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Optimization of urinary extracellular vesicles isolation and purification methods for diabetic patients

Master's Thesis

30 EAP

Signe Parts

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„Diabeeti põdevate patsientide uriinis leiduvate ekstratsellulaarsete vesiikulite isoleerimise ning puhastamise meetodite optimeerimine“

Rahvusvahelise Diabeedi Föderatsiooni (IDF) andmetel suri 2021. aastal diabeedi või diabeedi tüsistuste tagajärjel umbes 6.7 miljonit täiskasvanut inimest. Valitseb kriitiline vajadus paremate meetodite järele, et tõhusalt hinnata diabeedi ning diabeedi tüsistuste kulgu. Uudsed biomarkereid, nagu seda on ektratsellulaarsed vesiikulid (EVd), aitavad paremini mõista organismi patofüsioloogilistes tingimustes toimuvaid muutuseid. EVd on tuntud kui rakkude vahelise suhtluse mediaatorid ning neil on suur potentsiaal aidata tõhusamalt hinnata haiguse kulgu ka kliinilises praktikas. Siiski vajavad nii EV isoleerimise kui ka puhastamise meetodid optimeerimist ja normaliseerimist, et saavutada parem kliiniline rakendatavus.

Märksõnad: Ekstratsellulaarsed Vesiikulid, Diabeet, Biomarkerid

CERCS kood: Füsioloogia (B470)

„Optimization of urinary extracellular vesicles isolation and purification methods for diabetic patients “

According to International Diabetes Federation (IDF) in 2021 about 6.7 million adults have died as a result of diabetes, or its complications. We are in critical need to find better disease monitoring methods to assess the development of complications and the disease itself. Novel biomarkers such as extracellular vesicles (EVs) can provide a better understanding of the changes taking place in human body under pathophysiological conditions. EVs are known for their emerging role as cell-to-cell communication mediators and they have great potential to help assess disease development in clinical practice. Yet, EVs isolation and purification methods need to be optimized and normalized to reach the desired clinical applications.

Keywords: Extracellular Vesicles, Diabetes, Biomarkers

CERCS code: Physiology (B470)

TABLE OF CONTENTS

ABREVIATIONS

BSA	Bovine serum albumin
Cas9	CRISPR associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
DKD	Diabetic kidney disease
DN	Diabetic nephropathy
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
EV	Extracellular vesicle
HFD	Hydrostatic filtration dialysis
MISEV2018	Minimal information for studies of extracellular vesicles 2018
MVB	Multivesicular body
MWCO	Molecular weight cutoff
NTA	Nanoparticle tracking analysis
PAF	Platelet-activating factor
PROSPR	Protein Organic Solvent precipitation
SEC	Size exclusion chromatography
TALEN	Transcription activator-like effector nucleases
TEM	Transmission electron microscopy
THP	Tamm-Horsfall protein

TLR	Toll like receptor
uEV	Urinary Extracellular vesicle
UMOD	Uromodulin
UTI	Urinary tract infection
WT1	Wilms tumor 1
ZNF	Zinc-finger nucleases
ZP	Zona pellucida

INTRODUCTION

In 2021 approximately 537 million adults are living with diabetes according to the International Diabetes Federation (IDF) and about 6.7 million adults have died as a result of diabetes or its complications. In a very simplified definition diabetes is a disease where the body doesn't make enough insulin or insulin isn't used well, resulting in high blood glucose. Diabetes early symptoms include polyuria, polydipsia, and polyphagia but within years many complications such as diabetic neuropathy, nephropathy, or retinopathy can develop. Novel ways are developed to increase the diabetic patient's life and decrease the complications, but we are still in need to find more reliable biomarkers to assess the development of the disease.

One of such potential biomarkers are extracellular vesicles (EVs). EVs are lipid-bound vesicles that are secreted into the extracellular space by almost all cell types. EVs have been found in all isolated bodily fluids including blood, sweat, and urine. They are known as cell-to-cell communication mediators and EVs profiles vary depending on the environment and their origin. EVs have a great potential to work as biomarkers under pathophysiological conditions since their components such as proteins and mRNAs are versatile.

To research EVs potential, we need to optimize EVs isolation and purification methods. We desire non-invasive, sensitive, and fast methods to assess patients' health. Urine is a great bodily fluid where EVs from the kidneys, bladder, and urinary tract are combined but isolating these EVs populations comes with many challenges such as proteins, cells, and microorganisms. With a high-quality method to isolate EVs from urine, we desire to overcome these obstacles and to achieve high purity, yield, and EVs quality.

In this study, we aim to investigate different methods for the isolation of EVs from diabetic patients and healthy individuals' urine. We compared various purification methods to achieve the highest EVs yield with the least contamination. Optimized EVs isolation and purification method will be used for further studies of diabetic patients' EVs profiles and functionalities.

LITERATURE REVIEW

1.1. Introduction to Extracellular Vesicles

Extracellular vesicles (EV) are membrane-enclosed nanosized particles. Typically, their size varies from 30 nm to 1000 nm in diameter (Conde-Vancells et al., 2008) but some EVs populations may be larger, even up to 10 000 nm (Willms et al., 2018). They are released into the environment such as blood, urine, and saliva by eukaryotic and prokaryotic cells (van der Pol et al., 2012) and they are found in almost all biological fluids.

EVs are divided into three groups depending on their size and origins - apoptotic bodies, microvesicles, and exosomes (W. Zhang et al., 2016). Exosomes are bilayer 40-100 nm in diameter (Figure 1a) and they contain proteins, lipids, RNA from cell cytoplasm, and small molecules that are derived from the plasma membrane (Merchant et al., 2017). Exosomes formation is a multi-step mechanism where multivesicular bodies (MVBs) are formed which can be released as exosomes or degraded by lysosomes (Figure 1a) (Medeiros et al., 2020).

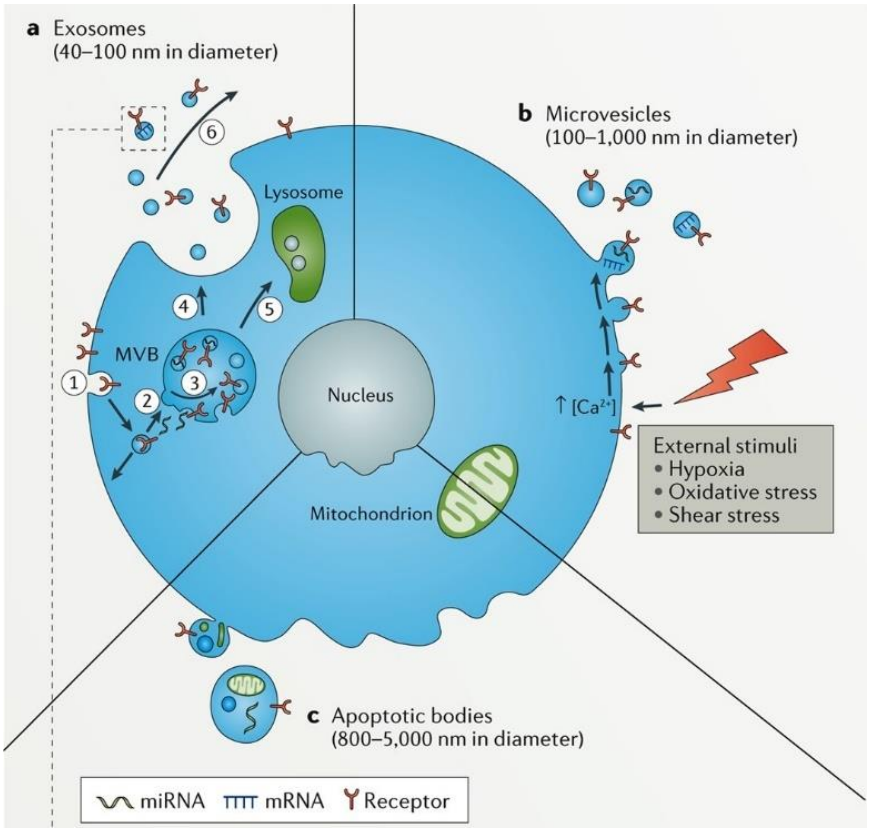

Figure 1: Exosomes are shown on the figure in part A. Exosomes are 40-100 nm bilayered vesicles. They contain proteins, lipids, RNA from cell cytoplasm, and other small molecules

from the plasma membrane. Exosome formation is a multistep process which includes membrane proteins endocytosis by cells, maturation of the endosomes, invagination of cell membrane which will lead to the formation of multivesicular bodies (MVBs). MVBs can fuse with the plasma membrane and lead to the release of intraluminal vesicles (exosomes). In case MVB fuse with a lysosome, their content degrades. Microvesicles are shown on figure part B. They are large (100-1000 nm) bilayered vesicles which need an extra stimulus for formation. They can contain plasma membrane lipids and proteins, and cytoplasmic lipids, proteins, and nucleic acids. In figure part C there are shown apoptotic bodies which contain organelle-specific proteins, nucleic acids, and lipids. The figure is adjusted from Nature Reviews Nephrology 12, 731-74 9(2017) by Michael L. Merchant et al.

Microvesicles are larger than exosomes with about 100 - 1 000 nm in diameter (Figure 1b). They are also bilayered and can contain plasma membrane lipids and protein, but they can also contain lipids, proteins, and nucleic acids from the cytoplasm (Merchant et al., 2017). The formation of microvesicles needs an external stimulus such as hypoxia, oxidative stress, shear stress, or any other kind of stimuli that leads to the shedding of the cell. Shedding of the cell can be mediated by Ca^{2+} or phospholipid-binding proteins and it causes a release of microvesicles from the plasma membrane (Merchant et al., 2017). Apoptotic (Figure 1c) bodies are usually the largest with 800 - 5000 nm in diameter. They can contain various plasma membrane-derived lipids, proteins, and cytoplasmic materials such as nucleic acids, lipids, and proteins from a specific organelle. (Merchant et al., 2017).

With increasing interest in EVs new subpopulations, such as exomeres, have been discovered. Exomeres are approximately 35 nm in diameter, non-membranous nanoparticles. These subpopulations each had unique N-glycosylation, protein, lipid, DNA and RNA profiles, and biophysical properties, which indicates distinct biological functions. (H. Zhang et al., 2018)

Despite the heterogeneity of EVs populations, each EV subtype has specific features depending on their origin. Recent studies have shown that EVs have various surface markers as well as cargo such as miRNAs, mRNAs, and proteins which could serve as promising biomarkers (Merchant et al., 2017). EVs are known for mediating cell-to-cell communication but more detailed mechanisms and functions for EVs are yet to be established. EVs

heterogenicity and role as a mediator make them ideal biomarkers for disease diagnostics and treatment monitoring.

1.2. Functional role

Novel understanding of the EVs functional role has been emerging with the increased interest in the field. Cargo-bearing vesicles are known to be cell-to-cell communication mediators, but the importance and precise mechanisms are yet to be discovered.

EVs potential as active biological components was identified during the 1980s and 1990s (Couch et al., 2021) but the existence of EVs was noted already prior to that in 1944 when Chargaff and West unknowingly isolated EVs and described their effect on blood clotting (Chargaff, 1945). It wasn't until 1967 when Peter Wolf captured so-called platelet dust with electron microscope (Wolf, 1967) which is now known as the first capturing of EVs. Since then, we have come a long way to describe EVs characteristics and functionality. According to PubMed.gov, a continuously rising number of publications is published. Only ten years ago the field gained just about under 900 publications per year whilst in 2021 over 6 500 publications were published (*Extracellular Vesicles - Search Results - PubMed*, n.d.).

With the fast development of EVs research, a novel understanding of their functionality has emerged. It is getting clear that EVs have a lot of potential as signaling mediators. They are known to play a key role in the disease development and in maintaining required physiological conditions. EVs have specialized functions as they participate in intercellular signaling and waste management as well as they can contribute to cell adhesion, protection against stress, and they can also have an antibacterial effect (van der Pol et al., 2012). EVs are known to deliver molecules from cell to cell and they can carry an excessive amount of protein, nucleic acid, and lipids. Their role in signaling pathways is becoming more popular subject in immunology, cancer research, hematology, and other biomedical fields. For example, EVs can play important role in immune suppression by transporting ligands and receptors, and they can transfer signaling components as well as present antigens (Berezin & Berezin, 2021). It has been found that EVs can present inflammatory mediators such as interleukin 1β, platelet-activating factor (PAF), TNF receptor 1, and Toll-like receptor (TLR) 4 (van der Pol et al., 2012).

Some exosomes can even mediate apoptosis by exposing suitable ligands like *FasL* ligand on exosomes which can mediate T-cell death (Anel et al., 2019).

It has been determined that EVs function is dependent on the host cell type. Cells from different human tissues of the body communicate through the secretion of EVs into proximal body fluids (Figure 2) (Yáñez-Mó et al., 2015). The diversity of EVs function, size, and locations where they are released makes it a fascinating research subject and opens new ways for diagnostics, prognosis, and therapies.

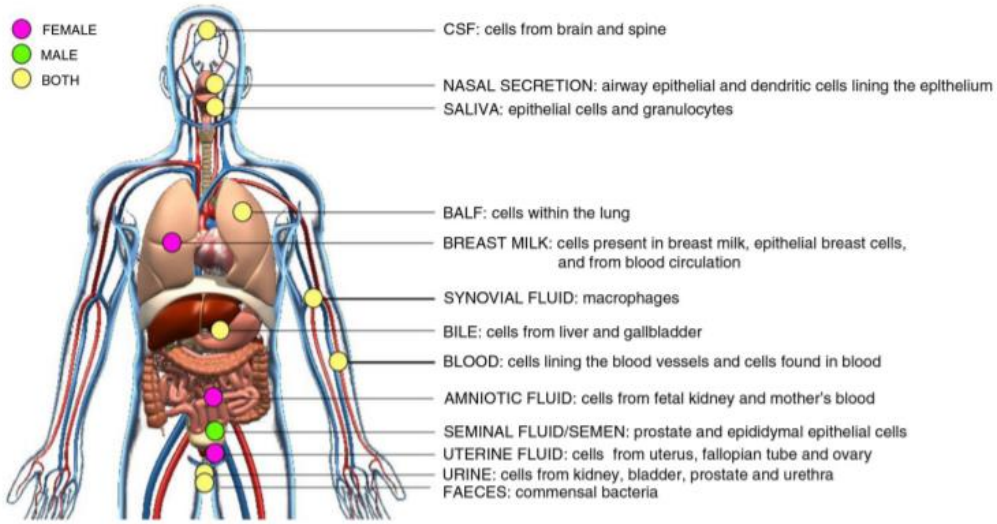

Figure 2 Schematic of in vivo derived EVs isolated from body fluids. Pink dots present EVs isolated from females, green from males, and yellow from both females and males. María Yáñez-Mó et al Biological properties of extracellular vesicles and their physiological functions (2015) , Journal of Extracellular Vesicles, 4:1, DOI: 10.3402/jev.v4.27066

1.3. Extracellular Vesicles as biomarkers

By definition biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to an intervention” (Downing, 2001). Functional biomarkers need to be certain condition-specific, and the results need to be measurable and reproducible (Byrnes & Weigl, 2018). Isolation of potential biomarkers can be done from bodily fluids, tissues, microorganisms, and cells but for disease prognostics and diagnostics, non-invasive methods of diagnostics are highly appreciated.

