Univerza v Ljubljani
Medicinska fakulteta

EXTRACELLULAR VESICLES: MEDIATORS AND BIOMARKERS OF PATHOLOGY

BOOK OF ABSTRACTS

Ljubljana, 05. 12. 2019

Univerza v Ljubljani
Medicinska fakulteta

Workshop

EXTRACELLULAR VESICLES: MEDIATORS AND BIOMARKERS OF PATHOLOGY

Ljubljana, 05. 12. 2019

Book of abstracts

Workshop **Extracellular vesicles: mediators and biomarkers of pathology**

Book of Abstracts
Electronic version

Edited by: Katja Goričar
Reviewed by: Marija Holcar, Metka Lenassi
Published by: University of Ljubljana, Faculty of Medicine
Cover design by: Katja Goričar
Organised by: University of Ljubljana, Faculty of Medicine, Institute of Biochemistry
Electronic version: Available at: https://www.mf.uni-lj.si/ibk/novice/simpozij-extracellular-vesicles-mediators-and-biomarkers-pathology
Electronic format: PDF
Available from: 05. 12. 2019

Kataložni zapis o publikaciji (CIP) pripravili v Narodni in univerzitetni knjižnici v Ljubljani
COBISS.SI-ID=302996480
ISBN 978-961-267-168-6 (pdf)

TABLE OF CONTENTS

OPENING ADDRESS

Dear Colleagues,

We are pleased to welcome you to the workshop "Extracellular vesicles: mediators and biomarkers of pathology" taking place on the 5th of December 2019 at the Institute of Biochemistry, Faculty of Medicine, University of Ljubljana.

Extracellular vesicles (EVs) are a heterogeneous population of membrane vesicles, which are shed from cells *in vitro* or are released into various body fluids *in vivo*, like blood plasma, cerebrospinal fluid, urine, and others. They consist of a lipid bilayer membrane that surrounds a small amount of cytosol and contain various typical proteins, lipids and nucleic acids, which mirror the composition and the state of the cell of origin. Thus EVs have great potential for human diagnostics and therapeutic applications. Importantly, EVs were also identified as mediators of physiological processes and various pathological conditions.

At this year's workshop, we will first learn about the current opportunities and challenges in EV studies, focusing on rigor and standardization of EV research and on novel discoveries that will help realize the full potential of EVs as biomarkers and modulators of health and disease (session 1). We will later hear more about the contributions of Slovenian researchers and clinicians in uncovering the role of EVs in pathogenicity (session 2) and as biomarkers of pathology (session 3). The stimulating atmosphere will hopefully promote new collaborations and research findings.

On behalf of the organising committee, we welcome you to the workshop on the topic of a rapidly expanding field of extracellular vesicles.

Assoc. Prof. Metka Lenassi, PhD

Chair of the organising committee

Assist. Prof. Katja Goričar, PhD

Member of the organising committee

Assist. Marija Holcar, PhD

Member of the organising committee

ORGANISATION

Organised by

Univerza v Ljubljani
Medicinska fakulteta

Organising committee

Metka Lenassi, chair
Katja Goričar
Marija Holcar

SPONSORS

Excellence in Routine and Science

Dolinškova 8, 1000 Ljubljana
tel. 01 4273213, 4283650, fax 01 4273191
el. pošta: omega@omega.si

PROGRAMME

08.30 – 09.00	*Coffee break*
09.00 – 09.10	**Opening address**
Session 1	**Introduction to extracellular vesicles**
09.10 – 09.50	Extracellular vesicles: the membrane's the limit for a budding field Kenneth W. Witwer, Johns Hopkins University, USA
09.50 – 10.10	Mechanisms of extracellular vesicles formation Veronika Kralj Iglič, UL ZF
10.10 – 10.30	Fungal extracellular vesicles and their relevance in ecology and pathogenicity Teja Lavrin, UL MF
10.30 – 11.10	*Coffee break*
Session 2	**Extracellular vesicles as mediators of disease pathology**
11.10 – 11.30	The role of extracellular vesicles and their synthetic analogues in the modulation of TLR4 activity Thai Van Ha, KI
11.30 – 11.50	Role of extracellular vesicles released after oxidative stress in activation of adaptive response Peter Pečan, KI
11.50 – 12.10	Altered surface protein profile of small plasma extracellular vesicles from patients with antiphospholipid syndrome Ula Štok, UKCL
12.10 – 12.30	Extracellular vesicle miRNAs in type 1 diabetes Tine Tesovnik, UKCL
12.30 – 13.30	*Buffet lunch*
Session 3	**Extracellular vesicles as biomarkers of disease pathology**
13.30 – 13.50	Enrichment of plasma extracellular vesicles for reliable quantification for biomarker discovery Marija Holcar, UL MF
13.50 – 14.10	Characteristics of plasma extracellular vesicles in pancreatic ductal adenocarcinoma David Badovinac, UKCL
14.10 – 14.30	MicroRNAs in extracellular vesicles as biomarkers in malignant mesothelioma Katja Goričar, UL MF
14.30 – 14.50	Assessment of extracellular vesicles directly in blood plasma Darja Božič, UL ZF
14.50 – 15.10	Extracellular vesicles from urine in kidney transplant recipients: optimization of the isolation method Ivana Sedej, UKCL
15.10 – 15.15	**Closing address:** Metka Lenassi, UL MF, Chair of the organising committee

