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# Prevalence of Apo A and Apo E in extracellular vesicles using different purification methods

Bachelor's Thesis (12 ECTS)

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## Prevalence of Apo A and Apo E in extracellular vesicles using different purification methods

**Abstract:**

Apolipoprotein (Apo), a protein portion of plasma lipoprotein, can attach to and distribute blood lipids to different tissues in the body for metabolism and utilization. It is primarily synthesized in the liver and, to a lesser extent, in the small intestine. High levels of apolipoproteins may affect blood lipid metabolism and use, causing the onset and progression of hyperlipidemia and atherosclerosis that might lead to cardiovascular and cerebrovascular disease. However, these lipid-protein molecules are also present in extracellular vesicles (EVs) samples. The study shows various extracellular vesicle purification methods to detect apolipoproteins as well as how the apolipoprotein yield correlates with apolipoproteins and cholesterol fractions measured by routine methods in a clinical laboratory.

**Keywords:**

EVs, Apolipoproteins, lipoproteins, atherosclerosis

**CERCS:** B230 BIOMEDICAL SCIENCES Microbiology, bacteriology, virology, mycology

## Apo A ja Apo E levimus rakuvälistes vesiikulites kasutades erinevaid puhastusmeetodeid

**Lühikokkuvõte:**

Apolipoproteiin (APO), plasma lipoproteiini valguosa, saab kinnitada ja levitada vere lipiidid erinevatesse kudedesse kehas ainevahetuse ja ärakasutamise jaoks. See on peamiselt sünteesitud maksas ja vähemal määral peensooles. Kõrge apolipoproteiinide tase võib mõjutada vere lipiide ainevahetust ja kasutamist, põhjustades hüperlipideemia ja ateroskleroosi teket ja edasiarenemist, mis võib viia südame-veresoonkonna ja tserebraalhaiguseni. Kuigi need lipiidvalgu molekulid esinevad ka rakuvälistes vesiikulite (EVs) proovides. Uuring näitab mitmesuguseid rakuväliseid vesiikulite puhastamisemeetodeid apolipoproteiinide tuvastamiseks ja samuti ka kuidas apolipoproteiini saagis korreleerub apolipoproteiinide ja kolesterooli fraktsioonidega, mis on mõõdetud kliinilises laboris rutiinse meetodiga.

**Märksõnad:**

Rakuväline vesiikul, Apolipoproteiinid, lipoproteiinid, ateroskleroos

**CERCS**: B230 BIOMEDITSIINILISED TEADUSED mikrobioloogia, bakterioloogia, viroloogia, mükoloogia.

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# TERMS, ABBREVIATIONS AND NOTATIONS

Apo - apolipoprotein

GAM - goat anti-mouse

GAR - goat anti-mouse

SDS -PAGE - sodium dodecyl sulphate–polyacrylamide gel electrophoresis

EV – extracellular vesicle

HDL – high density lipoprotein

LDL - low density lipoprotein

IDL - intermediate density lipoprotein

PBS - phosphate-buffered saline

PEG - polyethylene glycol

UC – ultracentrifugation

min - minutes

LPL - lipoprotein lipase

HTGL - hepatic triglyceride lipase

kDa – kilodalton

# INTRODUCTION

Extracellular vesicles (EVs) are categorized based on their biogenesis and size. EVs can be broken down into exosomes, microvesicles, and apoptotic bodies. All biological fluids, including blood, urine, amniotic fluid, saliva, and cerebrospinal fluid, contain EVs (Williams *et al.*, 2018). They are unique markers of pathological processes and cellular activation. They also spark considerable interest in the management of cardiovascular diseases and cancer (Oggero, Austin-Williams, and Norling, 2019).

Lipoproteins are lipid-protein particles that transport hydrophobic substances in plasma's hydrophilic environment. Their hydrated densities are used to classify them. Lipoprotein size, structure, and apolipoprotein content can all change as a result of the action of enzymes, including lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL).

The aims of the thesis are to isolate extracellular vesicles in blood samples using three purification methods and then detect the presence of apolipoproteins in the purified EVs. We also compare our findings with apolipoprotein A, B100 and different cholesterol fractions measured in a clinical laboratory.

The first part of the study gives the literature overview of extracellular vesicles, their various sizes, purification methods, analyzing methods that could be utilized; as well as lipoproteins, apolipoproteins and atherosclerosis. The experimental part of the study is based on the methods used to detect apolipoproteins after purification of EVs from selected blood samples. This study was performed in the prof. Reet Kurg laboratory in the Institute of Technology, University of Tartu.