EVs have become more popular research field resulting in novel diagnostic methods. EVs can be used as liquid biopsy markers since they carry molecules such as RNAs (mRNA, miRNA), lipids, and proteins from parental cells (Urabe et al., 2020). Many studies have confirmed EVs association with diseases such as cancer. It is noted that even in the early stages of cancer EVs markers are detectable (Kosaka et al., 2019).

1.4. Bodily fluids and model organisms as a source of biomarkers

EVs make promising biomarkers due to specific EVs functionality and secretion into bodily fluids (Yáñez-Mó et al., 2015). Working with human body fluids and tissues may be difficult because of the sample availability and ethical requirements. There are several bodily fluids, such as urine, saliva, semen, or milk, which can be collected with non-invasive methods. Even blood collection is considered a low-risk and routine method in medical practice making the samples more available.

Bodily fluids are a great source of potential biomarkers, but the research of specific EVs function or origin of EVs is challenging due to the rich EVs profiles in bodily fluids. In bodily fluids, EVs are present from various tissue types creating a heterogeneous EVs population (Yáñez-Mó et al., 2015). Model organisms such as mice, bacteria, or even tissue cultures can be used to achieve more detailed results from invasive procedures or stimulated conditions since almost all eucaryotic and procaryotic organisms produce EVs. Mice are widely used as model organisms in many fields of biology and medicine. They are relatively fast-growing, similar to humans, and can be genetically modified to achieve desired model properties. It is possible to use CRISPR (Clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR associated protein 9), ZNF (Zinc-finger nucleases), TALENS (Transcription activator-like effector nucleases), or other methods for precision gene editing and make mutant lines of mice or other model organisms (Barreiro et al., 2020). For example, CRISPR/Cas9 mechanism edited mice can develop renal dysfunction which can be compared to human diseases (Gómez-García et al., 2022). There are different models which imitate disease or medical conditions making research more tissue or organ-specific. Therefore, diabetic mice as model organisms create the possibility to investigate potential EVs roles and to determine EV functionality.

1.5. Extracellular Vesicles and Diabetic Kidney Disease

In 2020, approximately 4500 patients received diabetes diagnoses in Estonia (*Tervisestatistika Ja Terviseuuringute Andmebaas*, n.d.). According to the Estonian Diabetes Association, about 70 000 Estonians are already diagnosed with diabetes (Estonian Diabetes Association, 2022). Individual treatment plan is assigned to each diabetic patient in Estonia, and they undergo regular health assessments. Urine and blood samples are collected regularly to assess treatment efficiency and disease progression. In addition, many patients have to monitor their blood sugar daily to adjust treatment as necessary. It is vital to control kidney function during the routine health assessments in these patients as 35 to 50% of diabetic patients as a complication develop diabetic nephropathy (DN) (Estonian Diabetes Association, 2022).

Proteinuria, specifically microalbuminuria, is one of the main kidney dysfunction indicators (Abebe et al., 2019) in diabetic patients. Unfortunately, proteinuric urine indicates already progressed disease and earlier diagnostic methods are in need. To increase patients' quality of life new monitoring, prognostic and diagnostic markers need to be developed which could indicate even the smallest, yet specific, changes in the diabetes progression.

Exosomes and microvesicles are considered potential diabetic and kidney physiology biomarkers (Merchant et al., 2017). Lipids, proteins, RNAs, and other biochemical compounds derived from urinary extracellular vesicles (uEVs) participate in cell-to-cell communication and thus EVs could be used to assess treatment efficacy, disease diagnostics, and treatment evaluation in patients with kidney problems.

EVs found in urine enable better diagnostics of early IgA nephropathy and other forms of nephropathy for patients with microscopic hematuria (W. Zhang et al., 2016). All cell types in kidneys produce EVs and researchers have shown variations in EVs profiles in case of pathophysiological conditions (W. Zhang et al., 2016). Researchers have detected several potential biomarkers for DN. One of those is protein Wilms tumor 1 (*WT1*) derived from blood EVs, which is upregulated in most diabetic patients' samples but is absent in healthy individuals (Hashemi et al., 2021). In addition to proteins, EV-derived microRNAs are assessed as potential diabetic markers. Experiments done on the mouse models show that over 20

miRNAs are up or downregulated in the diabetic mouse model compared to the control group (Barutta et al., 2013).

In addition to work done on animal models or blood, isolation and analyzing urine EVs is a promising noninvasive diagnostic and prognostic tool. Discovering new biomarkers can make it more precise and universal method for disease control. EVs have become a popular research topic to find novel biomarkers of diabetic kidney disease (DKD) or DN (W. Zhang et al., 2016). DN is a microvascular complication in patients with both type I and II diabetes associated with poor glycemic control (Lu et al., 2020).

Several potential biomarkers have been found in the uEV, but the normalization of the results remains challenging. In diabetic patients' urinary exosomes biomarkers such as proteins *MLL3*, *AMBP*, *VDAC1,* and *WT1* (Figure 3) have been detected (W. Zhang et al., 2016). It has been noted that not only proteins could be EVs biomarkers but also diabetic patients' RNA profiling in uEVs can vary from healthy individuals. Already in 2013, it was shown that 22 microRNAs had altered levels in patients with microalbuminuria compared with patients without albuminuria. The most significant changes were miR-130a and miR-145 upregulation in patients with microalbuminuria and miR-155, miR-424 downregulation (Barutta et al., 2013). More research has been done on diabetic animal models, which support miR-145 potential as a biomarker for DKD (W. Zhang et al., 2016).

Biomarkers	DN type	Marker	Source/origion	Isolation method
miRNAs	Type 2 DN	Combined let-7i-5p, miR-15b-5p, miR-24-3p, and miR-27b-3p	Urinary extracellular vesicles	miRCURY™ Exosome Isolation Kits
	Type 2 DN	miR-362-3p, miR-877-3p, miR-150-5p, and miR-15a-5p	Urinary exosomes	Ultracentrifuge
	Type 2 early DN	miR-192	Urinary extracellular vesicle	Ultracentrifuge
	Type 1 DN	miR-130a,miR-145, miR-155, and miR-424	Urinary exosomes	Ultracentrifuge
	Type 2 DN	miR-320c	Urinary exosomes	Exosome precipitation reagent ExoQuick-TC
	DN rats	miR-451-5p	Urinary exosomes	Differential centrifugation
mRNAs	Type 1 and 2 DN	WT1 mRNA	Urinary exosomes	Ultracentrifuge
Proteins	Type 1 and 2 DN	Regucalcin	Urinary exosomes	Combination of an ultracentrifugation-based protocol with DTT treatment of the low-speed pell
	Type 1DN	WT1 protein	Urinary exosomes	Differential centrifugation method
	Type 1 and 2 DN	AMBP, MLL3, and VDAC1 protein	Urinary exosomes	Combination of an ultracentrifugation-based protocol with DTT treatment of the low-speed pell
	Type1 DN	Cystatin B and NGAL	Urinary exosomes	Hydrostatic filtration dialysis

Figure 3: Diabetic nephropathy (DN) biomarkers, sources of origin, and isolation methods. The figure is from Lu Y, Liu D, Feng Q and Liu Z (2020) Diabetic Nephropathy: Perspective on Extracellular Vesicles. Front. Immunol. 11:943

1.6. Challenges With Urinary EV biomarkers

EVs have been found in various bodily fluids such as blood, saliva, breastmilk, and urine. Urine is a promising source of biomarkers since it can be collected in large amount for non-invasive diagnostics and changes in urine can be detected daily (or even hourly). For early diagnosis of disease, some new urine biomarkers have been detected but more research needs to be done. Analyzing uEVs and detecting specific molecular contents may be useful for finding new biomarkers for kidney diseases (W. Zhang et al., 2016). All uEVs secreted in urine transport proteins, nucleic acid, and small metabolites from epithelial cells forming the nephron and lower urinary tract (Musante et al., 2020). Therefore, urine contains EVs from all sections of the nephron. It is possible to determine from which parent cell EVs originates because it contains markers of that parent cell. Such markers include *PC1*, *PC2* which indicates EV originates from cilia of kidney cells or *AQP-2* indicative of collecting duct origin, etc. (Erdbrügger & Le, 2016). It has been found that EVs are not only important diagnostic tools for assessing the damage done by the disease, but they can also contribute to the development of the disease too. EVs may have significant importance in renal health by

mediating cell to cell signals and by protecting the urinary tract from bacterial infection (antibacterial effect) (Erdbrügger & Le, 2016).

Characterizing isolated EVs can be challenging since we lack a single comprehensive method and have to analyze the heterogeneous population of EVs with various origins and functions. According to Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018) (Théry et al., 2018) nanoparticle tracking analysis (NTA) is widely used to assess the particle number. NTA method is measuring particles by light scattering and measuring the Brownian motion. Yet there are a few challenges that need to be faced when using NTA on uEVs samples and when interpreting the results. It is noted that traditional NTA particle concentration results are interfered by the abundant proteins present in urine such as Tamm-Horsfall Protein (THP) (Droste et al., 2021). THP, also known as uromodulin (UMOD), is produced in kidneys, but the function remains unclear. It is estimated that UMOD plays important role in salt transportation or in urinary tract infection (UTI) prevention (Devuyst et al., 2017). Even the isolation of uEVs can be challenging because of the UMOD. THP is a glycosylphosphatidylinositol-linked membrane protein which has a *zona pellucida* (ZP) domain of approximately 260 amino acids including 8–10 conserved cysteine residues (Fernández-Llama et al., 2010). Polymeric THP network can trap EVs and make isolation of uEVs inefficient. Therefore, removing THP is necessary for successful isolation and for the accuracy of the results.

1.7. Isolation Techniques of Urinary Extracellular Vesicles

Several methods to isolate uEVs have been developed but all of them have several disadvantages. Most isolation ways depend on the physicochemical properties of uEVs for their purification (Merchant et al., 2017). Methods can be separated into size-based or density-based isolations (Figure 4). Density-based separation methods are different centrifugations such as ultracentrifugation or differential centrifugation. Size-based methods include filtration and size exclusion chromatography (SEC). For the best result, a combination of different

methods should be used to isolate a larger part of the heterogenous EVs population (Liangsupree et al., 2021).

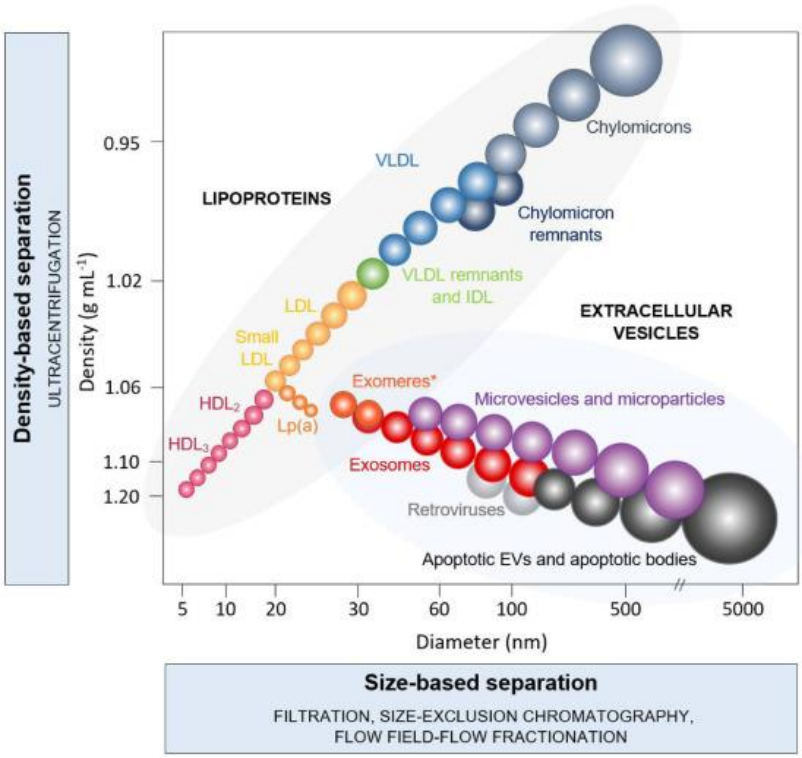

Figure 4: Extracellular vesicles (EVs) and lipoprotein subtypes (HDL: high-density lipoprotein, LDL: low-density lipoprotein, Lp(a): lipoprotein(a), IDL: intermediate-density lipoprotein, and VLDL: very-low-density lipoprotein) and their physical characteristics. From Liangsupree, T., Multia, E., & Riekkola, M.-L. (2021). Modern isolation and separation techniques for extracellular vesicles. Journal of Chromatography A, 1636, 461773.

Many studies use a density-based method as the first isolation method (Figure 5). One of many ways to isolate uEVs is a two or more step differential centrifugation process. The THP network is usually disrupted in the step where the sample is centrifuged at a low speed (17 000g). In that process, THP can be denatured by adding precipitants such as dithiothreitol (DTT), salt, acetone, or other organic solvents. DTT precipitation aims to denature the ZP domains resulting in the THP degradation. EVs remain in the supernatant and in the second faster spin (200 000 g) exosomes will accumulate at the bottom of the tube in a pellet.(Fernández-Llama et al., 2010). Various differential and ultracentrifugation methods are used but each lab has optimized the protocol to their needs. Protocols vary with centrifugation duration, speed, force, and added precipitation agents. The aim for all developed protocols is the same – to achieve the highest yield and purity of EVs in the sample.