LECTURE ABSTRACTS

Session 1: Introduction to extracellular vesicles

Extracellular vesicles: the membrane's the limit for a budding field

Kenneth W. Witwer

Johns Hopkins University School of Medicine in Baltimore, Maryland, USA

Extracellular vesicle research has decades-old roots, but has only recently achieved widespread international attention. The current opportunities in EV studies are the subject of this talk. Challenges of rigor and standardization are an ongoing focus of the International Society for Extracellular Vesicles (ISEV) as well as a proliferation of regional societies and local centers. One such initiative, the community-based update to the Minimal Information for Studies of EVs (MISEV2018) provides a structure for examining exciting frontiers in EV science. For example, new, orthogonal methods for single-particle analysis are opening doors to new discoveries, suggesting that we may soon realize the full potential of EVs as biomarkers and modulators of health and disease.

Mechanisms of extracellular vesicles formation

Veronika Kralj-Iglič[1], Gabriella Pocsfalvi[2], Henry Hagerstrand[3], Luka Mesarec[4], Vid Šuštar[5], Aleš Iglič[4]

[1]Laboratory of Clinical Biophysics, Faculty of Health Sciences, University of Ljubljana, Slovenia; [2]Institute of Biosciences and Bioresources, National Research Council of Italy, Italy; [3]Department of Biology, Abo Akademi University, Biocity, Finland; [4]Laboratory of Physics, Faculty of Electrical Engineering, University of Ljubljana, Slovenia; [5]Turku Biosciences, University of Turku, Finland

Introduction: A view on mechanisms of biological membrane budding and micro/nano-vesiculation is presented with the aim to expose physical parameters that are key to the processes taking place in cells in vitro and ex vivo.

Methods: Theoretical model of the budding and vesiculation mechanisms is described where the shape of the entities composed of the membrane and a fluid interior and exterior are determined by minimization of the free energy of the membrane at chosen constraints. The membrane free energy is obtained by summing the contributions of the single membrane constituent energies and considering principles of statistical physics.

Results: It is shown that the budding and micro/nano-vesiculation of the membrane is promoted by the intrinsic shape of the membrane constituents (so-called inclusions) and their lateral and orientational distribution. The budding and stability of nanovesicles of different shapes and sizes is explained in connection with their composition. A mechanism based on the direct interactions between inclusions to form lipid-protein complexes (e.g. membrane rafts) and consequent budding and formation of exo and endo – nanovesicles, is proposed. Experimental evidence that agrees with above mechanisms, as observed in cells, extracellular vesicles and phospholipid vesicles is shown.

Conclusions: It is suggested that exosomes are formed following membrane budding, in an essentially similar mechanism to microvesiculation, due to direct interaction of membrane constituents that segregate in the internal folds. Three mechanisms of membrane formation are distinguished: apoptosis, budding (microvesicles and exosomes) and fragmentation due to stress during processing of samples.