# 1 LITERATURE REVIEW

## 1.1 Extracellular vesicles (EVs)

Extracellular vesicles (EVs) are cell-derived nanovesicles generated from discrete subcellular compartments and delivered into extracellular space. EVs have a lipid bilayer enclosing both soluble cytosolic material and nuclear components, which have an essential role in intercellular communication and are involved in the transport of biological signals for regulating diverse cellular functions (Williams *et al.*, 2018). EVs exist in all biological fluids, including blood, urine, amniotic fluid, saliva, and cerebrospinal fluid. Their diversified composition and biological content represent specific signatures of pathological processes and cellular activation. EVs have raised significant interest in cardiovascular diseases and cancer management as diagnostic and prognostic biomarkers (Oggero, Austin-Williams and Norling, 2019). Various methods for their isolation, detection, and characterization are being developed.

### 1.1.1 Types of EVs

ll cells (prokaryotes and eukaryotes) release different types of extracellular vesicles (EVs) as part of their normal physiological processes. EVs can be broadly grouped into two categories, exosomes and ectosomes. Exosomes are a type of EVs that include constituents of DNA, RNA, and proteins of the cells that secrete them. They are released during multivesicular endosomal fusion with the plasma membrane. Their size is about 40–120 nm in diameter. Exosomes transfer molecules from one place to another by membrane vesicle trafficking, thereby exerting influence on the immune system, such as B cells and dendritic cells, and may have a functional effect in mediating adaptive immune responses to microbes and tumors. Exosomes can be released into urine by the kidneys and can be used as a diagnostic tool or as a treatment response marker in prostate cancer (Pisitkun, Shen and Knepper, 2004). Exosomes which are released from tumors into the blood may have diagnostic prospects. Exosomes are stable in bodily fluids strengthening their usefulness as reservoirs for disease biomarkers (Williams *et al.*, 2018).

Ectosomes, also known as microvesicles are budded from the surface of the plasma membrane. They include microvesicles (100–1000 nm in diameter), and apoptotic bodies (1–5

μm in diameter) produced by cell apoptosis (Sunkara, Woo and Cho, 2016). EVs are classified according to their biogenesis and size. Specific markers and analytic methods are currently being developed to distinguish the origins of the vesicles upon release into the extracellular matrix since it is quite difficult to differentiate between smaller size vesicles when they are isolated together (Sunkara, Woo and Cho, 2016).

At the point of their discovery, EVs were thought to be involved in cellular excretion of by-products. As such less attention was given to them until their participation in the immune response was revealed. In 2007, it was discovered that they intercede communication between cells due to the presence of functional RNAs. After that, research experiments on EVs has shot up exponentially concentrating on the growth of new methods for the isolation as well as analysis of their physiological functions and composition (Sunkara, Woo and Cho, 2016).

EV functions are dependent on their composition as well as their origin. Because they carry diverse membrane and cytosolic proteins, DNA, mRNA and miRNA, EVs are considered prospective biomarkers and are known to be involved in both disease progression and normal physiological processes in a pleiotropic manner, including the immune response, cell signaling, antigen presentation, intercellular communication *etc.* (Sunkara, Woo and Cho, 2016)

Patients with elevated EVs might have several health conditions, including atherosclerosis (Chironi *et al.*, 2006)

### 1.1.2 Purification Methods

EVs as potential biomarkers are becoming a growing interest, and various purification techniques have been recently developed (Liga *et al.*, 2015). Additionally, numerous research groups compared the efficiency of available purification techniques (Tauro *et al.*, 2012), (Caradec *et al.*, 2014), (Lane *et al.*, 2015). Three main classes of isolation techniques can be used based on the principle of separation: density, size, and immunoaffinity – based isolation.

**Density-based isolation**

This technique includes ultracentrifugation and regular centrifugation with precipitation reagents.

**Ultracentrifugation (UC)**

Low-density particles are separated by applying differential centrifugation by varying *g* forces. For decades, ultracentrifugation greater than 100,000 *g* has been used to isolate eukaryotic viruses, and bacteriophages due to their relatively low densities (McNamara and Dittmer, 2020). Ultracentrifugation has become a frequently utilized technique for EV isolation. The fact that ultracentrifugation is generally used in biochemistry and routine molecular biology, makes it a fast and reliable technique for EV isolation. Cells and debris are removed at a low *g* force, although with a high *g* force of approx. 200,000 *g* sediments of EVs (1.13–1.19 g mL−1) are obtained (Figure 1) (Sunkara, Woo and Cho, 2016). Even so, it takes approx. 5 h for isolating the exosomal pellet. Additionally, it requires special equipment, an ultracentrifuge, which might be unavailable in standard laboratory settings. Other drawbacks in this technique include insufficient isolation and low recovery.