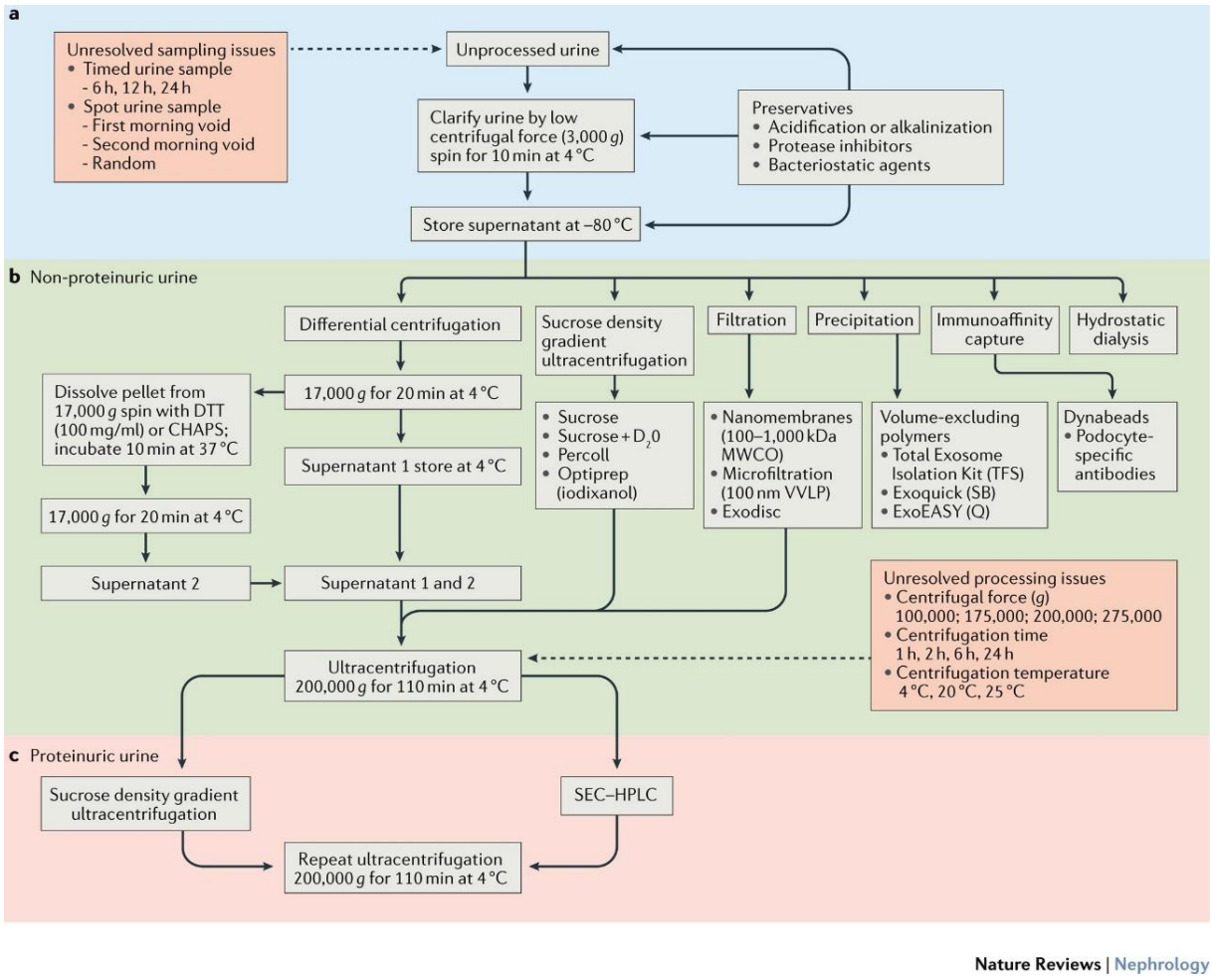

Figure 5: In part A it is shown the urine collecting step which can be as a spot urine or timed urine sample. Collected urine is unprocessed and needs to be clarified by low speed or force centrifugation step. In this step, some contaminants such as bacteria and cells are removed. In part B the urinary extracellular vesicle isolation takes place. In this step there are many different methods. Choosing the best isolation method depends on the type of urine sample (proteinuric or non-proteinuric) and what it is cleaned for (to analyze transcriptomics or proteomics or for other downstream analyses). These methods are differential centrifugation or ultracentrifugation, single step centrifugation using density gradient material (sucrose, Percoll), filtration or ultrafiltration, precipitation (for example, Exoquick), immunoaffinity capture and hydrostatic dialysis. For proteinuric urine from patients with nephrotic syndrome step C is necessary. This step additionally purifies EV sample from contaminants such as high molecular weight protein complexes. CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; D2O, deuterium oxide; DTT, dithiothreitol; HPLC, high performance liquid chromatography; MWCO, molecular weight cut-off; Q, Qiagen; SB, Systems Biosciences; TFS, ThermoFisher Scientific; VVLP, hydrophilic polyvinylidene difluoride. Figure is used from Nature Reviews Nephrology 12, 731-74 9(2017) by Michael L. Merchant et al.

The second way to isolate EVs is density gradient ultracentrifugation. To create a density gradient sucrose or Percoll is added to samples. It is known that exosomes can be further characterized by their ability to float on a sucrose gradient at a density of 1.13–1.19 g/ml (Merchant et al., 2017). Density gradient ultracentrifugation has single-step centrifugation where the EVs will float into the sucrose gradient and they can be separated based on their density. This method can be used to clean proteins and other unnecessary products from the sample. The pellet at the bottom of the tube will contain all the contaminants after ultracentrifugation. This method works only for specific EV types with suitable densities which allows them to float in sucrose. (Merchant et al., 2017). However, ultracentrifugation combined with density gradient can result in higher purity of EVs compared to regular ultracentrifugation protocols.

Other ways for EVs isolation include antibody-based affinity capture, ultrafiltration, and polymer-based precipitation (Figure 5). Ultrafiltration is a faster and simpler method to isolate uEVs (Merchant et al., 2017). Usually, for this method, a polyethersulfone nanomembrane filter with an approximately 100 kDa molecular mass cut-off is used. This method is used to isolate specific sized EVs such as exosomes from small volumes of urine (about 0.5 ml). It is noted that ultrafiltration is as effective as the ultracentrifugation method (Merchant et al., 2017). Unfortunately, ultrafiltration is not compatible with all downstream proteomic analyses (liquid chromatography-mass spectrometry) because some EVs can get stuck in nanomembrane and to release them from the membrane heated Laemmle buffer containing 400 mM DTT should be used. However, for non-proteinuric urine ultrafiltration has been shown high yields with similar RNA concentrations compared to the ultracentrifugation method and the RNA profiles of samples from both methods are also similar (Merchant et al., 2017). For proteinuric urine, ultrafiltration is not recommended method since it also retains and concentrates soluble proteins that are present in urine (Gonzales et al., 2008). These proteins can adhere to the nanomembrane which will reduce the efficiency of ultrafiltration. Therefore, ultrafiltration is a suitable method for RNA profiling, but ultracentrifugation should be preferred for studies of the proteome and for proteinuric urine.

The increasing use of co-precipitants has increased with the development of the methods. Polyethylene glycol combined with low-speed centrifugation has been more popular to gather exosomes. Precipitation methods such as ExtraPEG are inexpensive and they sufficiently

harvest total protein and RNA from vesicles (Rider et al., 2016). There are many precipitation isolation methods developed for total exosome isolation or for targeted EVs isolation. For targeted EVs isolation immunoprecipitation can be used since this method uses specific antibodies against EVs surface proteins such as CD63, CD99, and CD81(Markowska et al., 2017).

To achieve even better results and more optimized methods novel techniques of EVs isolation based on hydrostatic filtration dialysis (HFD) are developed. These methods claim to be quick, inexpensive, and simple. This makes HFD ideal for diagnostic tool (Barreiro et al., 2020). HFD method includes centrifugation, inhibition of bacterial growth and filtration with cellulose ester dialysis membrane with a molecular weight cutoff (MWCO) of 1000 (Figure 6). These steps concentrate the sample to approximately 5 ml after which comes membrane washing and HFDa fractioning. After these steps samples can be collected into tubes and used for downstream applications. The exact protocol for EVs isolation is available in Gnudi, L., & Long, D. A. "Diabetic Nephropathy. Methods in Molecular Biology" (2020).

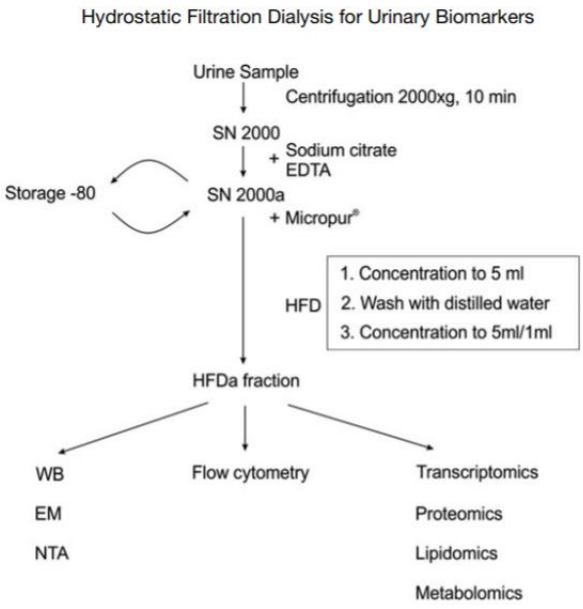

Figure 6 General protocol for hydrostatic filtration dialysis. EM electron microscopy, WB Western blot, NTA nanoparticle tracking analysis. From Gnudi, L., & Long, D. A. "Diabetic Nephropathy. Methods in Molecular Biology" (2020) page 181.

Besides the methods mentioned before, more commercialized new isolation kits have made it to the market. For example, Ymir Genomics has developed a novel precipitation method that does not require polyethylene glycol (Markowska et al., 2017). The Ymir Genomics kit is a simple and effective method to isolate uEVs and RNA. The method uses a precipitation reagent which depends less on concentration. Ymir kit has shown promising results with a lower concentration and is less laborious compared to ultracentrifugation. This method makes it easier to detect biomarkers in smaller volumes (Markowska et al., 2017).

Most of the methods used are isolating EVs based on their size (filtration, flow field-flow fractionation), density (ultracentrifugation, density gradient) (Figure 5), charge (Electrophoresis, dielectrophoresis), or affinity (precipitation, immunoprecipitation) (Liangsupree et al., 2021). For the best result, a combination of methods could be used. In this way a higher yield of EVs could be isolated and separated. One more common combination would be size-exclusion chromatography (SEC) with a filtration-based method (Liangsupree et al., 2021).

1.8. Comparison of different methods of EVs purification and isolation

An overview of the most commonly used methodologies, the time needed to perform the methodology, advantages and disadvantages of each method is depicted in Table 1. The most common method with proteinuric urine is differential centrifugation and ultracentrifugation but many other precipitation methods are used as well.

Table 1 Comparison of various EV isolation methods.

	Method	**Advantages**	**Disadvantages**	**References**
1	Differential centrifugation	Eliminate cellular debris, apoptotic bodies, and larger EVs	Rotor type and centrifugation time influence the yield and purity Does not allow high throughput of sample Time consuming Contaminants (Proteins)	(Cvjetkovic et al., 2014) (Musante et al., 2014)

			Depends on many factors (acceleration, rotor, viscosity of the sample)	
2	Ultra-centrifugation	First method to isolate EVs and is well established Reliable Isolation from large volumes Easy protocol	Contaminated with soluble proteins, such as the Tamm–Horsfall protein (uromodulin) Limited throughput Time consuming and limited sample size depending on the rotor Costly, equipment Isolates only small portion of the EVs Low RNA yield Can damage EVs (100 000–200 000 g for 1.5 h)	(Tomiyama et al., 2021) (Gámez-Valero et al., 2015) (Harding & Stahl, 1983) (Cheruvanky et al., 2007)
	Size filtration	Removes protein aggregates and cell debris	Can fracture larger EVs	(Cvjetkovic et al., 2014) (Liangsupree et al., 2021)
3	Density gradient	Separates contaminants such as protein and protein–RNA aggregates	Not suitable for all downstream applications	(Cheruvanky et al., 2007)
4	Ultrafiltration	As effective as the standard ultracentrifugation method	Loss of EVs into the filter interference by highly abundant soluble proteins	(Cheruvanky et al., 2007) (Rood et al., 2010)

		Can be used for urine samples from patients with proteinuria.	Lower limit of urine sample volume	
5	Hydrostatic dialysis	No need for UC step Suitable for highly diluted samples Very high throughput of samples Cost Standard sample concentration Downstream applications	Equipment Bacterial contamination	(Musante et al., 2014)
6	Size-exclusive chromatography (SEC), columns filled with polymers with heterogeneous pores	allows for identification of lower abundant microvesicular proteins in nephrotic urine without interference by highly abundant proteins Different fractions of EVs (Most EVs included)	non-EV-related contaminants could mask the presence of relevant EV markers time consuming 7 Limited sample volume Processing one sample at a times Time consuming	(Gámez-Valero et al., 2016)
8	Commercial kits: ExoQuick	High yield High purity Universal protocol and easy to use	Expensive Nonbiased information	(Brennan et al., 2020)

9	Commerical kits: Norgen	Easy to use and specific protocols Fast	Expensive Nonbiased informationn	(Tataruch-Weinert et al., 2016)
10	Precipitation with sodium chloride, 0.1 M acetate	Simple Cheap High purity	Contamination Time consuming	(Kosanović & Janković, 2014)
11	Precipitation of proteins with organic solvent (PROSPR), cold acetone	Simple Cheap High purity Smaller amount of proteins	Aggregation in multivesicles Small volumes Time consuming Low yield	(Gallart-Palau et al., 2015)
12	DTT treatment	Simple Cheap Higher yield of EVs	THP can still be present Not suitable for functional studies	(Gámez-Valero et al., 2015)

Based on overview of the uEVs isolation processes at the Table 1, most promising methods are the differential centrifugation with precipitation, HFD or SEC with filtration. One of HFDs biggest advantages in isolating EVs is its compatibility with proteinuric urine, it is not affected by urinary salts or urine concentration, pigments, or proteins like THP glycoprotein. It makes HFD suitable for diabetes and proteinuric urine research. This method is most efficient if working with large amounts of urine because it concentrates sample volume from 1 to 2 L per 24 hours to 2-3 mL of useful urine. Using HFD is practical for diagnostics, prognostics and biomarkers search purposes because it remains all the important EV classes. (Barreiro et al., 2020). In addition, HFD can be used for any downstream application such as NTA, Western blotting, and transmission electron microscopy (TEM). Western blotting is one of the main methods used to characterize EVs and there are many researched markers available. For diabetes patients, Raman spectroscopy could be used to distinguish diabetic patients (with good and unsatisfactory glycemic control) from healthy subjects (Roman et al., 2019). HFD

method protocols have been improved over time and there are rather few articles to show results. Another disadvantage of this method is it is a rather long protocol and with many steps compared to some other methods. It is simple but efficiency depends on the protocol which is used.