Fungal extracellular vesicles and their relevance in ecology and pathogenicity

Teja Lavrin[1], Rok Kostanjšek[2], Samo Hudoklin[3], Peter Veranič[3], Boris Rogelj[4], Nina Gunde Cimerman[2], Ana Plemenitaš[1], Metka Lenassi[1]

[1]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia; [2]Biotechnical Faculty, University of Ljubljana, Slovenia; [3]Institute of Cell Biology, Faculty of Medicine, University of Ljubljana, Slovenia; [4]Department of Biotechnology, Jozef Stefan Institute, Slovenia

Introduction: Extracellular vesicles (EVs) are newly identified mediators of intercellular communication within the same organism or between different species. EVs are namely released by organisms of all kingdoms, including fungi. Fungal EVs have generally similar characteristics, but are enriched in proteins of adaptive mechanisms, pigments, cell wall components and other virulent factors. In our study, we aimed to isolate and characterise EVs of extremophilic and pathogenic fungi and evaluate their role in adaptation to environmental stress and pathogenicity.

Methods: Experiments were performed on the following fungi: extremophilic *Wallemia ichtyophaga* and extremotolerant/pathogenic *Hortaea werneckii* and *Exophiala dermatitidis*. Fungi were cultured in defined media until exponential growth phase, without or with added melanin biosynthesis inhibitor tricyclazole. Next, extracellular particles (EP) were isolated from conditioned media by sequential centrifugation or by further separation on sucrose density gradient. Isolated EPs were characterised for purity and morphology by TEM, concentration and size by NTA, and molecular content (proteins, melanin) by immunoblotting and spectrophotometry (WB, A_{562}, A_{400}). Pathogenic potential of fungal EPs was tested *in vitro* on neuroblastoma cells by cytotoxicity assay.

Results: We established a protocol for isolation of fungal EPs, which was successfully used in all three fungi. TEM micrographs showed heterogeneous nature of isolated EPs, with typical cup-shaped EVs representing different shares in the three fungi. EPs size and abundance differed between fungi: average mode diameter of 154 nm and concentration of 5.9×10^7 particles per mL of media for *Wallemia*, 97 nm diameter and 1.7×10^9 particles/mL for *Hortaea*, and 90 nm diameter (*R*g) and 1.0×10^7 particles/mL for *Exophiala*. Fungal EPs carried typical EV marker proteins Hsp70, α-tubulin and GAPDH as shown by immunoblotting, with *Hortaea* EPs additionally packing protein Hog1, the main kinase in osmotic stress signalling. Subpopulation of *Hortaea* and *Exophiala* EPs contained melanin and therefore separated in fractions with higher buoyant density, as EPs isolated from cultures in the presence of melanin inhibitor redistributed to less dense fractions. Furthermore, *Exophiala* EPs containing melanin were neurocytotoxic for neuroblastoma cell line (20.9% viability compared to control), as the effect was lost in the presence of EPs without melanin.

Conclusions: The three studied fungi release a large amount of heterogeneous EPs that could act as mediators in adapting to stress environments (such as high osmolarity) and in promoting pathogenicity in fungal human infections.

Session 2: Extracellular vesicles as mediators of disease pathology

The role of extracellular vesicles and their synthetic analogues in the modulation of TLR4 activity

Van Thai Ha, Mateja Manček Keber

Department of Synthetic Biology and Immunology, National Institute of Chemistry, Slovenia

Introduction: Toll-like receptor 4 (TLR4) and its co-receptor MD-2 play a critical role in innate immunity associated with lipopolysaccharide (LPS)-mediated responses. In addition, TLR4/MD-2 is also activated by endogenous factors, generally known as damage associated molecular patterns (DAMPs) that promote sterile inflammation in diseases such as: ischemia/reperfusion injury (IRI), atherosclerosis, rheumatoid arthritis (RA)... Under oxidative stress, which is a hallmark of sterile inflammation, increased levels of extracellular vesicles (EVs) were detected. Additionally, several enzymes, among them 15-lipoxygenase (15-LO) and secreted phospholipase A_2 ($sPLA_2$) are induced and were suggested to play an important role in the activation of Toll-like receptor 4 (TLR4) signaling.

Methods: StressEVs were produced from HEK293 cells exposed to 10 uM A23187 and isolated with ultracentrifugation. SynEVs prepared from natural PLs, individual PLs, lysoPLs or 20:4 lysoPI were oxidized for 10 min with 15-LO. Additionally, synEVs from AAPE were prepared, oxidized with 15-LO and hydrolyzed with sPLA2. Activity was measured by qPCR on wt and TLR4-KO macrophages. 15-LO oxidized 20:4 lysoPI was analyzed by mass spectrometry. sPLA2 activity was measured in synovial fluid from RA patients using fluorometric assay.

