONE_AGEING_2" (Ref: COMPETE2030-FEDER-01175000).

Analysis of small extracellular vesicles in severe preeclampsia

Eva Drmota[1], Katja Goričar[3], Metka Lenassi[3], Miha Lučovnik[1,2]

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Introduction: Severe preeclampsia is a life-threatening pregnancy disorder characterized by endothelial dysfunction and placental pathology. Small extracellular vesicles (sEVs) are important mediators of intercellular communication and may contribute to disease pathophysiology. However, data on sEVs in severe preeclampsia remain limited.

Methods: This case–control study included women with severe features of preeclampsia (n = 17), healthy pregnant controls (n = 30), and healthy non-pregnant women (n = 30). Cord blood plasma samples were also collected from preeclamptic (n = 17) and control pregnancies (n = 30). sEVs were isolated from plasma by ultracentrifugation over sucrose cushion and analysed using nanoparticle tracking analysis (NTA) to determine concentration and size distribution. Profiling of 37 surface proteins was performed using a bead-based multiplex flow cytometry assay (MACSPlex). Group differences in sEV concentration, size and surface proteins were assessed.

Results: Maternal sEV concentration differed significantly between groups, with higher levels in severe preeclampsia compared to healthy pregnant controls (Padj = 0.034) and non-pregnant women (Padj < 0.001), while no difference was observed between healthy pregnant and non-pregnant groups (Padj = 0.127). Cord blood sEV concentration was higher in preeclampsia compared to controls ($p < 0.001$). No significant differences in sEV size were observed between groups. Statistical analysis of EV surface proteins is ongoing.

Conclusions: Severe preeclampsia is associated with increased sEV concentration in both maternal and fetal circulation, without changes in vesicle size. The lack of difference between healthy pregnant and non-pregnant women suggests a disease-specific effect, supporting the potential role of sEVs as biomarkers of severe preeclampsia.

Acknowledgements: The authors thank all participants and the clinical and laboratory staff for their support in sample collection and processing.

Urinary extracellular vesicle protein profiling enables identification and stratification of kidney allograft injury

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Introduction: Early and non-invasive detection of kidney allograft injury remains a major challenge, as conventional clinical biomarkers often fail to identify subclinical damage. Urinary extracellular vesicles (uEVs), largely originating from the kidney, represent a promising source of biomarkers reflecting ongoing pathophysiological processes within the allograft. We investigated uEV surface proteins as potential biomarkers for differentiating kidney allograft injury phenotypes.

Methods: Thirty-eight kidney transplant recipients underwent protocol or indication biopsy at a median of 942 days after transplantation. Patients were classified into kidney injury (KI; n = 28, 74%) or normal histology (NH; n = 10, 26%) groups. KI cases included recurrent glomerulonephritis (rGN; n = 10), BK virus nephropathy (BKVN; n = 8), and chronic antibody-mediated rejection (cABMR; n = 10). uEVs were isolated from 20 mL of second-morning urine and analyzed by nano-flow cytometry after staining with antibodies targeting nephron segment markers (proximal tubule - CD13, distal tubule - CD24, podocyte - PODXL, collecting duct - AQP2), endothelial cells (CD31, CD90), and urothelial cells (UPIIIa). In addition, tetraspanin-positive EVs were characterized using the MACSPlex Exosome Kit (Miltenyi Biotec).

Results: Compared with NH, KI group showed significant differences in the proportion of endothelial- ($p = 0.003$) and urothelial-positive uEVs ($p = 0.0009$), as well as in normalized concentrations of distal tubule- ($p = 0.0427$) and urothelial-positive uEVs ($p = 0.0163$). Tetraspanin-positive EV analysis further revealed significant differences in leukocyte-associated markers (CD4, CD8, CD14, CD19, CD45, CD56), leukocyte activation markers (CD40, CD69), antigen presentation markers (HLA-ABC, HLA-DRDPDQ), platelet marker CD62P, endothelial markers (CD31, CD105), and tubular marker CD326 (all $p < 0.05$). Several markers also discriminated between individual KI subgroups.

Conclusions: uEV surface protein profiles differed between patients with normal histology and kidney allograft injury, with several marker groups showing potential to distinguish specific injury phenotypes. These exploratory findings support further validation of uEV profiling as a non-invasive approach for detecting and stratifying kidney allograft injury.