Another more traditional method is differential ultracentrifugation. It is suitable for large volume isolation and compatible with proteinuric urine. With this method, it is possible to eliminate additional chemicals and with DTT treatment can be made more efficient. This method is widely used for biomarker isolation. Compared to HFD it has more disadvantages such as low RNA yield and potential for exosome damage. Ultracentrifugation efficiency is affected by the rotor, force, sample properties and so on (Cvjetkovic et al., 2014). Differential ultracentrifugation gives an adequate overview of isolated uEVs, but the quality of samples depends on the protocol which is used.

Isolation of uEVs by differential centrifugation can be challenging because of THP, which is abundant in urine and is present in large amounts. THP is a glycosylphosphatidylinositol-linked membrane protein which has a ZP domain of approximately 260 amino acids including 8–10 conserved cysteine residues (Fernández-Llama et al., 2010). Polymeric THP network can trap exosomes and make isolation of uEVs inefficient. Therefore, removing THP is recommended for the successful purification of exosomal proteins. To precipitate urinary mucoproteins 0.58M sodium chloride (NaCl) could be used (Chen et al., 2020).

EXPERIMENTAL PART

2. AIMS OF THE STUDY

EVs isolation from urine is challenging due to many variable factors such as urine concentration, collection method and additional contaminants such as proteins, cells, and microorganisms. To assure a high-quality analysis of the EVs and their function, effective purification methods need to be established. The main aim of this thesis was to establish the most effective method to purify EVs from urine to achieve high purity, yield, and EV physical quality.

Aims of this investigation were achieved by attaining the following objectives:

- To compare different purification methods of uEVs
- To analyze the concentration of EVs from various purification methods to achieve the highest EV yield
- To characterize purified EVs with imaging methods

3. MATERIALS AND METHODS

3.1. Collection of urine samples

90 ml of morning urine was collected from six subjects at the University of Tartu Hospital (Ethical Committee licence 290/T-20). Urine was collected from healthy people and patients with diabetes. In this study, participants were advised on how to properly collect urine (*Ida-Tallinna Keskhaigla*, 2018). Urine was collected into sterile urine beakers (VACUETTE®) and distributed evenly into 10 ml Greiner BIO-ONE urine tubes with no additives (VACUETTE®). Information about the urine color was recorded as an indicator of urine concentration. Urine color was assessed with indicators from Table 2.

Table 2 Urine color indicators and indicator values.

Indicator	**Indicator value**		
Urine color	Light yellow *Healthy, less concentrated*	Yellow to amber *Healthy, normal urine*	Dark brown *Dehydrated, more concentrated*
Presence of blood	No blood *Normal*		Blood *Not Healthy*
Clear	Clear *Normal*	Cloudy *Not healthy*	Sediment *Not healthy*

Urine samples were collected from male and female patients from various age groups. Information about the patients' age, gender, diagnosis, and disease duration was collected. Information about additional diseases was also collected. An overview of used urine samples is shown in Table 3.

Table 3 Information about participants used in the study.

	Subject 1	**Subject 2**	**Subject 3**	**Subject 4***	**Subject 5***	**Subject 6***
Gender	Male	Male	Female	Male	Male	Male
Age	50	67	28	39	60	72

Urine color	Light yellow, clear	Yellow, clear	Yellow, Clear	Light yellow, Cloudy, Sediment	Dark brown, Cloudy, blood	Dark brown, Clear
Diabetes	No	Type II	Type I	Type I	No	Type II
Duration	-	16 years	27 years	8 years	-	16 years
Additional information	Healthy	-	-	-	UTI Obesity	-

3.1. Sample preparation

After collection, the urine was stored in the fridge at 4º C for up to 12 h. After that, urine samples were stored at – 20º C until further application. Urine samples (~10 ml) were thawed under running warm water for a minute and put on ice until completely thawed. Samples were transferred into clean 15 ml tubes and processed further for the purification and isolation of EVs.

3.2. Purification and isolation of Extracellular Vesicles

In this study, we used a combination of different methods to assure the highest yield of EVs and the purity of the obtained samples. Four different combinations of differential centrifugation, SEC, salt precipitation and PROSPR (Protein Organic Solvent precipitation) methods was used:

1) Differential centrifugation + Filtration + SEC
2) Differential centrifugation + Salt precipitation with NaCl + SEC
3) Differential centrifugation + PROSPR with acetone
4) Differential centrifugation + PROSPR with acetone + SEC

3.2.1. Differential centrifugation

Once urine was transferred into new 15 ml tubes, differential centrifugation steps were performed. Samples were centrifuged at 400 g for 10 minutes at 4 ^{o}C. The supernatant was transferred to a new tube. The supernatant was centrifuged at 2000 g for 20 minutes at 4 ^{o}C. The supernatant was moved to another tube leaving apoptotic bodies and other possible contaminants in the pellet. Finally, the supernatant was centrifuged at 18 000 g for 20 minutes at 4 ^{o}C.

3.2.2. Filtration

After differential centrifugation (Materials and Methods 3.2.1) clean syringe with 0.2 µm filter was prepared. Urine was passed through the filter and transferred into a clean 15 ml tube.

3.2.3. Size Exclusion Chromatography

Purified samples were concentrated down to 500 µl using Amicon® Ultra-15 centrifugal filter devices with a 10 kDa cut-off. EVs from urine samples were isolated using size exclusion chromatography (SEC) benchtop columns (Econo-pac® Disposable chromatography column, Bio-Rad, Berkeley, CA, USA). Chromatography columns were filled with a cross-linked 4% agarose matrix of 90 µm beads (Sepharose 4 fast flow™, GE HealthCare Bio-Sciences AB, Uppsala, Sweden) and columns were stored in the fridge at 4^{o}C for at least 24 hours. The chromatography column was drained from the remaining ethanol and subsequently, the columns were washed with 10 ml of Milli-Q water, and 10 ml of 1x Dulbecco's phosphate-buffered saline (DPBS) (Sigma® Life Science, St. Louis, MO, USA). A concentrated urine sample (500 µl) was loaded on the SEC column. To assure stable flow during fraction collection 10 ml of DPBS (Sigma® Life Science, St. Louis, MO, USA) was added after the sample had passed through. 20 fractions of 500 µl fluid was collected into 1.5 ml tubes. NTA was performed to measure EV concentration in all fractions.

3.2.4. Salt precipitation

Thawed urine samples (~10 ml) were transferred into 15 ml tubes and differential centrifugation steps at 400 g for 10 minutes at 4° C, and 2000 g for 20 minutes at 4° C were performed. After each centrifugation step supernatant was transferred into a clean 15 ml tube to eliminate cell debris and apoptotic bodies, respectively. Sodium chloride (NaCl) was added to the supernatant to a concentration of 0.58M. Samples were incubated for 2 hours at 4° C to let the salt precipitate proteins in the sample. After the precipitation samples were centrifuged at 18 000 g for 25 minutes. The supernatant was collected into a new tube, leaving the pellet with contaminants untouched.

3.2.5. Protein Organic Solvent precipitation

Urine samples were purified from cell debris, dead cells, apoptotic bodies, and other contaminants by using differential centrifugation steps described previously in Materials and Methods section 3.2.1. Samples were concentrated to 500 µl using 10kDA Amicon® Ultra-15 centrifugal filter units and the sample was placed into a new 15 ml tube. Cold (-20 °C) acetone was added to the sample with 1:4 ratio (1 part sample and 4 parts acetone). The sample was gently mixed by inverting the tube. The centrifugation step at 3000g for 5 minutes at 4 °C was followed immediately after mixing. The supernatant was pipetted into clean 2 ml tubes and samples were concentrated using a vacuum concentrator to a total volume of 500 µl. In the vacuum concentrator tube lids were left open and final samples were pooled together into 1.5 ml tube for further application.

3.3. Characterization methods

3.3.1. Nanoparticle tracking analysis

EVs size and concentration was detected with the ZetaView PMX 110 NTA instrument (Particle Metrix GmbH, Inning am Ammersee, Germany). Standard manufacturers' protocol was followed for the nanoparticles size distribution and concentration measurements. NTA machine was calibrated before measurements with a 100 nm particle standard *(*Applied Microspheres BV, Leusden, The Netherlands). The measurement cell was cleaned before each sample injection with Milli-Q® water and DPBS (Sigma® Life Science, St. Louis, MO, USA). Three

cycles of measurements were performed to each sample with 11 frames per cycle. The machine was set to sensitivity 85, shutter speed 70 and frame rate 30 frames per second.

3.3.2. Transmission electron microscopy

Isolated and purified EVs were concentrated to 150 µl using Amicon® Ultra 2 centrifugal filter units (10 kDa) (Merck Millipore Ltd., USA). TEM analysis was performed from a droplet of the purified EVs samples on Formvar-carbon-coated 200 mesh copper grids (Agar Scientific, Essex, UK). The sample was allowed to set for 20 minutes and after fixed on a grid in 2% paraformaldehyde (Sigma-Aldrich, USA) and 1% glutaraldehyde (Polysciences, Warrington, PA, USA), contrasted in uranyl oxalate (a mixture of 4% uranyl acetate (Polysciences, Warrington, PA, USA) and 0.15 M oxalic acid (Sigma-Aldrich, Germany)). Samples were embedded in a mixture of methylcellulose (Sigma-Aldrich, USA) and uranyl acetate (Polysciences, Warrington, PA, USA). Transmission electron microscope JEM 1400 (JEOL Ltd. Tokyo, Japan) was used to visualize the samples at 80 kV. Images were captured with a numeric camera (Morada TEM CCD camera, Olympus, Germany).

TEM imaging samples were prepared for TEM by Signe Parts and the imaging was done in collaboration with prof. Aneta Andronowska from Polish Academy of Sciences in Olsztyn by Mohammad Mehedi Hasan and Qurat Ul Ain Reshi from prof. Fazeli group at the University of Tartu. Images were analyzed, edited, and selected by Signe Parts.

3.3.3. Protein quantity measurement (Bradford assay)

The protein concentration of each sample was determined with the Quick Start™ Bradford Protein Assay (Bio-Rad, California, USA) according to the manufacturer's protocol. The assay was done on a 96-well microplate with the volume of 5 µl of the bovine serum albumin (BSA) standard (2mg/ml, Sigma-Aldrich, USA), negative control (DPBS (Sigma® Life Science, St. Louis, MO, USA)) or samples. 95µl of Bradford Reagent (Ref: B6916, Sigma-Aldrich, USA) was added to the wells. On each plate 1.4, 1.0, 0.5, 0.25 ja 0.125 mg/ml BSA standard (2mg/ml, Ref: P0834, Sigma-Aldrich, USA) dilutions were made for calibration curve. Samples were incubated under foil cover for 15 minutes. Samples were measured with a spectrophotometer

(Ledetect 96 Microplate Reader, Biomed Dr. Wieser GambH, Austria) at 620nm. A calibration curve was constructed based on the previously mentioned BSA standard dilutions and the protein concentrations were calculated according to the calibration curve. Each sample was analyzed in three replicates.

3.4. Statistical analysis

All data from NTA was transformed from text (.txt) files into Microsoft® Excel® worksheets (.xlsx files) using a self-made Python program (Appendix 1). Data analysis was conducted using Microsoft® Excel® (2019, version 2204) and R (version 4.0.3.0). Statistical significance was calculated with an unpaired two-tailed student t-test. P-values lower than 0.05 were considered significant.

Sample protein concentrations were analyzed using Microsoft® Excel® (2019, version 2204) where the average of three replicates light absorption was calculated, a calibration curve was prepared, and final protein concentrations were calculated in mg/ml.

4. RESULTS AND DISCUSSION

4.1. Method optimization

To achieve the highest yield and purity of EVs, various methods were combined. It is important to discard one of the most abundant proteins in urine – Tamm-Horsfall protein (THP). According to the literature review following methods were combined for optimization (Materials and Methods 3.2):

- Differential centrifugation with filtration and SEC
- Differential centrifugation with salt precipitation and SEC
- Differential centrifugation with PROSPR
- Differential centrifugation with PROSPR and SEC

Optimization included evaluating differential centrifugation steps, precipitation time and concentration and selection of precipitating agents. As a result, 4 methods protocols were created to isolate EVs from diabetic patients' and healthy subjects' urine.

Protocol for method 1 was created to use differential centrifugation with filtration and SEC (Figure 7).