Results: During oxidative stress, endogenous stress-derived EVs (stressEVs) were found to activate TLR4 with a gene profile different from bacterial lipopolysaccharide (LPS). Additionally, concerted activity of 15-LO and secreted $sPLA_2$ were needed for the formation of TLR4 agonists, which we determined as lysophospholipids (lysoPLs) with oxidized unsaturated fatty acid. Hydroxy, hydroperoxy and keto products of 20:4 lysophosphatidylinositol (lysoPI) were determined by mass spectrometry and they activated the same gene pattern as stressEVs. Injection of sPLA2-IIA to mice promoted K/BxN serum induced arthritis in TLR4-dependent manner.

Conclusions: 15-LO and $sPLA_2$ are enzymes induced during inflammation, which opens the opportunity to design specific inhibitors that could limit sterile inflammation but will not globally affect systemic innate immunity.

Role of extracellular vesicles released after oxidative stress in activation of adaptive response

Peter Pečan, Thai Van Ha, Mateja Manček Keber

Department of Synthetic Biology and Immunology, National Institute of Chemistry, Slovenia

Introduction: Adaptive response is an important defense function, which helps cells to fight against oxidative stress. Many studies showed that low levels of oxidized species (among them also oxidized phospholipids) enhance defense capacity of cells by inducing the expression of antioxidant enzymes such as heme oxygenase 1 (HO-1). We investigated the capability of extracellular vesicles (EVs), released after oxidative stress (sEVs), to induce Mox phenotype with characteristics of adaptive response in naïve macrophages. We were also interested in the role sEVs might play by triggering adaptive response in cardiomyocytes, which can protect the cells from the forthcoming tissue injury resulting from hypoxia/reoxygenation.

Methods: StressEVs were produced from HEK293 cells exposed to 10 uM A23187 and isolated using ultracentrifugation. Using qPCR and WB analysis we determined the changes in expression of genes, relevant in response to oxidative stress, after stimulation with sEVs. Cytotoxicity after hypoxia/reoxygenation was determined by measuring the activity of lactate dehydrogenase (LDH), which is released from damaged cells.

Results: Our results showed that sEVs trigger polarization of macrophages to M1 phenotype and not Mox phenotype. On the other hand, we were able to show induction of HO-1 expression in rat cardiomyocytes after sEV stimulation. Moreover, preincubation of cardiomyocytes with sEVs decreased release of lactate dehydrogenase after hypoxia/reoxygenation leading to increased cell survival, which was further promoted when proinflammatory signaling was inhibited.

Conclusions: Our results suggest that sEVs can trigger adaptive immune response and most likely represent one of the mechanisms present in remote ischemic conditioning.

Altered surface protein profile of small plasma extracellular vesicles from patients with antiphospholipid syndrome

Ula Štok[1,2], Elizabeta Blokar[1], Metka Lenassi[3], Marija Holcar[3], Katja Perdan Pirkmajer[1], Aleš Ambrožič[1], Matija Tomšič[1,4], Andreja Erman[1,5], Nataša Resnik[1,5], Snežna Sodin Šemrl[1,6], Saša Čučnik[1,2] and Polona Žigon[1,6]

[1]University Medical Centre Ljubljana, Department of Rheumatology, Slovenia; [2]University of Ljubljana, Faculty of Pharmacy, Slovenia; [3]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Slovenia; [4]University of Ljubljana, Faculty of Medicine, Chair of Internal Medicine, Slovenia; [5]University of Ljubljana, Faculty of Medicine, Institute of Cell Biology, Slovenia; [6]University of Primorska, Faculty of Mathematics, Natural Sciences and Information Technologies, Slovenia

Introduction. Antiphospholipid syndrome (APS) is a systemic autoimmune disease, which is characterized by thromboses and/or obstetric complications with the presence of antiphospholipid antibodies. Extracellular vesicles (EVs) have been suggested to play a role in the pathogenesis of APS, however no information to date has been published about small extracellular vesicles (sEVs) in patients with APS. The purpose of this study was to determine plasma levels of sEVs in APS patients vs. healthy blood donors (HBD) and investigate their surface protein profiles.

Methods. Whole blood was collected from APS patients (n=16) and HBDs (n=7) and processed to obtain platelet poor plasma. Sucrose cushion ultracentrifugation was utilized to obtain sEVs for the analysis of their number and size by Nanoparticle Tracking Analysis. The presence of sEVs was confirmed by Transmission Electron Microscopy. In order to determine the expression of 39 different protein markers on sEVs, immune-magnetic anti-CD63 beads were used for isolation, coupled to MACSPlex multiplex flow cytometry.