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Determination of extracellular vesicles in canine whole blood, plasma and serum

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Introduction: Extracellular vesicles (EVs) are mediators of intercellular communication by exchanging the material and information within cellular fragments. EVs are the subject of increasing interest as they are considered potential indicators of the body's response to the disease and effectors in systemic metabolic regulation. To better understand the underlying mechanisms, large population and clinical studies should be performed which requires simple and high-throughput methods.

Methods: We analysed 200 samples of diluted blood, 250 samples of plasma and 150 samples of serum from 190 dogs treated at the Small Animal Clinic, Veterinary Faculty, University of Ljubljana. The samples were diluted with physiological solution. The number density and hydrodynamic diameter was assessed by Interferometric Light Microscopy (ILM), and protein and nucleic acid content given by the ratio of absorbances at 280 nm and 260 nm (A280/A260) was assessed by UV-visual spectroscopy.

Results: The average number density of EVs in whole blood was $(28.48\pm37.27)\times10^9$/mL, in plasma $(34.66\pm37.81)\times10^9$/mL, and in serum $(33.19\pm40.04)\times10^9$/mL. The average hydrodynamic diameter in blood was (168 ± 39)nm, in plasma (154 ± 42)nm and in serum (156 ± 41)nm. We found statistically highly significant positive correlations ($p<10^{-4}$) between number densities of EVs in blood and plasma, and plasma and serum, but not between blood and serum. We found statistically highly significant correlations ($p<10^{-8}$) between hydrodynamic diameter of all three sample populations. We found statistically highly significant negative correlation between the number density of EVs in blood and A280/A260 in blood ($p<10^{-8}$), but not in plasma and serum.

Conclusions: ILM yielded similar number average densities and hydrodynamic diameters of EVs in plasma and serum, but standard deviations of the number density were comparable to measurement values themselves. The protocol for sample preparation and dilution for ILM should therefore be further elaborated.

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Diagnostic potential of extracellular vesicles and miRNAs in advanced renal cell carcinoma

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Introduction: Renal cell carcinoma is diagnosed at an advanced stage in approximately one third of patients and is associated with an unfavourable prognosis. Immune checkpoint inhibitors are now standard treatment; however, reliable biomarkers are needed to identify patients most likely to benefit. We investigated the association between extracellular vesicle (EV) characteristics, EV miRNA expression in blood and urine, and treatment outcomes during the first 16 weeks of immunotherapy.

Methods: This prospective study included patients with advanced renal cell carcinoma treated with first- or second-line immunotherapy. During the first 16 weeks, four blood samples and two urine samples were collected. Treatment response was assessed using CT scans. EVs were isolated from urine by size-exclusion chromatography and from plasma by ultracentrifugation. EV concentration and size were determined by nanoparticle tracking analysis. MiRNAs were isolated, transcribed to cDNA, and analysed using quantitative PCR.

Results: Fifty-nine patients were included; mean age was 66.3 years, 48 were male and 11 female. According to International Metastatic Renal Cell Carcinoma Database Consortium risk groups, 13 patients had favourable-risk, 35 intermediate-risk, and 11 poor-risk disease. Mean EV concentration was 1.28×10^9 EV/mL in plasma and 1.64×10^9 EV/mL in urine, with mean vesicle size 185.9 nm and 159.9 nm, respectively. Baseline plasma EV concentration differed across prognostic groups, with highest values in the poor-risk group, but without statistically significant correlation. However, longitudinal EV concentration changes were not significantly associated with CT response or adverse effects. Fifteen candidate EV miRNAs were tested, and eight were selected for final analysis, which is ongoing.

Conclusions: Plasma EV concentration may reflect baseline prognostic risk and early treatment dynamics, but EV concentration alone was not a consistent biomarker of treatment response or toxicity. Final miRNA analyses are needed to determine whether EV-derived miRNAs provide stronger predictive value.

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Posters abstracts

Surface binding mechanisms of extracellular vesicles and model liposomes under flow conditions

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Introduction: Our group investigates molecular interactions at biological interfaces, including those that govern the behaviour and function of vesicles. This study investigates the binding behaviour of EVs and liposomes under controlled physicochemical conditions, with emphasis on distinguishing specific marker-mediated recognition from non-specific membrane interactions.