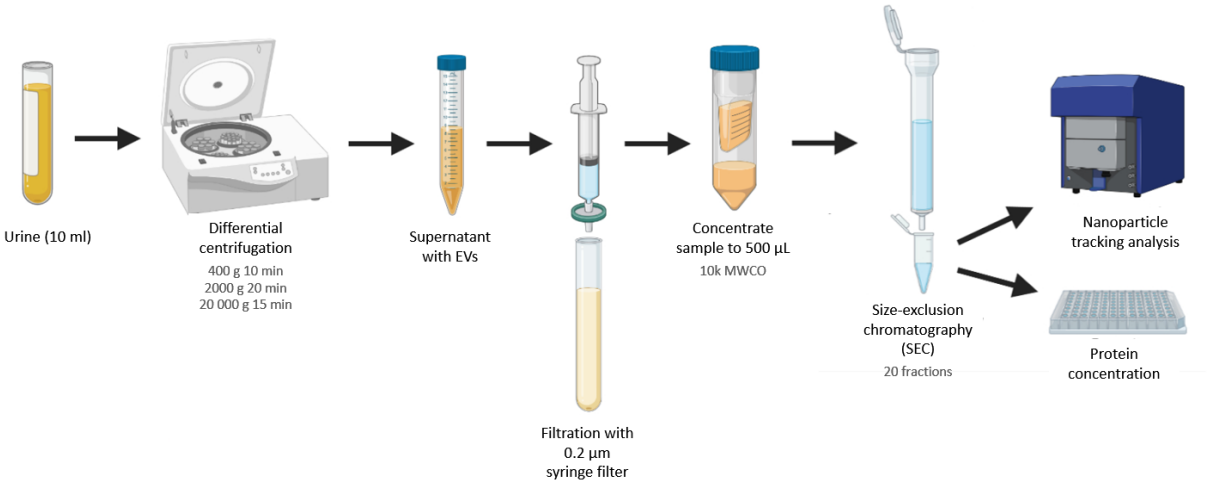

Figure 7 Optimized workflow for EVs isolation and purification with differential centrifugation, filtration and SEC referred to as Method 1. Optimized protocol is created for 10 ml of urine. With differential centrifugation acceleration applied is shown in g units and time in min (minutes). Filtration with a syringe is shown in µm and concentrating samples were done with a 10K molecular weight cutoff (MWCO) concentrator. EVs – Extracellular vesicles

Two precipitation agents were used with the combinations of EVs isolation and purification methods. In the optimization of precipitation protocols, the main aim was to discard proteins such as THP and albumin. A healthy persons' urine has relatively low to no protein concentration, but diabetic patients can have proteinuria. It is necessary to assure these purification methods are appropriate for clinical applications therefore optimization was done to work with both healthy and proteinuric urine.

For method 2 sodium chloride (NaCl) was used as a precipitating agent. According to the literature, to precipitate the proteins in the sample, the most optimal concentration of NaCl is 0.58 M (Literature review 1.8) with a precipitation time of 2 hours.

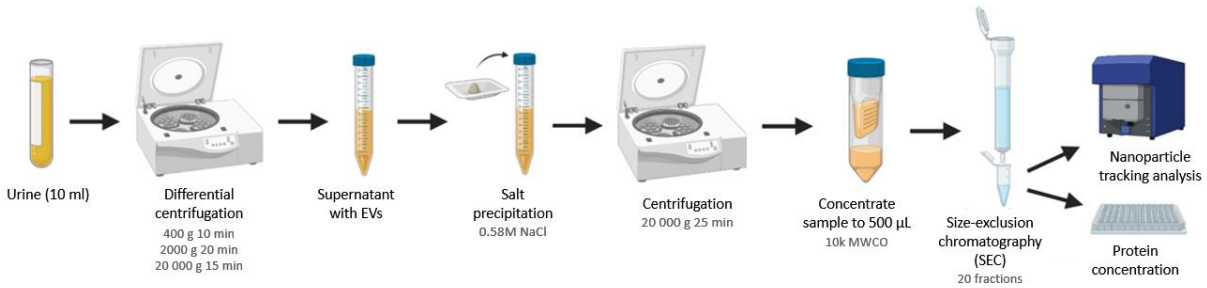

Figure 8 Method 2 workflow with differential centrifugation and salt precipitation with NaCl and size-exclusion chromatography (SEC). 10 ml of urine is used for the protocol. For differential centrifugation acceleration applied is shown in g units and time in min (minutes). The salt concentration is in molar concentration (M) and samples were concentrated with 10k molecular weight cutoff concentrator.

For method 3 acetone was selected as the precipitation agent. Most proteins are insoluble in acetone resulting in setting into a pellet after the centrifugation process. It is mentioned in several papers that cold acetone works the best for protein precipitation (Baghalabadi & Doucette, 2020).

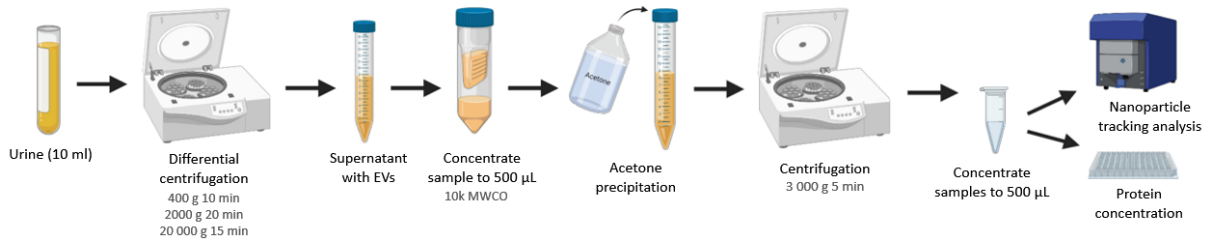

Figure 9 Method 3 optimized workflow for differential centrifugation and PROSPR with acetone. Protocol was optimized for 10 ml of urine. For centrifugation acceleration applied is shown in g units and time in min (minutes). Samples were concentrated with 10k molecular weight cutoff concentrator.

For the 4th method, we used acetone precipitation with SEC. Due to acetone's possible lowering effect on the yield, using additionally SEC could result in a very low concentration of EVs. Yet using SEC could help us select specific sized particles and therefore enables isolation of high purity heterogeneous population of EVs and discard possible contaminants.

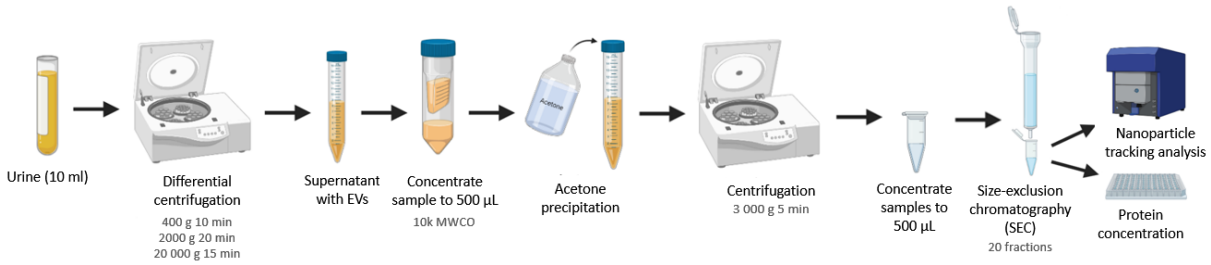

Figure 10 Method 4 optimized workflow for differential centrifugation and PROSPR with acetone and SEC. 10 ml of urine was used for the protocol. Centrifugation acceleration applied is shown in g units and time in min (minutes). Samples were concentrated with the 10k molecular weight cutoff concentrator. Samples were run through the size-exclusion chromatography (SEC) column.

4.2. EV concentration

EV concentrations were measured in purified samples with NTA. EV concentration is an important indicator of isolation methods efficacy since we desire the highest possible EV yield. We measured EV concentration of 4 EV isolation methods for all 6 participants (Figure 11). Pooled together EV fractions were used for the measurement. EV concentration was highest for participant 1 sample purified with method 2 (Differential centrifugation with salt

precipitation and SEC). Participant 1 sample was the healthy person sample. Method 2 gave the best results for both participants 1 and 2 while having promising results for all the samples. Method 2 was also very stable and gave the best results for participants 4 and 6.

Method 3 (Differential centrifugation and PROSPR with acetone) results depended on each participants. Method 3 seems to work better for diabetic patients' urine and less for healthy participant. More research should be done to confirm method 3 efficacy. The lowest yield for all participants was with method 4 (Differential centrifugation with PROSPR and SEC).

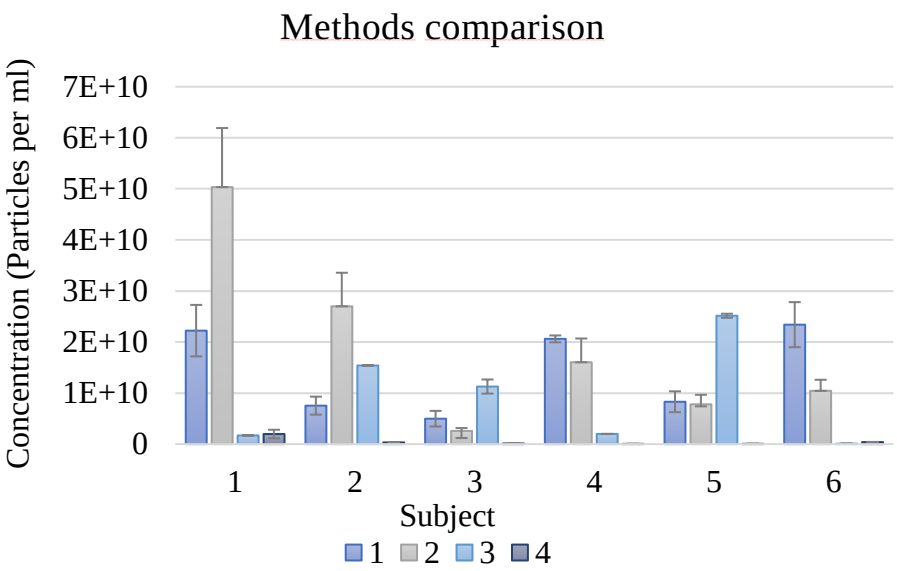

Figure 11 Methods comparison between individuals. Bar plot is created for 4 methods (different colored bars) for 6 participants (on the x-axis). EV concentration of pooled together extracellular vesicle fractions is shown in particles per ml on the y-axis. Error bars are SD of pooled together fractions.

Additional statistical analysis was performed to assess each method's results. Two-tailed student t-test showed significant difference between methods 1 and 4 (p = 0.002241151), and for 2 and 4 (p = 0.026347925) (Figure 12). No statistically significant difference was detected between methods 1 and 2.

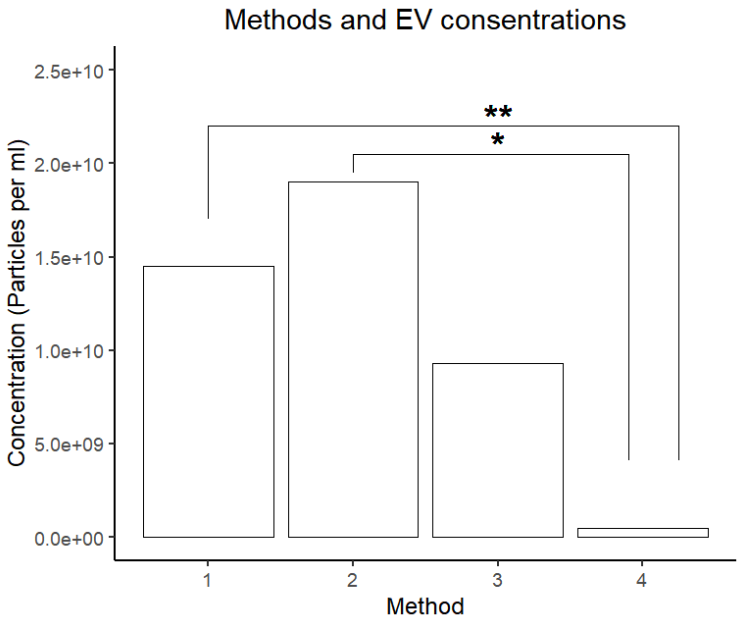

Figure 12: Methods and EV concentrations student t-test results. Two-tailed student t-test was performed to compare each methods EV concentration (n = 6). Method 1: Differential centrifugation with filtration and SEC. Method 2: Differential centrifugation with salt precipitation and SEC. Method 3: Differential centrifugation and PROSPR with acetone. Method 4: Differential centrifugation and PROSPR with acetone and SEC. Difference between Method 1 and 4 was considered significant as well as difference between Method 2 and 3. * p-value < 0.05, ** p-value < 0.005.

These results confirmed that method 4 resulted in a relatively low yield of EVs. It is understandable since protein precipitation with PROSPR can lower the EV yield as shown on Figure 12 for method 3 and method 4. In addition to method 3, method 4 had SEC which eliminated an even bigger fraction of EVs compared to method 3.

Considering only EV yield, more efficient EV isolation methods are method 1 and method 2. Both methods had a statistically significant difference ($p < 0.05$) with our least efficient method (method 4), indicating relatively high concentrations of EVs. In contrast to method 4, which also used SEC, methods 1 and 2 show that using SEC can give us high yield if we don't over-process the samples.

4.3. Protein concentration

Protein concentrations were measured for all isolated EV fractions with the Bradford assay. All samples contained less than 0.5 mg/ml of protein (Figure 13) indicating that all samples were with high purity. Some samples (Figure 13 P2.1, P5.1, P5.2) showed a slight increase in protein levels after the 13th fraction. It is considered as protein fractions for SEC since EV-sized particles have passed through the column before and leaving proteins or other contaminants to later fractions. In these fractions, there was no change in NTA measured particle concentration and therefore there were no significant amount of EVs present. No detectable EV fraction was apparent for method 4 participant 5 (Figure 13 P5.4). We cannot see the protein or EV fraction-based concentration distribution for method 3 due to not including SEC as a method since we received fractions of EVs after the EVs isolation with SEC. For the highest concentration of EV samples, fractions 4-7 were pooled together according to the NTA EV concentration in fractions (Figure 13).

The purification method 4 with PROSPR and SEC resulted in low yield and untypical EV fraction distribution. It eliminates all the necessary contaminants but leaves us with low yield EVs. Method 4 proves to be unsteady with large SD variations shown as error bars on our plot chart (Figure 13). In contrast methods 1 and 2 show smaller SD values with a less fraction-based variation. Even the purity of these two methods is high and stable for all the participants.