Results. APS patients had significantly higher number of sEVs, as compared to HBD. Significant differences in expression of CD63 positive sEV surface protein markers HLA-DRDPDQ, CD24, CD62P, CD133/1 and CD326 was found between APS patients vs. HBD indicating activation of the immune system, increased cellular adhesion potential, as well as altered endothelium.

Discussion. Elevated numbers of sEVs and their specific surface protein profiles in plasma of APS patients could represent an important disease phenomenon.

Extracellular vesicle miRNAs in type 1 diabetes

Tine Tesovnik[1,2], Jernej Kovač[2], Katka Pohar[3], Samo Hudoklin[4], Katarina Trebušak Podkrajšek[2,5], Maruša Debeljak[2], Peter Veranič[4], Alojz Ihan[3], Tadej Battelino[1,6]

[1]University Medical Centre Ljubljana, University Children's Hospital, Department of Pediatric Endocrinology, Diabetes and Metabolic Diseases, Slovenia; [2]University Medical Centre Ljubljana, University Children's Hospital, Clinical Institute of Special Laboratory Diagnostics, Slovenia; [3]University of Ljubljana, Faculty of Medicine, Institute of Microbiology and Immunology, Slovenia; [4]University of Ljubljana, Faculty of Medicine, Institute of Cell Biology, Slovenia; [5]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Slovenia; [6]University of Ljubljana, Faculty of Medicine, Chair of Paediatrics, Slovenia

Introduction: Type 1 diabetes is an autoimmune disease associated with pancreatic Langerhans beta-cells destruction, resulting in a lack of endogenous insulin, which dramatically reduces the quality of patients' life and contributes to the development of diabetes complications. The etiology of the disease is still not well established due to the inaccessibility of the affected targeted cells. However, the insight into the physiological processes in the affected beta-cells can be achieved with the study of extracellular vesicles as biomarkers and mediators in the development of the disease.

Methods: EVs were isolated using the precipitation reagent, and isolated EVs fractions were characterized by the transmission electron microscopy. Blood plasma EVs RNA fractions of type 1 diabetes individuals and Langerhans islets transplantation patients were isolated and sequenced using next-generation sequencing technology. The selected differentially expressed miRNA were further investigated with the *in vitro* study using flow cytometry to assess their immunomodulatory effect.

Results: The images of transmission microscopy revealed EVs in human blood plasma, containing proteins expressed in Langerhans islets beta-cells. The next-generation sequencing analysis revealed several differentially expressed miRNA in type 1 diabetes individuals and individuals with transplanted Langerhans islets. The *in vitro* vesicle derived miRNAs were accumulated in the endolysosomal pathway of phagocytes, which is reflected in the increased degranulation and proliferation of NK and T-cells.

Conclusions: Our study shows the presence of beta-cells derived extracellular vesicles of Langerhans islets in human blood plasma, which confirms the intercellular EVs communication of endocrine pancreas with other tissues and the immune system. The differentially expressed miRNAs in type 1 diabetes derived with vesicles are accumulated in the endolysosomal pathway of phagocytes triggering TLR7 and/or TLR8 signaling. The TLR7/8 signaling results in the increased downstream activation of the immune system with NK, as well as T-cell proliferation and cytotoxicity. Our study provided data describing the importance of a complex involvement of EVs-derived human miRNAs in the regulation of the immune system, and potentially in the development of T1D autoimmunity. The results also emphasize the implications for developing strategies for the prevention and treatment of T1D-related immune processes.

Session 3: Extracellular vesicles as biomarkers of disease pathology

Enrichment of plasma extracellular vesicles for reliable quantification for biomarker discovery

Marija Holcar[1], Jana Ferdin[1], Simona Sitar[2], Magda Tušek-Žnidarič[3], Vita Dolžan[1], Ana Plemenitaš[1], Ema Žagar[2], and Metka Lenassi[1]

[1]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia; [2]Department of Polymer Chemistry and Technology, National Institute of Chemistry, Slovenia; [3]Department of Biotechnology and System Biology, National Institute of Biology, Slovenia

Introduction: Extracellular vesicles (EVs), phospholipid bilayer-enclosed particles, are secreted by all cell types and abundantly released into body fluids. EVs' morphology and molecular cargo reflect (pato)physiological state of their cell-of-origin, making them ideal candidates for minimally invasive biomarkers. However, they are still largely unexplored due to a lack of established isolation methods from complex fluids. Here, we propose an EV-enrichment method from relatively small volumes of blood plasma to reliably quantify small EVs for biomarker discovery.