Methods: Surface plasmon resonance (SPR) was used to compare vesicle binding to immobilised antibodies. Anti-CD81 was selected as a positive EV marker, while anti-Calnexin was used as a negative reference. EVs were isolated from Jurkat cells, and they were compared with liposomes POPC:Chol:SM (60:20:20).

Results: SPR measurements showed that EVs preferentially bound to anti-CD81 and formed a stable interaction, consistent with the presence of CD81 on the EV surface. In contrast, EVs showed negligible binding to anti-Calnexin, supporting the specificity of the observed anti-CD81 response. Liposomes displayed a different behaviour: they interacted with both anti-CD81 and anti-Calnexin surfaces, but the binding was transient and did not remain stable after the flow conditions were changed. This suggests that liposomes mainly undergo non-specific or weak surface interactions rather than marker-specific recognition.

Conclusion: EVs show stable, marker-dependent attachment to anti-CD81, whereas liposomes interact more broadly and reversibly with antibody-coated surfaces. These findings support further investigation of molecular interactions at biological interfaces, including those governing vesicle behaviour, by combining SPR with Prometheus Panta-based stability analysis under physiologically relevant flow, pH, and ionic strength conditions.

Plasma extracellular vesicles as non-invasive biomarkers of kidney allograft injury

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Introduction: Detection of kidney allograft injury is challenging, as needle biopsy is invasive and traditionally used biomarkers are nonspecific and may miss subclinical injury. Extracellular vesicles (EVs) are released by all cell types and circulate in body fluids, including plasma. Previous research has shown that urinary EVs can reflect kidney allograft status. We evaluated whether kidney-specific EVs can be detected in plasma and whether the concentration of EVs with specific surface markers and their size reflect the kidney allograft status.

Methods: Plasma samples from 37 kidney transplant recipients were analysed. Patients were classified based on Banff and MMDx criteria as having normal histology (NH; n=10) or kidney injury (KI; n=27), and the later were further stratified into chronic antibody-mediated rejection (cABMR; n=10), recurrent glomerulonephritis (rGN; n=10), and BK virus nephropathy (BKVN; n=7) subgroups. EVs were isolated from 900 µL plasma by sucrose cushion ultracentrifugation as described previously. Next, EVs were labelled with antibodies against renal structure-associated markers: podocalyxin for podocytes, aquaporine 2 for collecting ducts, CD13 for proximal tubule, and CD24 for distal tubule, and their concentration measured with nano-flow cytometry.

Results: Total EV concentration did not differ significantly between NH and KI groups ($p = 0.606$) or among the four diagnostic injury groups ($p = 0.503$). However, the median EV size was significantly higher in KI than in NH group (73.20 vs. 71.20 nm, $p = 0.045$), with a significant difference also observed across the four groups ($p = 0.027$). Analyses of kidney-marker-positive EV subsets are ongoing.

Conclusions: Plasma EVs can be successfully isolated and characterized in kidney transplant recipients. Although total EV concentration did not distinguish between injury phenotypes, plasma EV size and kidney-specific EV subpopulations may provide additional information on allograft pathology.

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Visualization of tiny particles in biological samples: from early studies to new minimally invasive methods

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Introduction: Manipulation of waves in 17th century (invention of the microscope) enabled insight into the structures that are not directly visible to human eye, however a possibility to reach the resolution that enabled observation of particles smaller than a micrometer was given by the discovery of the wave properties of matter (de Broglie, 1924) and by construction of electron microscope in the twenties and thirties of the 20th century. Images of biological samples revealed particles smaller than cells and existence of such particles could then be extrapolated also by observing samples with optical microscope.

Methods: We have observed samples from different natural and synthetic sources with Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM). For SEM, we have used a new, minimally invasive method for sample preparation. Strongly hydrophobic surface was created on the alluminia stands enabling quick drying of liquid samples which were then immediately observed in low vacuum. We paralleled our images with historical images of different types of nanoparticles from biological samples.

Results: We envisaged extracellular vesicles, apoptotic bodies, exosomes, extracellular particles engineered by microalgae and coacervates. We present the micrographs and parallel them by early results of imaging featuring the work of pioneers. Electron dense nanoglobules are abundant in samples from different natural sources and correspond to the coacervation mechanism.