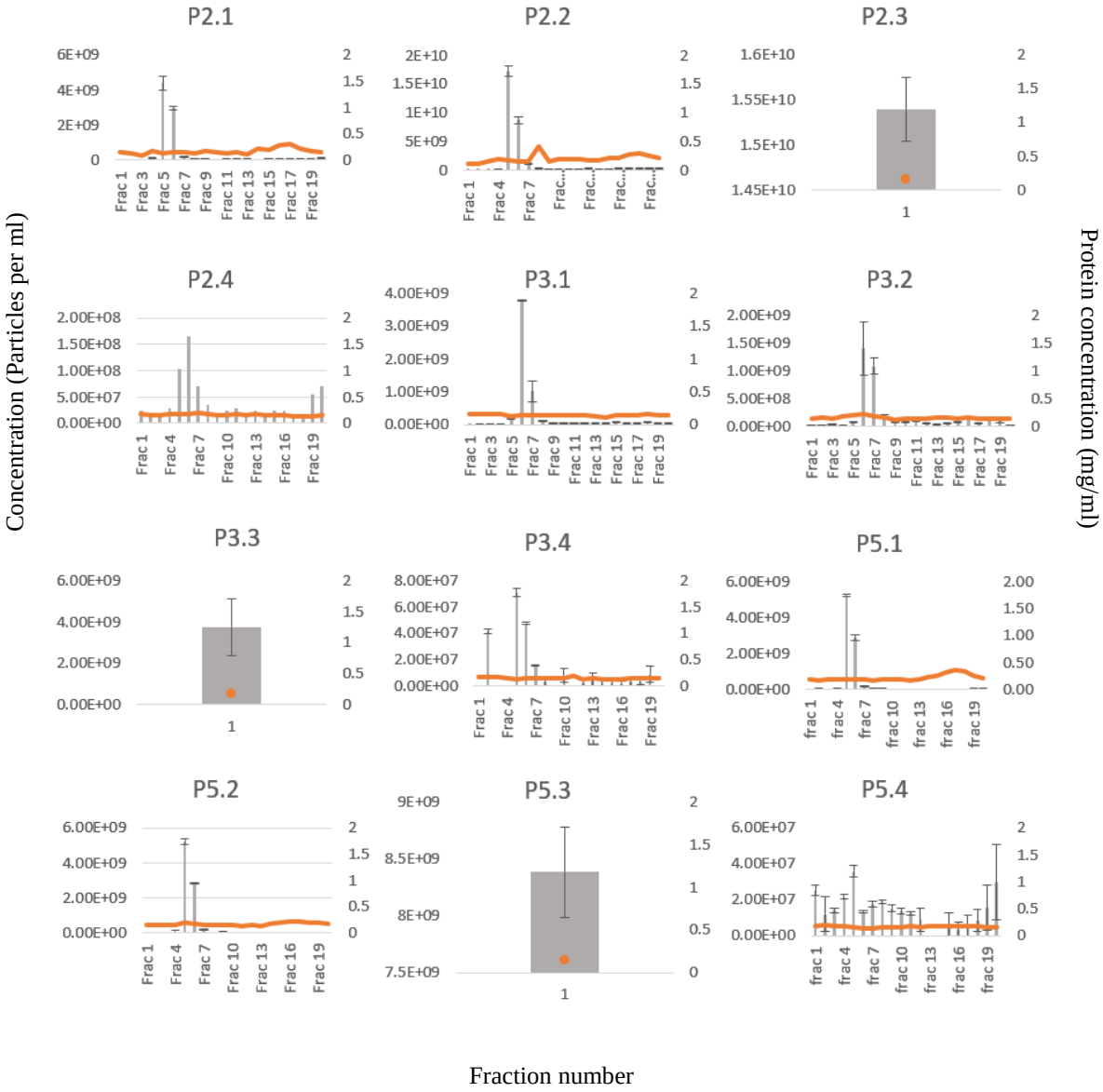

Figure 13 Protein concentration for randomly selected participants for all isolation methods. On the left axis for the bar plot is EV concentration in particles per ml and on the right axis is protein concentration in mg/ml. Protein concentration is shown with orange. Error bars are SD of the fraction.

4.4. Size distribution of isolated EV

Typical EV size varies from 30 nm to 1000 nm. Size distribution indicates the concentration of different-sized particles in the sample.

In our study, most EVs range between 15 to 350 nm and the NTA measurement size distribution for EVs is bell-shaped with a peak at around 135 nm (Figure 14). Sample concentration varies between individuals, but the size distribution of the particles is similar for all participants. EV size ranges for samples A, B, and C from 15 nm to 315 nm. Up to 350nm size particles were in samples D and E. Smaller range was for sample F from 15 to 255 nm. Interestingly, method 3 PROSPR and method 4 acetone PROSPR with SEC has a smaller particle range for all the samples. For all samples, the range for these methods is up to 200nm. A smaller size range indicates that some EVs populations are eliminated from the samples.

Due to low EV yield, method 4 was discharged from further analysis and characterization.

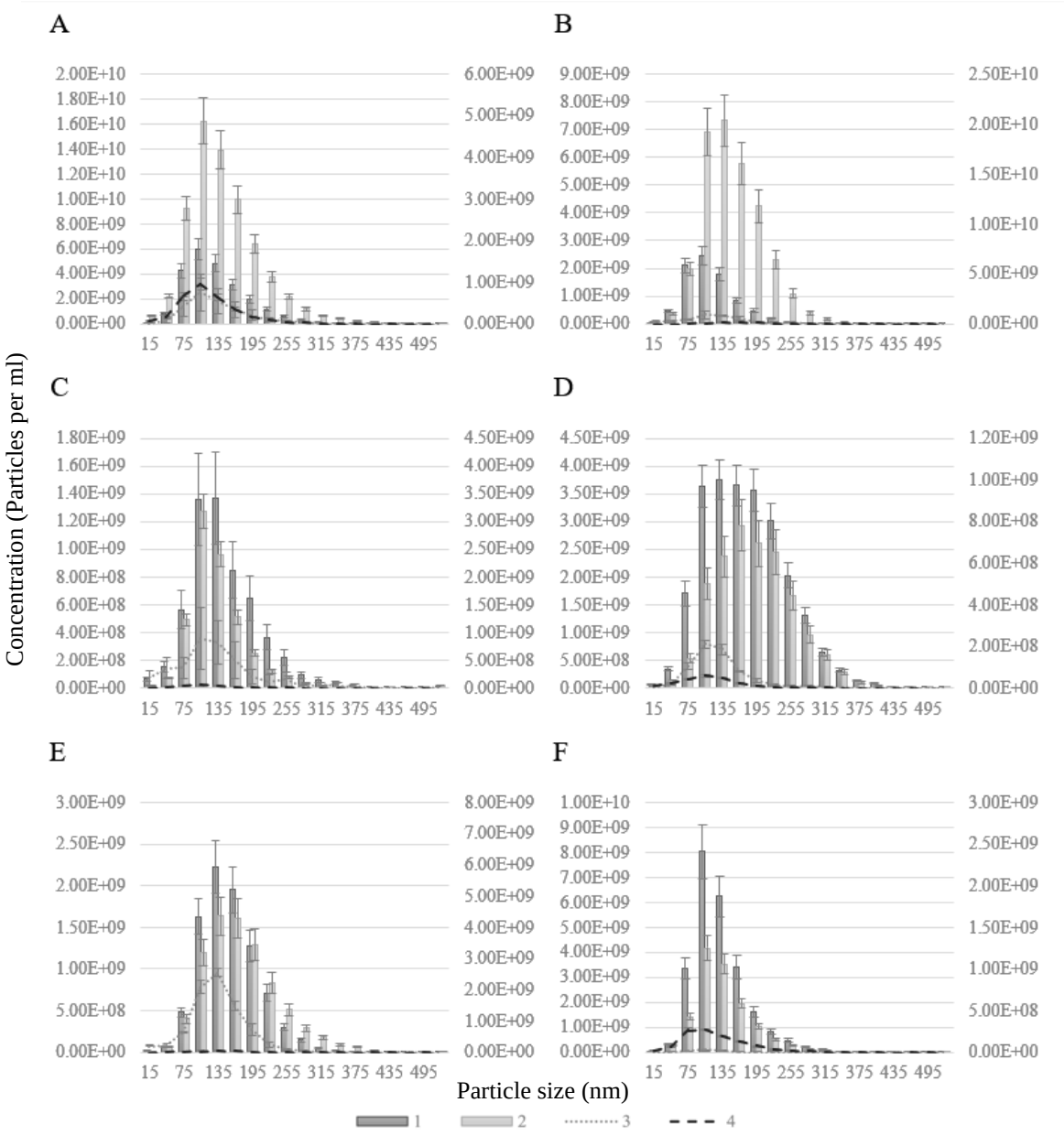

Figure 14 EV size distribution with different methods measured with NTA. Figure A presents results from person 1, B person 2, C person 3, D person 4, E person 5, and F person 6. Particle size is shown on the x-axis in nm and concentration in the y-axis as particles per ml. Methods: 1 - Differential centrifugation + Filtration + Size-exclusion chromatography (SEC); 2 - Differential centrifugation + Salt precipitation with NaCl + SEC; 3 - Differential centrifugation + PROSPR with acetone; 4 - Differential centrifugation + PROSPR with acetone + SEC. Methods 1 and 2 concentrations are shown on the left and for methods 3 and 4 are shown on the right axes. Error bars are SD of the same size range particles.

4.5. EV characterization with TEM

TEM was used to image the purified EVs samples and to describe particles measured with NTA. EVs are naturally spherical particles and with TEM are captured as cup-shaped particles. Comparing the same individual EVs samples with TEM images will show if the sample contains EVs or not but it cannot be used as a quantitative method to measure the concentration of EVs.

With all methods, EV particles were present (Figure 15) but for method 3 low count of particles was seen. Interestingly, for method 3 all particles were of similar size. TEM image showed high purity for all 3 methods as the background of the imaged samples was clean and no additional contaminants were detected.

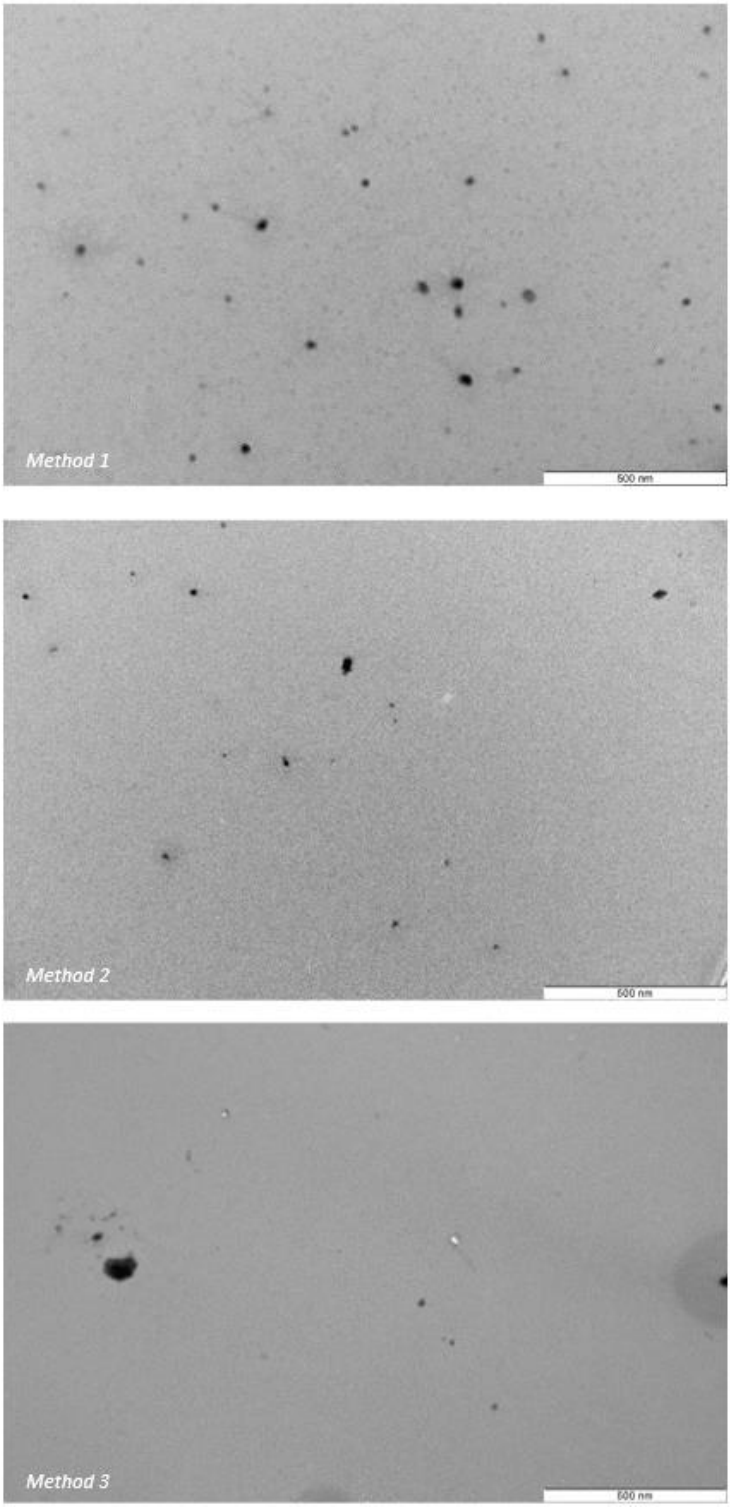

Figure 15 Transmission electron microscopy images of methods 1, 2 and 3. Method 1 and method 2 contain more extracellular vesicles compared to method 3. Images were done in collaboration with Aneta Andronowska from the Polish Academy of Sciences in Olsztyn and with Mohammad Mehedi Hasan and Qurat Ul Ain Reshi from prof. Fazeli group at the University of Tartu. On the left side of the images is 500 nm scale.

TEM image for method 1 showed smaller vesicle populations from 30 nm to 80 nm (Figure 16). Method 2 image shoved a more heterogeneous population as particle size varied from 30 nm to 100 nm (Figure 17) with several larger particles. Both methods had clear spherical structured particles which are described as EVs.