Methods: Small EVs were enriched from the plasma of 10 healthy subjects, using density-based (ultracentrifugation on 20% sucrose cushion; sUC) and size-based (size exclusion chromatography; SEC) methods. Size, concentration, and purity of isolates were determined with nanoparticle tracking analysis (NTA), asymmetric flow field-flow fractionation with multi-angle light scattering (AF4-MALS), transmission electron microscopy (TEM) and miRNA levels with qPCR. Repeatability of the chosen method (sUC) and effect of proteinase K and DNase-I treatment on size and concentration of particles have also been analyzed. The study was approved by the National Medical Ethics Committee.

Results: While the average size (mode diameter) of enriched particles in the samples was comparable between two isolation methods, SEC generally led to a higher number but lower quality of isolates compared to sUC ($P<0.001$). Based on TEM, this is due to contamination with lipoproteins and protein aggregates. The sUC method was highly repeatable and resulted in purer EV isolate with more miRNA cargo. NTA detected $3.11*10^9$ particles (mean size 109 nm) and AF4-MALS $0.66*10^9$ particles ($2*R_{geom}$ 195 nm) after sUC isolation from 1 mL of plasma. Digestion of sUC-enriched EVs with proteinase K and DNase-I did not significantly affect the size and concentration of EVs in the sample, implying that potential impurities in the EV isolate do not contribute to the measurements.

Conclusions: The sUC method led to higher yield and purity of EVs in the enriched samples of human plasma and could be used to explore EVs' size and concentration as potential biomarkers.

Characteristics of plasma extracellular vesicles in pancreatic ductal adenocarcinoma

David Badovinac[1], Katja Goričar[2], Hana Zavrtanik[1], Miha Petrič[1], Teja Lavrin[2], Nina Mavec[2], Vita Dolžan[2], Aleš Tomažič[1,3], Metka Lenassi[2]

[1]Department of Abdominal Surgery, University Medical Centre Ljubljana, Slovenia; [2]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia; [3]Department of Surgery, Faculty of Medicine, University of Ljubljana, Slovenia

Introduction: Pancreatic ductal adenocarcinoma (PDAC) is one of the cancers with worst prognosis, thus better preoperative characterization of patients would aid in treatment optimization. Extracellular vesicles (EVs) are promising and largely unexplored liquid biopsy biomarkers in PDAC. This study aimed to evaluate if plasma EV characteristics are associated with PDAC clinical characteristics and overall survival (OS).

Methods: The prospective cohort included 34 patients with PDAC who underwent surgery with curative intent. Patient data and plasma samples were collected preoperatively, intraoperatively and one month postoperatively. All subjects provided informed consent and the study was approved by the Slovenian Committee on Human Research. Plasma EV concentration and size were determined by nanoparticle-tracking analysis. Mann-Whitney test, Spearman's rho and Cox regression were used in statistical analysis.

Results: Preoperatively, patients with poorly differentiated tumors had significantly larger plasma EVs when compared to patients with well/moderately differentiated tumors (mean diameter 176.9 vs. 149.2 nm, p=0.021), the EV size even enabling discrimination of the two groups (AUC=0.742, 95% CI=0.560-0.923). Plasma EV characteristics were also a significant predictor of OS in multivariable analysis. Patients with >33.8% increase in EV concentration had 7.2 months shorter median OS (HR=10.21, 95% CI=2.33-44.67, p=0.002), while patients with >28.0% decrease in EV size had 9.2 months shorter median OS (HR=0.24, 95% CI=0.06-0.97, p=0.045, respectively), compared to patients below these cutoff values.

Conclusions: Plasma EV concentration and size correlate with tumor differentiation and may predict OS in PDAC patients. Further studies are needed to evaluate EVs as biomarkers for preoperative assessment of tumor grade and prediction of OS.