Conclusions: Imaging reveals the identity of EPs and also the process of their formation. Coacervation is a common process in biological samples as evidenced in blood, microalgae and plant samples.

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Synergistic Effects of Lactobacillus salivarius and SCAP-Derived Exosomes on Osteogenic Differentiation in 3D SCAP Cultures

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Background: Regenerative approaches in bone tissue engineering increasingly rely on combinatorial strategies involving stem cell–derived extracellular vesicles and bioactive microbial stimuli. This study investigated the effects of *Lactobacillus salivarius* (*L.s.*) preconditioning and stem cells from the apical papilla (SCAP)-derived exosomes, individually and in combination, on osteogenic differentiation in a 3D scaffold model.

Methods: SCAPs were seeded onto nano-hydroxyapatite-based scaffolds and subjected to four treatment conditions: control, *L.s.* preconditioning, exosome supplementation, and combined treatment. Osteogenic differentiation was evaluated after 7 days using Alizarin Red S (ARS) staining, immunocytochemistry for osteocalcin (OCN), and gene expression analysis of key osteogenic markers (BMP4, RUNX2, ALP, and OCN).

Results: All treatments promoted osteogenic differentiation compared to control, with the most pronounced effects observed in the combined *L.s.* + exosome group. ARS staining demonstrated significantly increased calcium deposition, particularly in the combination group. Immunocytochemical analysis revealed enhanced OCN expression, with strongest signal intensity in exosome-treated and combined groups, indicating advanced osteogenic maturation. Interestingly, osteocalcin (OCN), typically considered a late osteogenic marker, was upregulated as early as day 7, suggesting an accelerated osteogenic differentiation process induced by the treatment. Gene expression analysis showed that *L.s.* preconditioning significantly upregulated early osteogenic markers (ALP, RUNX2, BMP4), while the addition of exosomes did not result in further statistically significant increases in these genes. A trend toward higher OCN expression was observed in the combined treatment.

Conclusion: *L. salivarius* and SCAP-derived exosomes exert complementary effects on osteogenesis in 3D cultures, with a synergistic impact primarily reflected at the level of mineralization and osteogenic maturation. These findings support their combined application as a promising strategy for bone regeneration.

Influence of Isolation Methods on the Characteristics of Mesenchymal Stromal Cell-Derived Extracellular Vesicles

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Introduction: Mesenchymal stromal cell (MSC)-derived extracellular vesicles (MSC-EVs) have emerged as promising mediators of intercellular communication and potential therapeutic agents due to their immunomodulatory and regenerative properties. However, the heterogeneity of EVs (their cellular origin, size, and molecular cargo), as well as the lack standardised isolation protocols, remain major challenges limiting their clinical application. The aim of this study was to compare the yield and the concentration of specific molecules in MSC-EVs from three different tissue sources (umbilical cord, adipose tissue, and bone marrow) using three isolation approaches.

Methods: MSCs derived from all three tissue sources were cultured under standardised conditions and maintained in serum-free medium for 48 h after reaching 70% confluence. Conditioned media were collected and stored at −80 °C until isolation. MSC-EVs were isolated using three methods: single ultracentrifugation, additional ultracentrifugation on an OptiPrep density gradient, and size-exclusion chromatography. Isolated MSC-EVs were characterised by imaging flow cytometry to determine EV concentration with specific marker expression (CD9, CD81, CD63, CD73, CD90, and CD146). Molecular cargo profiling (BDNF, CNTF, EGF, GDNF, IDO, IL-6, IL-7, IL-8 (CXCL8), IL-10, NGF beta, PDGF-BB, VEGF-A) was performed using ProcartaPlex assays on Luminex technology.

Results: Imaging flow cytometry enabled the MSC-EVs quantification and characterization. Preliminary results showed variability in EV concentration depending on the isolation method, while molecular cargo profiling indicated that tissue source and isolation method both affected the concentration of some MSC-associated biomolecules.

Conclusions: The results highlight the influence of both biological origin and isolation strategy on the composition of MSC-EVs, which may help improve standardisation of MSC-EV production and further support the development of EV-based therapeutics.