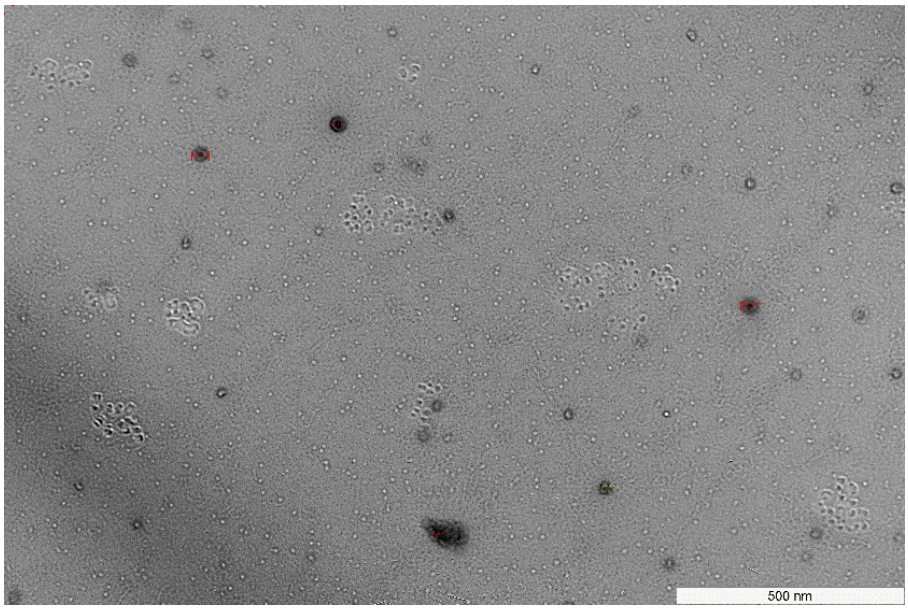

Figure 16 Method 1 transmission electron microscopy image. Image scale 500 nm is shown on the right bottom of the image. Images were done in collaboration with Aneta Andronowska from the Polish Academy of Sciences in Olsztyn and with Mohammad Mehedi Hasan and Qurat Ul Ain Reshi from prof. Fazeli group at the University of Tartu.

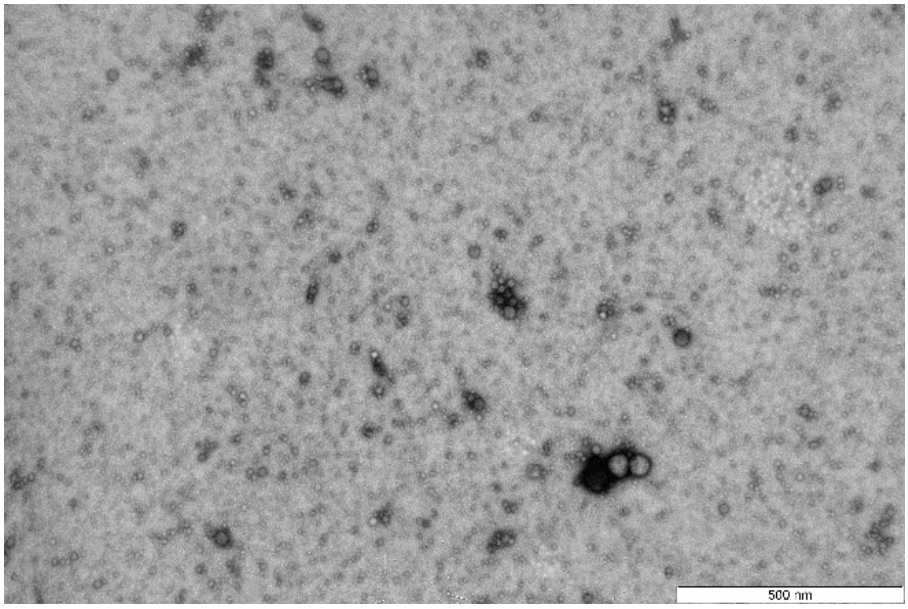

Figure 17 Method 2 transmission electron microscopy image. Image scale 500 nm is shown on the right bottom corner. Images were done in collaboration with Aneta Andronowska from

the Polish Academy of Sciences in Olsztyn and with Mohammad Mehedi Hasan and Qurat Ul Ain Reshi from prof. Fazeli group at the University of Tartu.

4.6. Comparison of methods

Four methods were used to isolate and purify EV urine samples from healthy individuals and diabetic patients. Method 4 was the least reliable with low yield, long sample processing time and low EV quality (Table 4). Method 3 was also less reliable compared to other methods since its yield depended on the subject but always resulted in high purity. In addition, it was the less time-consuming method, but the reproducibility of isolation efficiency was unstable since the yield varied a lot. Both of the methods showed a small shift in the particle range (Figure 14) resulting in a smaller EV size range. Leaving out some size range of EVs indicates that entire populations of EVs have been eliminated during the sample processing. Even the fraction-based distribution of EVs showed changes in EV fractions and low-quality results for method 4 (Figure 13). Interestingly the EVs were so damaged that the proper bell-shaped curve did not form of our measurement results. With these results, we can say that methods 3 and method 4 are not the best suited to analyze uEVs.

Table 4 Comparison of isolation methods. Comparison of time consumption, yield, purity and adjustability to proteins in urine.

	Method 1	**Method 2**	**Method 3**	**Method 4**
Time consumption	3h-4h	3-4h	2-3h	3-4h
Yield	++	++	-	- -
Purity	+	++	++	+
Adjustable	-	+	+	+

The most promising results were for methods 1 and 2 which both had similar time consumption per sample. Both of these isolation methods resulted in high yield and purity. Both methods showed rich TEM images of EVs (Figure 16, Figure 17), where EVs had desired cup-shaped. TEM images confirmed the presence of EVs in our samples purified with methods 2 and 3. Our NTA measurement results aligned well with what we saw on TEM images since

both indicated higher particle count for methods 1 and 2 meanwhile method 3 had fewer particles.

For final comparison, we looked at the methods' adjustability to contaminants. Method 2 advantage was its adjustability depending on the presence of proteins since it can be used for both proteinuric and non-proteinuric urine by adjusting the salt concentration. Both method 1 and method 2 worked well for urine EVs isolation resulting in high yield and purity but for diabetic patients, the salt precipitation works better as it results in higher purity for all sample types.

SUMMARY

According to the International Diabetes Federation (IDF) every 1 in 10 people are living with diabetes making it a global health issue. About 100 years ago first insulin injection made by Leonard Thompson helped us a huge step forward in this disease management but even now we lack effective methods to assess the development of the disease. We are in critical need for more sensitive biomarkers to assess the progression of complications and the disease itself even better.

Extracellular vesicles (EVs) are novel nano-sized biomarkers which mediate cell-to-cell communication. EVs carry many bioactive molecules such as proteins and mRNA. Changes in EV transported molecules are dependent on the cell types, pathophysiological conditions, and environment. Analyzing EV components can help us find more specific and sensitive biomarkers to better understand disease development and drug efficacy.

We aimed to investigate 4 different methods for isolation of EVs from diabetic patients and healthy individuals' urine. Firstly, in our study, we combined and optimized EVs purification and isolation methods to achieve EV presence in the samples with the least contaminants. As a result of protocol optimizations, we created protocols for method 1 (differential centrifugation with filtration and SEC), method 2 (differential centrifugation with salt precipitation and SEC), method 3 (differential centrifugation with PROSPR) and lastly method 4 (differential centrifugation with PROSPR and SEC).

After the protocol optimizations, we compared all the methods for EV concentrations, protein concentrations and physical characteristics. Our study showed a statistically significant differences between methods 1 and 4, and methods 2 and 4. Method 2 proved to be the most effective in the overall comparison of sample purity, EV yield and adjustability to sample type since the method is suitable for non-proteinuric urine and proteinuric urine. In this study, we concluded that method 2 can be used for further studies of diabetic patients' EV profiles to analyze EVs potential role as biomarkers.

„Diabeeti põdevate patsientide uriinis leiduvate ekstratsellulaarsete vesiikulite isoleerimise ning puhastamise meetodite optimeerimine“

Signe Parts

Resümee

Rahvusvahelise Diabeedi Föderatsiooni (IDF) andmetel kannatab iga kümnes inimene diabeedi all ning tegemist on ülemaailmse terviseprobleemiga. Umbes 100 aastat tagasi Leonard Thompsoni polt tehtud esimene insuliinisüst on aidanud meid selle haiguse ohjamisel suure sammu edasi, kuid isegi praegu puuduvad meil tõhusad meetodid haiguse arengu jälgimiseks. Vajame tundlikumaid biomarkereid, et hinnata veelgi paremini tüsistuste ja haiguse enda kulgu.

Ekstratsellulaarsed vesiikulid (EV) on uudsed nano-suuruses biomarkereid, mis on rakkudevahelist suhtluse vahendajateks. EV-d kannavad palju bioaktiivseid molekule, nagu seda on valgud ja mRNAd. Muutused EV kantavates molekulides sõltuvad rakutüüpidest, patofüsioloogilistest tingimustest ja ka keskkonnast. EV komponentide analüüsimine võib aidata meil leida tundlikumaid ja spetsiifilisemaid biomarkereid, et paremini mõista haiguste arengut ja ravimite tõhusust.

Meie eesmärk oli uurida 4 erinevat EV-de eraldamise meetodit diabeediga patsientide ja tervete inimeste uriinist. Kõigepealt kombineerisime ning optimeerisime EV-de puhastamise ja eraldamise meetodeid, et saavutada minimaalse saastetega EV-de esinemine proovides. Optimeerimise tulemusena koostasime neli kombineeritud metoodikatega protokolli – 1. meetodi (diferentsiaaltsentrifuugimine filtreerimise ja suuruseralduskromatograafiaga (SEC)), 2. meetodi (diferentsiaaltsentrifuugimine soolaga sadestamise ja SEC-iga), 3. meetodi (diferentsiaaltsentrifuugimine PROSPR-iga) ja meetodi 4 (diferentsiaaltsentrifuugimine koos PROSPR-i ja SEC-iga). Pärast protokolli optimeerimist võrdlesime kõigi meetodite EV kontsentratsioone, valgu kontsentratsioone ja EVde füüsikalisi omadusi. Meie uuring näitas statistiliselt olulist erinevust meetodite 1 ja 4 ning meetodite 2 ja 4 vahel. Meetod 2 osutus proovi puhtuse, EV saagise ja valgu kontsentratsioonist tingitud kohaldatavuse alusel kõige tõhusamaks, kuna meetod sobib nii valku sisaldava kui ka valguvaba uriini jaoks. Antud uuringus jõudsime järeldusele, et meetodit 2 saab kasutada diabeediga patsientide EV-profiilide edasisteks uurimiseks, et analüüsida EV-de potentsiaalset rolli biomarkeritena.

BIBLIOGRAPHY

Abebe, M., Adane, T., Kefyalew, K., Munduno, T., Fasil, A., Biadgo, B., Ambachew, S., & Shahnawaz, S. (2019). Variation of Urine Parameters among Diabetic Patients: A Cross-Sectional Study. *Ethiopian Journal of Health Sciences*, *29*(1), 877–886. https://doi.org/10.4314/ejhs.v29i1.9

Anel, A., Gallego-Lleyda, A., de Miguel, D., Naval, J., & Martínez-Lostao, L. (2019). Role of Exosomes in the Regulation of T-Cell Mediated Immune Responses and in Autoimmune Disease. *Cells*, *8*(2), 154. https://doi.org/10.3390/cells8020154

Baghalabadi, V., & Doucette, A. A. (2020). Mass spectrometry profiling of low molecular weight proteins and peptides isolated by acetone precipitation. *Analytica Chimica Acta*, *1138*, 38–48. https://doi.org/10.1016/j.aca.2020.08.057

Barreiro, K., Huber, T. B., & Holthofer, H. (2020). Isolating Urinary Extracellular Vesicles as Biomarkers for Diabetic Disease. *Methods in Molecular Biology (Clifton, N.J.)*, *2067*, 175–188. https://doi.org/10.1007/978-1-4939-9841-8_13

Barutta, F., Tricarico, M., Corbelli, A., Annaratone, L., Pinach, S., Grimaldi, S., Bruno, G., Cimino, D., Taverna, D., Deregibus, M. C., Rastaldi, M. P., Perin, P. C., & Gruden, G. (2013). Urinary exosomal microRNAs in incipient diabetic nephropathy. *PloS One*, *8*(11), e73798. https://doi.org/10.1371/journal.pone.0073798

Berezin, A. E., & Berezin, A. A. (2021). Antigen-presenting cell-derived extracellular vesicles in accelerating atherosclerosis. *Biomedical Research and Therapy*, *8*(3), 4258–4266. https://doi.org/10.15419/bmrat.v8i3.664

Brennan, K., Martin, K., FitzGerald, S. P., O'Sullivan, J., Wu, Y., Blanco, A., Richardson, C., & Mc Gee, M. M. (2020). A comparison of methods for the isolation and separation of

extracellular vesicles from protein and lipid particles in human serum. *Scientific Reports*, *10*(1), 1039. https://doi.org/10.1038/s41598-020-57497-7

Byrnes, S. A., & Weigl, B. H. (2018). Selecting analytical biomarkers for diagnostic applications: A first principles approach. *Expert Review of Molecular Diagnostics*, *18*(1), 19–26. https://doi.org/10.1080/14737159.2018.1412258

Chargaff, E. (1945). Cell structure and the problem of blood coagulation. *Journal of Biological Chemistry*, *160*(1), 351–359.

Chen, Q.-G., Chen, L., Zhong, Q.-H., Zhang, L., Jiang, Y.-H., Li, S.-Q., Qin, T.-Y., Sun, F., You, X.-H., Yang, W.-M., Huang, B., & Wang, X.-Z. (2020). Optimization of urinary small extracellular vesicle isolation protocols: Implications in early diagnosis, stratification, treatment and prognosis of diseases in the era of personalized medicine. *American Journal of Translational Research*, *12*(10), 6302–6313.

Cheruvanky, A., Zhou, H., Pisitkun, T., Kopp, J. B., Knepper, M. A., Yuen, P. S. T., & Star, R. A. (2007). Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator. *American Journal of Physiology-Renal Physiology*, *292*(5), F1657–F1661. https://doi.org/10.1152/ajprenal.00434.2006

Conde-Vancells, J., Rodriguez-Suarez, E., Embade, N., Gil, D., Matthiesen, R., Valle, M., Elortza, F., Lu, S. C., Mato, J. M., & Falcon-Perez, J. M. (2008). Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. *Journal of Proteome Research*, *7*(12), 5157–5166. https://doi.org/10.1021/pr8004887

Couch, Y., Buzàs, E. I., Vizio, D. D., Gho, Y. S., Harrison, P., Hill, A. F., Lötvall, J., Raposo, G., Stahl, P. D., & Théry, C. (2021). A brief history of nearly EV-erything–The rise and rise of extracellular vesicles. *Journal of Extracellular Vesicles*, *10*(14), e12144.