MicroRNAs in extracellular vesicles as biomarkers in malignant mesothelioma

Katja Goričar[1], Marija Holcar[1], Nina Mavec[1], Metoda Dodič-Fikfak[2], Alenka Franko[2], Viljem Kovač[3], Metka Lenassi[1], Vita Dolžan[1]

[1]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Slovenia; [2]University Medical Center Ljubljana, Clinical Institute of Occupational Medicine, Slovenia; [3]Institute of Oncology Ljubljana, Slovenia

Introduction: Malignant mesothelioma (MM) is a rare cancer of the pleura or the peritoneum that is mostly associated with exposure to asbestos. It is usually diagnosed in the advanced stages and is characterized by poor prognosis and short survival. Extracellular vesicles (EVs) are membrane-bound particles released from many cell types into different body fluids. EVs can transport various molecules such as RNAs, proteins and metabolites and their molecular composition reflects the characteristics of the origin cell. EVs or their cargo could therefore serve as new minimally invasive biomarkers that would enable earlier detection of MM or better prediction of treatment response. Our aim was to evaluate miRNAs enriched in serum extracellular vesicles as potential biomarkers in patients with MM.

Methods: We included in the study 10 control subjects that were occupationally exposed to asbestos but did not develop any asbestos-related diseases and 20 MM patients. In MM, good response was defined as overall survival of more than 18 months, while poor response was defined as overall survival of less than 10 months. EVs were isolated from serum samples before and after treatment using ultracentrifugation on 20% sucrose cushion. Levels of miRNAs miR-103-3p, miR-126-3p and miR-625-3p from EVs were analysed using qPCR. MiR-425-5p and let-7i-5p were used for normalisation. Nonparametric tests and survival analysis were used to evaluate the biomarker potential of miRNAs from EVs.

Results: In the EVs isolated from serum of MM patients, relative expression of miR-103-3p was significantly lower compared to healthy asbestos-exposed controls (P=0.001), while relative expression of miR-126-3p was significantly higher (P=0.001). MiRNA expression before treatment was not associated with survival of MM patients. After treatment, relative expression of miR-625-3p and miR-126-3 increased only in MM patients with poor treatment response (P=0.012 and P=0.036, respectively), while no differences were observed in patients with good response (P=0.173 and P=0.374, respectively). Relative increase in miR-625-3p expression after treatment for more than 3.2% was associated with much shorter progression-free survival (7.5 vs 19.4 months, P=0.024) and overall survival (12.5 vs 49.1 months, P=0.043) of MM patients.

Conclusions: MiRNAs from EVs could serve as diagnostic or prognostic biomarkers in MM and could contribute to a more personalized treatment of these patients.

Assessment of extracellular vesicles directly in blood plasma

Darja Božič[1,#], Simona Sitar[2], Ita Junkar[3], Roman Štukelj[4], Manca Pajnič[4], Ema Žagar[2], Veronika Kralj-Iglič[4], Ksenija Kogej[1]

[1]Department of Chemistry and Biochemistry, University of Ljubljana, Faculty of Chemistry and Chemical Technology, Slovenia; [2]Department of Polymer Chemistry and Technology, National Institute of Chemistry, Slovenia; [3]Department of Surface Engineering and Optoelectronics, Josef Stefan Institute, Slovenia; [4]Biomedical Research Group, University of Ljubljana, Faculty of Health Sciences, Slovenia

[#]Current address: Biomedical Research Group, University of Ljubljana, Faculty of Health Sciences, Slovenia and Laboratory of Physics, University of Ljubljana, Faculty of Electrical Engineering, Slovenia

Introduction: In order to enable exploitation of extracellular vesicles (EVs) as diagnostic markers, the methods for their examination still need to be improved. According to the literature, the pre-analytical treatment of samples has a great effect on the results. Minimal sample handling is therefore desirable to minimize the risk of sample change and the time and cost of analysis. In this contribution, a combination of static (SLS) and dynamic light scattering (DLS) was applied directly to the blood plasma [1].

Methods: The results for blood plasma were compared with those for EV isolates and an exosome standard (HBM-PEP, HansaBioMed LCC, Estonia). Flow cytometry was used to detect the cell residuals and large vesicles in blood plasma. The DLS/SLS results for small particles were compared to those obtained by the asymmetrical flow field-flow fractionation. Atomic force microscopy was used to visualize the samples.