Microglial Nef-containing extracellular vesicles uptake monitoring by fluorescent microscopy and live cell imaging

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Introduction: Despite effective combination antiretroviral therapy, 20–50% of people living with HIV continue to develop HIV-associated neurocognitive disorders (HAND). Persistent, partially active viral reservoirs in microglial cells are believed to contribute to this pathology. Microglia were shown to express the HIV protein Nef, which was shown to contribute to HAND. Nef was found to be present in released microglial extracellular vesicles (Nef-EVs), enabling it to affect neighboring cells. However, the mechanisms governing Nef-EVs transport and endocytic uptake by recipient cells remain insufficiently defined.

Methods: Human microglial crude EVs, containing recombinant Nef-EGFP fusion protein (Nef-EVs), were enriched using ultracentrifugation and characterized by nanoparticle tracking analysis and nano-flow cytometry (Nano-FC). Nef-EVs were added to adherent HeLa and microglial HTHU immortalized human cell lines. Nef-EVs uptake by treated cells was monitored up to 20h using two live-cell imaging systems and live confocal microscopy. Additional samples were fixed on glass slides with 4 % paraformaldehyde at the 2-hour time point and examined by fluorescence microscopy. When required, EVs and/or cells were labelled with fluorescent dyes (MemGlow, ExoBrite, Apotracker, DiI, DAPI) following manufacturers' instructions.

Results: Nano-FC confirmed high levels of stained EVs with MemGlow, ExoBrite and Apotracker, however fluorescent signals did not always colocalize with EGFP on micrographs. The most intensive Nef-EVs fluorescence was observed between 30 min and 4 h after the treatment in both HeLa and microglial HTHU cell lines. Confocal microscopy further verified internalization of Nef-EGFP into cells of both lines, with intracellular EGFP signal persisting for several hours.

Conclusions: Using live cell imaging and fluorescence microscopy, we successfully monitored the uptake of microglial Nef-EVs by HeLa and HTHU cells. Live confocal imaging confirmed that Nef-EGFP is internalized and remains detectable within recipient cells.

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Comprehensive molecular and functional characterization of Nef-containing EVs released by human microglia: implications for HAND

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Introduction: HIV-1 infection remains associated with comorbidities, despite effective combination antiretroviral therapy. Importantly, HIV-associated neurocognitive disorders (HAND) affect 20–50 % of infected treated individuals, likely due to chronic neuroinflammation. Still underresearched contributors to neuroinflammation are defective but »active« microglial HIV reservoirs, which were shown to express viral proteins like Nef even under complete viral suppression. In this study, we aimed to characterize human microglia (h-microglia) release of viral Nef into the extracellular space (Nef-EVs) and investigate their impact on human neural progenitor cells (hNPC) during differentiation into astrocytes.

Methods: We established a h-microglia model with stably integrated Nef.GFP transgene under doxycycline (DOX)-inducible promoter. Small EVs were isolated from conditioned media by sequential centrifugation and purified using iodixanol density gradient. EV yield, size distribution, protein composition, and Nef.GFP content were analyzed using NTA, nano-flow cytometry, immunogold TEM, immunoblotting, and an in-house *nano*-Nef ELISA. For functional assays, hNPCs were exposed to EVs derived from Nef-expressing or GFP-control cells, as well as recombinant Nef, while inducing differentiation with CNTF. Cellular responses were assessed by measuring metabolic activity, proliferation, cell death, and lineage marker expression.

Results: DOX-induced h-microglia culture expressed Nef.GFP in over 95 % of cells at 48 h, which retained high cell viability (>95 %). Nef.GFP expression significantly increased EV release up to 4.5-fold compared to GFP controls, with comparable size distributions (157.2 ± 6.3 nm vs. 144.9 ± 6.1 nm). A substantial fraction of Nef-EVs (45.5 ± 15.8 %) contained Nef.GFP detectable with nano-flow cytometry, which was packed inside of EVs as shown by immuno-TEM. *nano*-Nef ELISA further quantified an average of 97.2 Nef.GFP molecules per individual EV. Nef-positive EVs were enriched in raft-associated lipids and canonical EV proteins and enriched in fractions characteristic of small EVs. Functionally, microglia-derived Nef-EVs modulated hNPC behavior, promoting differentiation towards an astrocytic phenotype and altering proliferation and metabolic activity, without inducing apoptosis or necrosis.