Cvjetkovic, A., Lötvall, J., & Lässer, C. (2014). The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. *Journal of Extracellular Vesicles*, *3*(1), 23111. https://doi.org/10.3402/jev.v3.23111

Devuyst, O., Olinger, E., & Rampoldi, L. (2017). Uromodulin: From physiology to rare and complex kidney disorders. *Nat Rev Nephrol*, *13*(9), 525–544. https://doi.org/10.1038/nrneph.2017.101

Downing, G. (2001). Biomarkers definitions working group. Biomarkers and surrogate endpoints. *Clin Pharmacol Ther*, *69*, 89–95.

Droste, M., Tertel, T., Jeruschke, S., Dittrich, R., Kontopoulou, E., Walkenfort, B., Börger, V., Hoyer, P. F., Büscher, A. K., & Thakur, B. K. (2021). Single Extracellular Vesicle Analysis Performed by Imaging Flow Cytometry and Nanoparticle Tracking Analysis Evaluate the Accuracy of Urinary Extracellular Vesicle Preparation Techniques Differently. *International Journal of Molecular Sciences*, *22*(22), 12436.

EH01: Esmashaigusjuhud soo ja vanuserühma järgi. (n.d.). Tervisestatistika Ja Terviseuuringute Andmebaas. Retrieved May 22, 2022, from http://statistika.tai.ee/pxweb/et/Andmebaas/Andmebaas__02Haigestumus__01Esmashaigestumus/EH01.px/

Erdbrügger, U., & Le, T. H. (2016). Extracellular Vesicles in Renal Diseases: More than Novel Biomarkers? *Journal of the American Society of Nephrology: JASN*, *27*(1), 12–26. https://doi.org/10.1681/ASN.2015010074

Estonian Diabetes Association. (2022). *Mis on diabeet ?*

Extracellular vesicles—Search Results—PubMed. (n.d.). Retrieved May 22, 2022, from https://pubmed.ncbi.nlm.nih.gov/?term=Extracellular%20vesicles&sort=date&ac=no&timeline=expanded

Fernández-Llama, P., Khositseth, S., Gonzales, P. A., Star, R. A., Pisitkun, T., & Knepper, M. A. (2010). Tamm-Horsfall protein and urinary exosome isolation. *Kidney International*, *77*(8), 736–742. https://doi.org/10.1038/ki.2009.550

Gallart-Palau, X., Serra, A., Wong, A. S. W., Sandin, S., Lai, M. K. P., Chen, C. P., Kon, O. L., & Sze, S. K. (2015). Extracellular vesicles are rapidly purified from human plasma by PRotein Organic Solvent PRecipitation (PROSPR). *Scientific Reports*, *5*(1), 14664. https://doi.org/10.1038/srep14664

Gámez-Valero, A., Lozano-Ramos, S. I., Bancu, I., Lauzurica-Valdemoros, R., & Borràs, F. E. (2015). Urinary Extracellular Vesicles as Source of Biomarkers in Kidney Diseases. *Frontiers in Immunology*, *6*. https://www.frontiersin.org/article/10.3389/fimmu.2015.00006

Gámez-Valero, A., Monguió-Tortajada, M., Carreras-Planella, L., Franquesa, M., Beyer, K., & Borràs, F. E. (2016). Size-Exclusion Chromatography-based isolation minimally alters Extracellular Vesicles' characteristics compared to precipitating agents. *Scientific Reports*, *6*(1), 33641. https://doi.org/10.1038/srep33641

Gómez-García, F., Martínez-Pulleiro, R., Carrera, N., Allegue, C., & Garcia-Gonzalez, M. A. (2022). Genetic Kidney Diseases (GKDs) Modeling Using Genome Editing Technologies. *Cells*, *11*(9), 1571. https://doi.org/10.3390/cells11091571

Gonzales, P., Pisitkun, T., & Knepper, M. A. (2008). Urinary exosomes: Is there a future? *Nephrology, Dialysis, Transplantation: Official Publication of the European Dialysis and Transplant Association - European Renal Association*, *23*(6), 1799–1801. https://doi.org/10.1093/ndt/gfn058

Harding, C., & Stahl, P. (1983). Transferrin recycling in reticulocytes: PH and iron are important determinants of ligand binding and processing. *Biochemical and Biophysical Research Communications*, *113*(2), 650–658. https://doi.org/10.1016/0006-291X(83)91776-X

Hashemi, E., Dehghanbanadaki, H., Baharanchi, A. A., Forouzanfar, K., Kakaei, A., Mohammadi, S. M., Zeidi, S., & Razi, F. (2021). WT1 and ACE mRNAs of blood extracellular vesicle as biomarkers of diabetic nephropathy. *Journal of Translational Medicine*, *19*(1), 299. https://doi.org/10.1186/s12967-021-02964-6

Kosaka, N., Kogure, A., Yamamoto, T., Urabe, F., Usuba, W., Prieto-Vila, M., & Ochiya, T. (2019). Exploiting the message from cancer: The diagnostic value of extracellular vesicles for clinical applications. *Experimental & Molecular Medicine*, *51*(3), 1–9. https://doi.org/10.1038/s12276-019-0219-1

Kosanović, M., & Janković, M. (2014). Isolation of urinary extracellular vesicles from Tamm-Horsfall protein–depleted urine and their application in the development of a lectin-exosome-binding assay. *BioTechniques*, *57*(3), 143–149. https://doi.org/10.2144/000114208

Liangsupree, T., Multia, E., & Riekkola, M.-L. (2021). Modern isolation and separation techniques for extracellular vesicles. *Journal of Chromatography A*, *1636*, 461773. https://doi.org/10.1016/j.chroma.2020.461773

Lu, Y., Liu, D., Feng, Q., & Liu, Z. (2020). Diabetic Nephropathy: Perspective on Extracellular Vesicles. *Frontiers in Immunology*, *11*. https://www.frontiersin.org/article/10.3389/fimmu.2020.00943

Markowska, A., Pendergrast, R. S., Pendergrast, J. S., & Pendergrast, P. S. (2017). A novel method for the isolation of extracellular vesicles and RNA from urine. *Journal of*

Circulating Biomarkers, *6*, 1849454417712666. https://doi.org/10.1177/1849454417712666

Medeiros, T., Myette, R. L., Almeida, J. R., Silva, A. A., & Burger, D. (2020). Extracellular Vesicles: Cell-Derived Biomarkers of Glomerular and Tubular Injury. *Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology*, *54*(1), 88–109. https://doi.org/10.33594/000000207

Merchant, M. L., Rood, I. M., Deegens, J. K. J., & Klein, J. B. (2017). Isolation and characterization of urinary extracellular vesicles: Implications for biomarker discovery. *Nature Reviews. Nephrology*, *13*(12), 731–749. https://doi.org/10.1038/nrneph.2017.148

Musante, L., Bontha, S. V., La Salvia, S., Fernandez-Piñeros, A., Lannigan, J., Le, T. H., Mas, V., & Erdbrügger, U. (2020). Rigorous characterization of urinary extracellular vesicles (uEVs) in the low centrifugation pellet—A neglected source for uEVs. *Scientific Reports*, *10*(1), 3701. https://doi.org/10.1038/s41598-020-60619-w

Musante, L., Tataruch, D. E., & Holthofer, H. (2014). Use and Isolation of Urinary Exosomes as Biomarkers for Diabetic Nephropathy. *Frontiers in Endocrinology*, *5*. https://www.frontiersin.org/article/10.3389/fendo.2014.00149

Rider, M. A., Hurwitz, S. N., & Meckes, D. G. (2016). ExtraPEG: A Polyethylene Glycol-Based Method for Enrichment of Extracellular Vesicles. *Scientific Reports*, *6*, 23978. https://doi.org/10.1038/srep23978

Roman, M., Kamińska, A., Drożdż, A., Platt, M., Kuźniewski, M., Małecki, M. T., Kwiatek, W. M., Paluszkiewicz, C., & Stępień, E. Ł. (2019). Raman spectral signatures of urinary extracellular vesicles from diabetic patients and hyperglycemic endothelial cells as

potential biomarkers in diabetes. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *17*, 137–149. https://doi.org/10.1016/j.nano.2019.01.011

Rood, I. M., Deegens, J. K. J., Merchant, M. L., Tamboer, W. P. M., Wilkey, D. W., Wetzels, J. F. M., & Klein, J. B. (2010). Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome. *Kidney International*, *78*(8), 810–816. https://doi.org/10.1038/ki.2010.262

Tataruch-Weinert, D., Musante, L., Kretz, O., & Holthofer, H. (2016). Urinary extracellular vesicles for RNA extraction: Optimization of a protocol devoid of prokaryote contamination. *Journal of Extracellular Vesicles*, *5*, 10.3402/jev.v5.30281. https://doi.org/10.3402/jev.v5.30281

Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., Antoniou, A., Arab, T., Archer, F., Atkin-Smith, G. K., Ayre, D. C., Bach, J.-M., Bachurski, D., Baharvand, H., Balaj, L., Baldacchino, S., Bauer, N. N., Baxter, A. A., Bebawy, M., … Zuba-Surma, E. K. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles*, *7*(1), 1535750. https://doi.org/10.1080/20013078.2018.1535750

Tomiyama, E., Fujita, K., & Nonomura, N. (2021). Urinary Extracellular Vesicles: Ultracentrifugation Method. In S. Salvi & V. Casadio (Eds.), *Urinary Biomarkers: Methods and Protocols* (pp. 173–181). Springer US. https://doi.org/10.1007/978-1-0716-1354-2_15

Urabe, F., Kosaka, N., Ito, K., Kimura, T., Egawa, S., & Ochiya, T. (2020). Extracellular vesicles as biomarkers and therapeutic targets for cancer. *American Journal of Physiology-Cell Physiology*, *318*(1), C29–C39. https://doi.org/10.1152/ajpcell.00280.2019

van der Pol, E., Böing, A. N., Harrison, P., Sturk, A., & Nieuwland, R. (2012). Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacological Reviews*, *64*(3), 676–705. https://doi.org/10.1124/pr.112.005983

Willms, E., Cabañas, C., Mäger, I., Wood, M. J. A., & Vader, P. (2018). Extracellular Vesicle Heterogeneity: Subpopulations, Isolation Techniques, and Diverse Functions in Cancer Progression. *Frontiers in Immunology*, *9*. https://www.frontiersin.org/article/10.3389/fimmu.2018.00738

Wolf, P. (1967). The Nature and Significance of Platelet Products in Human Plasma. *British Journal of Haematology*, *13*(3), 269–288. https://doi.org/10.1111/j.1365-2141.1967.tb08741.x

Yáñez-Mó, M., Siljander, P. R.-M., Andreu, Z., Zavec, A. B., Borràs, F. E., Buzas, E. I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colás, E., Cordeiro-da Silva, A., Fais, S., Falcon-Perez, J. M., Ghobrial, I. M., Giebel, B., Gimona, M., Graner, M., Gursel, I., … De Wever, O. (2015). Biological properties of extracellular vesicles and their physiological functions. *Journal of Extracellular Vesicles*, *4*, 27066. https://doi.org/10.3402/jev.v4.27066

Zhang, H., Freitas, D., Kim, H. S., Fabijanic, K., Li, Z., Chen, H., Mark, M. T., Molina, H., Martin, A. B., Bojmar, L., Fang, J., Rampersaud, S., Hoshino, A., Matei, I., Kenific, C. M., Nakajima, M., Mutvei, A. P., Sansone, P., Buehring, W., … Lyden, D. (2018). Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nature Cell Biology*, *20*(3), 332–343. https://doi.org/10.1038/s41556-018-0040-4

Zhang, W., Zhou, X., Zhang, H., Yao, Q., Liu, Y., & Dong, Z. (2016). Extracellular vesicles in diagnosis and therapy of kidney diseases. *American Journal of Physiology - Renal Physiology*, *311*(5), F844–F851. https://doi.org/10.1152/ajprenal.00429.2016

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International Diabetes Federation Diabetes Atlas

https://diabetesatlas.org/ (Accessed on 30.05.2022)

PubMed:

https://pubmed.ncbi.nlm.nih.gov/?term=Extracellular%20vesicles&sort=date&ac=no&timeline=expanded (Accessed on 30.05.2022)

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Signe Parts

30.05.2022

APPENDIX 1. Python code used to convert NTA results.

```
# Code used for NTA file transformation

# Code written and used with PyCharm 2021.2 by JetBrains

import xlsxwriter
import os
import glob

# all the files from the folder
folder_path = input("Folder location: ")


filename = glob.glob(os.path.join(folder_path, '*.txt'))
print(filename)

# Create an new Excel file and add a worksheet.
andmed = xlsxwriter.Workbook(input("New file name with .xlsx: "))
worksheet = andmed.add_worksheet()
palju_file = len(filename)

# Open file with data
file_nr = 0
while file_nr < palju_file:
    file = open(filename[file_nr])
    sisu = str(file.read())
    lines = sisu.splitlines()

    # sample name
    sample_location = lines[5]
    sample = sample_location[8:]
    print(sample)

    # Dillution
    dil = sisu.find("Dilution")
    lahjendusfaktor = float(sisu[
                      (dil + 11):(dil + 17)])  # find the dilution factor.
    # skip first and last lines
    last_word1 = sisu.find("Size / nm")
    last_word2 = sisu.find("-1.000000  -1.000000  -1.000000  -1.000000")
```

```
    text = sisu[last_word1:last_word2]
    skip_line = text.replace('\n', '\t')

    # Size column
    number_cols = len(skip_line.split('\t'))
    columns = skip_line.split('\t')

    row = 0
    col = 0

    s = 0

    while s <= number_cols:
        worksheet.write(row, col, columns[s])  # write size to excel
        if row < number_cols:
            row = row+1
            # print(columns[s])
        if row == number_cols:
            row = 0

        s = s+4
    col = file_nr+1
    row = 0

    data = sample
    c = 2
    while c <= number_cols:
        if c > 2:
            data = float(columns[c])*lahjendusfaktor
        worksheet.write(row, col, data)  # write to excel
        if row <= number_cols:
            row = row+1

            if row > number_cols:
                row = 0
        c = c+4

# Close all the files
    file.close()
    file_nr = file_nr+1
```

```
andmed.close()

# Code written and used with PyCharm 2021.2 by JetBrains
```