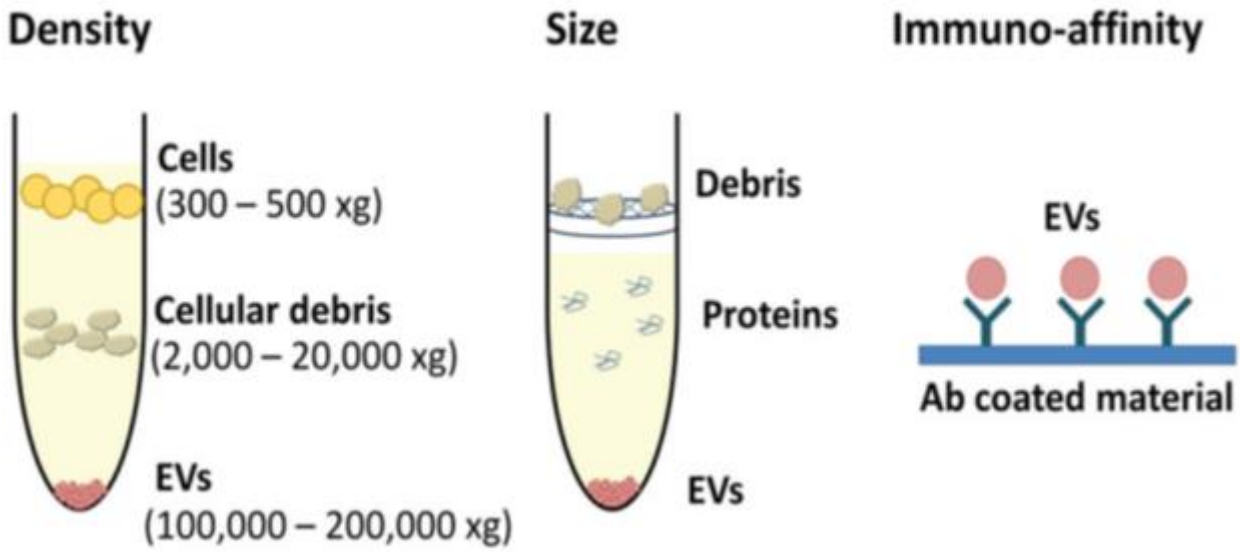


Figure 1. Isolation of EVs (Sunkara, Woo and Cho, 2016)

**Precipitation reagents**

In recent years several commercial kits which include precipitation reagents have been made available for EV isolation. The reagents decrease the solubility by lowering the hydration of EVs and lead to precipitation. Polyethylene glycol (PEG) has been utilized for many years as a crowding reagent to precipitate complex protein and macromolecule structures out of solutions. EVs precipitate at lower speed centrifugation (McNamara and Dittmer, 2020). PEG is the principal active element in many exosome precipitation reagents that are available commercially. Since viruses and smaller EVs such as microvesicles and exosomes have relatively similar biophysical properties, PEG precipitation has become a generally adopted method for EV isolation from fluids such as tissue culture supernatant and plasma. The use of PEG in a plasma solution or tissue culture supernatant can be viewed as a molecular fishing net that grabs larger molecules (depending on the molecular weight of the PEG) into a dense bundle at the same time allowing smaller molecules to be left in the solution. This provides for low-speed centrifugation (< 2000 *g*) to pellet the virus and or EV aggregates (Peterson *et al.*, 2015; Hurwitz *et al.*, 2016; Rider, Hurwitz and Meckes, 2016).

Precipitation using PEG can be done at lower speeds using desktop centrifuges and can hold larger volumes (100ml – 1 L) than ultracentrifuge units. From a cost-of-production viewpoint, precipitation with crowding reagents has a great advantage compared to other methods. Moreover, the principal underlying condition is that the use of crowding reagents with EVs are known to take long hours of precipitation prior to centrifugation (McNamara and Dittmer, 2020).

**Size-based isolation**

EVs can be separated from cells and large debris by using nano-sized membrane filters. Filtration is an effective procedure for the removal of large debris, including large vesicles of above 200 nm in diameter, subcellular fractions, plasma proteins, protein–nucleic acid aggregates, or protein aggregates (Sunkara, Woo and Cho, 2016). Membranes pores of sizes of 0.2, 0.22, and 0.45 μm and materials such as polyvinylidene fluoride and track etched polycarbonate are widely used to remove large debris. The purity of EVs is enhanced by a combination of filtration and ultracentrifugation. Muller *et al.* reported that the combined

method is preferable to using an ultracentrifugation-based technique, differential centrifugation, or sucrose density gradient alone (Muller *et al.*, 2014).

Furthermore, apart from membrane filtration, several approaches have been proposed for size-based separation of EVs. These techniques include acoustic waves and micro pillar-based methods. Santana *et al*. used geometry to isolate vesicles by using the principles of deterministic lateral displacement (DLD). Lee *et al.* used sound waves to purify EVs in a contact-free manner. Wang *et al*. used ciliated micro pillars for multi-scale filtration by removing cellular debris as well as proteins and other small objects (Sunkara, Woo and Cho, 2016).