Conclusion: HIV-1 Nef promotes the release of small EVs in h-microglia carrying intraluminal Nef. These Nef-containing EVs modulate hNPC differentiation toward astrocytes and alter their viability, supporting the potential contribution of Nef to chronic neuroinflammation and HAND pathogenesis.

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Influence of radiotherapy on cytokine profile in plasma extracellular vesicles in DCIS patients

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Introduction: Ductal carcinoma *in situ* (DCIS) is a non-invasive form of breast cancer treated with surgery followed by radiotherapy (RT). RT induces both direct and indirect cellular effects, including DNA damage and oxidative stress, which activates signalling pathways involved in inflammation and the release of extracellular vesicles (EVs). EVs are key mediators of intercellular communication and RT-induced systemic effects. The aim of this study was to isolate plasma EVs with two different methods and characterize their protein profile in patients with DCIS during longitudinal follow up, with the objective of assessing whether changes in EVs protein profiles may be associated with RT-induced adverse events.

Methods: This study will include DCIS patients treated with adjuvant RT. EVs will be isolated from plasma using two methods: magnetic bead separation and ultracentrifugation on a sucrose gradient. For analysis of size and distribution of EVs in plasma we will do nanoparticle tracking analysis (NTA). The EVs protein profile will be analysed using enzyme-linked immunosorbent assay (ELISA), with a focus on inflammatory cytokines.

Results: The results will provide insight into the impact of RT on EVs, including changes in their size and distribution across all time points (before RT, after RT, at 6 months, and at 2-year follow-up). In addition, the study will characterize RT-associated changes in EVs inflammatory protein profiles. These findings will allow assessment of longitudinal changes in EV-associated biomarkers in response to RT and their potential association with RT-induced adverse events.

Conclusion: Evaluation of inflammatory cytokines in EVs will contribute to better understanding of biological processes involved in the response to RT, as well as the development of RT-induced adverse events in patients with DCIS.

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Inflammatory profiling of extracellular vesicles released from human microglia expressing viral protein Nef

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Introduction: HIV-1 infection is associated with neurocognitive disorders driven by neuroinflammation and microglial senescence. Extracellular vesicles (EVs) released from microglia expressing viral protein Nef (Nef-EVs) may contribute to these processes, yet their inflammatory composition remains unclear. The aim of our study is to characterize the inflammatory profile of Nef-EVs and to evaluate how different EV isolation methods influence its composition.

Methods: EVs were isolated from human microglial cell line expressing Nef fused with enhanced green fluorescent protein (Nef.EGFP) under doxycycline-inducible promoter using differential ultracentrifugation (dUC), size exclusion chromatography (SEC), and ultracentrifugation in combination with density gradient (UC-DG). The proportion of EGFP-positive cells was quantified by flow cytometry. EV characterization was performed by immunoblotting, nanoparticle tracking analysis (NTA) and nano-flow cytometry (nano-FC). The inflammatory profile was assessed using semi-quantitative immune-based cytokine array.

Results: Flow cytometry analysis showed high proportion of EGFP-positive events (>90 %), suggesting stable expression of Nef.EGFP in microglia cultured in the presence of doxycycline. Successful EV isolation from culture media by all three methods was confirmed by the presence of CD9 and absence of calnexin as shown by immunoblotting. Nef-EGFP was detected in EVs derived from Nef-expressing cells as revealed by nano-FC and immunoblotting, confirming Nef association with EVs. SEC and UC-DG fractions enriched in EVs were identified, though EV and protein yield of SEC-isolated EVs varied depending on membrane cut-off during concentration step. Cytokine array analysis of Nef-EV samples isolated by dUC revealed distinct increases in specific inflammatory factors, notably Nexin and ICAM-1, compared to controls (EV samples from microglia expressing EGFP). Upcoming experiments will include the inflammatory profiling of EVs isolated by SEC or DG-UC methods for comparison.

Conclusions: Nef-EVs exhibit a distinct inflammatory signature compared to control EVs, suggesting role of Nef in determining inflammatory EV cargo. Further studies will examine whether the choice of isolation method significantly affects the observed EV inflammatory profile, as well as inflammatory factor localization. These findings may improve understanding of HIV-1–related neuroinflammation and senescence induced by Nef-EVs.