Results: The size distributions of the particles in blood plasma obtained by DLS were most often tri-modal. The particles with hydrodynamic radius, R_h, below 10 nm were assigned to proteins, while two populations were found in the size range expected for EVs: population 1 with R_h 10–50 nm (diameter 20–100 nm), and population 2 with R_h 100–150 nm (diameter ~200–300 nm). For the latter, the radius of gyration, R_g, was determined as well. The shape parameter ρ (= R_g/R_h) of population 2 was found to be ρ = 0.94–1.1 in the exosome standard (considering the viscosity of the medium for R_h evaluation to be that of water, *i.e.* 0.89 mPa/s) and ρ = 0.7–1.2 in blood plasma and EV isolates (considering the viscosity of the medium for the vesicles in blood plasma to be 1.2 mPa/s). These ρ values agree well with the theoretical value for vesicle topology (ρ = 1).

Conclusions: The DLS/SLS technique was found very promising. However, individual characteristics of biological samples must be kept in mind in data interpretation, mainly the viscosity of blood plasma and its effect on sedimentation rates and shear stress in the step of cell removal, and its effect on the size parameters determined in light scattering experiments.

[1] D. Bozic, S. Sitar, I. Junkar, R. Štukelj, M. Pajnič, E. Žagar, V. Kralj-Iglič, K. Kogej, Viscosity of plasma as a key factor in assessment of extracellular vesicles by light scattering, *Cells* **2019**, *8*(9), 1046; https://doi.org/10.3390/cells8091046

Extracellular vesicles from urine in kidney transplant recipients: optimization of the isolation method

Ivana Sedej[1], Magda Tušek Žnidarič[2], Vita Dolžan[3], Miha Arnol[1], Metka Lenassi[3]

[1]Department of Nephrology, University Medical Centre Ljubljana, Slovenia; [2]National Institute of Biology Ljubljana, Slovenia; [3]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia

Introduction. Although the management of kidney transplant recipients has improved over recent decades, the assessment of individual risks remains imperfect. Individualized strategies are necessary to early recognize and prevent immune-mediated allograft injury and to fine-tune immunosuppression, with the overall goal to improve allograft and patient outcomes. Kidney biopsy is currently recognized as a "gold standard" for the assessment of allograft injury. However, the diagnosis of subclinical injury requires multiple surveillance biopsies. Given that biopsy procedures are invasive, complications may occur; furthermore, sampling errors may jeopardize their diagnostic usefulness. Therefore, we focused on characterization of DNA, RNAs and extracellular vesicles (EVs) in blood and urine as potential non-invasive biomarkers of kidney allograft injury. As the first step, we aimed to establish and optimize the method for isolation of pure urinary EVs (uEVs).

Methods. The second morning spot urine samples (25 mL) were collected from 7 kidney transplanted patients. Urine samples were processed within 4 hours following four variations of the protocol to account for oxalate precipitation, variability in pH, uromodulin polymerization, high protein concentration and sample dilution. Isolated uEVs were characterized for morphology and purity by Transmission Electron Microscopy (TEM) and for size and concentration by Nanoparticle Tracking Analysis (NTA). uEVs specific proteins and micro RNAs were analyzed by Western blot and qRT-PCR, respectively.

Results. After optimizing different steps, the optimal protocol for preparation of EVs isolates included centrifugation of urine samples at low speed (2.000 x g, RT) to remove cells and storage at -80°C prior to further analyses. After thawing the urine at room temperature, the addition of EDTA prevented cryoprecipitate formation as well as uromodulin polymerization, while the addition of concentrated PBS helped to neutralize pH. Next, the filtration of samples through 0.22 µm pores removed bacteria and larger particles and concentrated the sample for separation of particles on size-exclusion chromatography (SEC; qEVoriginal, Izon Q). Out of the 20 fractions that were collected, the ones that tested negative for protein impurities (A_{280}) were pooled and concentrated to smaller volume (70-80 µl). TEM micrographs of these isolates demonstrated high purity and typical “cup shaped” morphology of uEVs. According to NTA, the average mean size of uEVs was 129,9 nm and concentration was in the range of $1x10^9$ particles per ml of urine. Western blot results showed that uEVs were positive for the tested marker proteins Hsc70, flotillin, tubulin, GADPH and CD63. qRT-PCR confirmed the presence of miRNAs in isolated uEVs, with C_T for miR let-7i at 20.

Conclusions. By using TEM, NTA, Western blot and qRT-PCR, we confirmed successful isolation of pure population of EVs from urine. The established method will be used to evaluate uEVs as non-invasive urinary biomarkers of allograft injury in kidney transplant recipients.