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Optimization of extracellular vesicle isolation from recombinant *Lactococcus cremoris*

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Introduction: *Lactococcus cremoris* is a well-established Gram-positive bacterium widely used as a platform for recombinant protein expression. Recombinant *L. cremoris* represents a promising platform for the production of engineered extracellular vesicles enriched with functional and bioactive proteins. However, efficient and reproducible isolation of EVs from *L. cremoris* remains a critical bottleneck, with current methods often yielding low recovery and poor scalability. In this study, we aimed to optimize the isolation of EVs from *L. cremoris*, focusing on improving yield and reproducibility through evaluation of different isolation strategies.

Methods: Two approaches for EVs isolation were compared, namely a standard ultracentrifugation protocol (130.000 × *g*, 2 h), and tangential flow filtration (TFF) combined with size-exclusion chromatography (SEC). To assess factors influencing EV production, *L. cremoris* cultures were grown under varying conditions, including different stress stimuli and cultivation volumes. EVs were isolated during different bacterial growth phases. Obtained EVs were systematically compared using flow cytometry to assess particle-associated signals and relative abundance.

Results: Systematic variation of growth conditions and downstream processing steps demonstrated that growth conditions only slightly influence EV production. On the contrary, classical ultracentrifugation resulted in higher and more reproducible vesicle recovery compared to TFF/SEC.

Conclusions: Our study confirms the potential of using *L. cremoris* for the production of EVs highlighting the importance of establishing an optimal workflow for EVs isolation. These advances contribute to the development of Gram-positive EV-based platforms for biotechnological and therapeutic applications.

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Natural Nanocarriers from Bovine Milk: Small Extracellular Vesicles for Nutraceutical Delivery

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Introduction: Extracellular vesicles (EVs) are membrane-bound nanoparticles involved in intercellular communication and are increasingly studied as natural drug delivery systems due to their biocompatibility and stability. Bovine milk represents a scalable and cost-effective source of EVs. Oleuropein aglycone (OA), a bioactive compound from olive plants, exhibits antioxidant and anti-obesity properties but suffers from low bioavailability due to extensive metabolic degradation. This study investigates bovine milk-derived EVs as carriers for OA, comparing loading strategies and evaluating their *in vitro* effects on fat cells.

Methods: EVs were isolated from skimmed milk *via* differential centrifugation, casein removal, and ultracentrifugation. Morphology was assessed by TEM, while size and concentration were analysed by NTA. EV identity was confirmed by Western blotting. OA was loaded using sonication or passive incubation, and encapsulation efficiency (EE%) was measured by UV-VIS spectroscopy. Differentiated murine 3T3-L1 cells were used to assess lipogenesis and lipolysis.

Results: EVs retained typical morphology after loading, with a slight size increase, more evident in passively loaded samples, indicating successful cargo incorporation. NTA confirmed a shift toward larger particle sizes in loaded EVs. EE % was significantly higher with overnight passive loading and sonication compared to short passive incubation. EVs were efficiently internalised by cells. EV-mediated OA delivery reduced cell viability at higher concentrations, depending on the loading method. Passively loaded EVs showed lower lipid accumulation, significantly reducing intracellular triglycerides at all doses, while sonicated EVs had weaker, non-dose-dependent effects. In lipolysis assays, sonicated EVs reduced glycerol release, whereas passively loaded EVs maintained or slightly increased lipolysis.

Conclusions: OA delivery *via* EVs is strongly influenced by the loading strategy. Passive loading enhances functional efficacy, reducing triglyceride accumulation while preserving lipolysis, whereas sonication results in weaker metabolic effects.

Comparison of concentration and size of extracellular vesicles after electroporation with 100 µs and bipolar pulses

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Introduction: Electroporation is a technique in which short electric pulses increase cell membrane permeability. It is used in medical applications like electrochemotherapy (ECT) and pulsed electric field (PEF) ablation. Depending on pulse parameters, electroporation can be reversible or irreversible; it can cause pores formation in plasmalemma, affects cell homeostasis, damages membrane lipids and proteins, activates membrane resealing processes, and can lead to cell death. These processes may also stimulate extracellular vesicle (EV) release.

Methods: We compared the concentration and size of EVs released from Chinese hamster ovary (CHO) cells 2 and 4 hours after electroporation using two protocols: ECT (eight 100 µs pulses, 1 Hz) and PEF (50 bursts of 50 bipolar 2 µs pulses). EV size distribution and concentration were measured by nanoparticle tracking analysis (NanoSight LM10).

Results: All electroporation protocols increased EV release compared to control cells (5.74×10^6 particles/ml). The highest EV concentration was observed after PEF pulse protocol (86.56×10^6 particles/ml), significantly exceeding that after ECT (21.54×10^6 particles/ml). The concentration of released EVs using PEF protocol was significantly lower after 4 h of incubation than after 2 h. The size of EVs does not significantly differ between the protocols or the incubation times.

Conclusions: The electroporation protocol does have some effect on the concentration of EVs, implying that EVs might play a role in the results of electroporation applications. Further experiments need to be done to evaluate the size and concentration of EVs with other commonly used electroporation protocols.

Chlorella sorokiniana nanoalgosomes as sinks for heavy metals Cu and Mn

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Introduction: Microalgae and plants are the silent majority in planet resiliation mechanisms and one health concept. While extensive work on extracellular particles (EPs) was focused on mammalian sources, recently, the interest has been expanded to the fields of microorganisms and plants. Here, we report on the response of microalgae to heavy metals (Cu and Mn). We hypothesize that exogenously added metals would enter the cell where they would be packed into exosomes and released from the cell following the endosome – based process.

Methods: *Chlorella sorokiniana* cultures were treated with 1mM $MnCl_2$ and $CuCl_2$ at the early stationary phase (20 days old). During 4 days after treatment, supernatants and pellets (obtained by centrifugation) were observed using Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) with Energy Dispersive Spectroscopy (EDS). We assessed number density n and hydrodynamic diameter D_h of EPs by Interferometric Light Microscopy (ILM). We performed mass photometry and Raman spectroscopy.

Results: Metal-treated microalgae were increasingly surrounded by amorphous mucilage. SEM revealed that Electron Dense Globules (EDGs) were formed in Cu-treated samples, controls, and medium with added phosphates (without microalgae), but not in Mn-treated samples and controls. Cu content was increased in EDGs. Raman spectroscopy yielded signal of beta carotene in all samples, however, the image did not reveal localized EPs. UV-vis spectroscopy indicated poor content of proteins and nucleic acids in all samples. Mass photometry showed small amount of molecules between 100 and 200 kDa.

Conclusions: Our results considering Cu-treated samples support the assumption of accumulation of metal in EPs/EDGs. As EDGs were found in samples composed of water and phosphates, it is indicated that also in microalgae-containing samples they are not formed in the cells in the process leading to exosomes, but are rather assembled extracellularly.

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Proteomic and physicochemical analysis of erythrocyte membrane vesicles from fresh and frozen blood

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Introduction: Erythrocyte membrane vesicles (EMVs) are promising nanocarriers for drug delivery due to their biocompatibility and immune evasion through retention of erythrocyte surface proteins. Clinical translation is limited by dependence on freshly drawn blood. Long-term storage of EDTA-anticoagulated blood at −80°C without cryoprotectant is an accessible alternative, but its effect on EMV physicochemical properties and protein composition has not been investigated.

Methods: An identical preparation protocol, hypotonic lysis, sonication, sequential membrane extrusion, and ultracentrifugation, was applied to both fresh and frozen blood. Resulting fractions were characterized by dynamic light scattering, nanoflow cytometry, cryo-TEM, negative staining TEM, SDS-PAGE, and quantitative mass spectrometry-based proteomics.

Results: EMV size, surface charge, and morphology did not differ significantly between fresh and frozen preparations. Extrusion normalized particle size despite increased heterogeneity in frozen ghost cell fractions, and both preparations yielded structurally intact vesicles. Freezing reduced proteomic complexity in membrane fractions and selectively depleted cytosol-associated proteins. The core membrane proteome, including Band 3, remained stable. Glycophorin A showed the largest reduction in frozen preparations, and CD47 levels were reduced in the ultracentrifuged fraction.

Conclusions: Frozen EDTA blood is a viable source for EMV production when immediate processing is not feasible. Minor proteomic differences, particularly in proteins relevant to immune evasion and surface charge, should be considered in applications where biological activity is critical.

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