**Affinity-based isolation**

Affinity purification of antibody or specific protein-based capture systems such as streptavidin, is a way to biochemically purify a discrete population from a heterogeneous mixture. The method can be used to separate viruses and EVs given the differences in membrane of capsid composition (McNamara and Dittmer, 2019). The common exosomal markers are tetraspanins including CD9, CD63, and CD81. These markers are specific to exosomes, and the methods aid in the specific isolation of exosomes depending on the capture antibodies used.

To purify EVs and study their relationship with cancer, new isolation techniques utilizing microfluidics including paper-based microfluidic devices, immunomagnetic beads, photosensitizer beads etc. are being developed, based on immunoaffinity capture with antibodies specific to EVs (McNamara and Dittmer, 2020). Most of these capture beads make use of the tetraspanin proteins present on the surface of EVs. Tetraspanins are incorporated onto the EV membrane during trafficking through the endosomal recycling pathway. The tetraspanin-coated EVs (exosomes) are biologically active post-capture or elution from the beads and are competent for uptake by recipient cells (Chugh *et al.*, 2013). Affinity purification remains the best standard for isolating a homogenous entity from a heterogeneous input, including the successful separation of virus particles from EVs and vice versa.

### 1.1.3 Analytical methods

**Size characterization**

Morphological information and size can be obtained from high-resolution imaging with electron microscopy using transmission electron microscopy (TEM) or scanning electron microscopy (SEM). Electron microscopy is an effective tool to determine the morphology, size, and existence of surface markers; however, this technique cannot measure the concentration of EVs. Nanoparticle tracking analysis (NTA), is another technique that uses particle tracking to measure the concentration of EVs and size distribution.

Surface markers and protein analysis can be done utilizing methods like ELISA, western blotting, Bradford's assay, and flow cytometry. However, these purification techniques require standardized and optimized protocols. Enzyme-linked immunosorbent assay (ELISA) technique provides information about the presence of surface markers. Western blotting (WB) is an effective method to visualize the presence of exosomal proteins where isolated EVs are lysed, and proteins within EVs are analyzed. Bradford's assay technique can determine the total protein content of purified EVs. However, these techniques cannot be applied to unpurified samples containing protein contaminants (Sunkara, Woo and Cho, 2016).

Other analytical methods might include molecular analysis such as fluorescence imaging; this method employs a lipophilic dye to capture antibodies in the EVs' lipid bilayer. The lipophilic dyes used consist of green fluorescent protein (GFP) or red fluorescent protein (RFP) labelled exosomal marker CD63 (Sunkara, Woo and Cho, 2016). Bioanalyzers, quantitative reverse transcription polymerase chain reaction (qRT PCR), miRNA arrays, and mass spectrometry are generally used for profiling exosomal cargo. Furthermore, reports have been made that EVs contain proteins and diverse RNA cargo that include short sequences of about 20 – 200 nucleotides. (Sunkara, Woo and Cho, 2016).

## 1.2 Apolipoprotein (Apo)

In order to understand what apolipoproteins are, we must briefly describe what lipoproteins are and their relation to apolipoprotein. Lipoproteins are lipid-protein particles that carry hydrophobic substances in the hydrophilic environment of plasma (Dominiczak and Caslake, 2011). They are classified according to their hydrated densities; very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and

high-density lipoproteins (HDL). Lipoproteins differ in size, lipid composition, and apolipoprotein content. These characteristics change due to the action of enzymes such as lipoprotein lipase (LPL), hepatic triglyceride lipase (HTGL), lecithin-cholesterol acyltransferase (LCAT), and cholesteryl ester transfer protein (CETP) (Dominiczak and Caslake, 2011).

Apolipoproteins (apo) describe amphipathic molecules with both hydrophilic and hydrophobic properties and are therefore able to interact with both lipoprotein lipids and aqueous plasma. Together with phospholipids and cholesterol, apolipoproteins form lipoprotein particles into which several different lipids can be packed. Apolipoproteins also control cellular uptake of lipoproteins by binding to membrane-specific lipoprotein receptors.

### 1.2.1 Types of apolipoprotein

Apolipoprotein A1 (ApoA1) is a protein composed of 243 amino acids. The apo A gene is located on chromosome 11 and synthesized in the liver and intestine. ApoA1 constitutes 70% of HDL apolipoproteins, it activates LCAT, and is also an anti-inflammatory molecule and an antioxidant.

Apolipoprotein AII (ApoAII) is mainly expressed in the liver and accounts for approximately 20% of HDL protein. It consists of 77 amino acids and has a molecular mass of 17,400 Da. ApoAII concentration is determined by its rate of production and is known to inhibit LPL activity, and as a co-factor for CETP and LCAT (Dominiczak and Caslake, 2011).

Apolipoprotein AIV (ApoAIV) is synthesized in the intestines and incorporated into nascent chylomicrons. It is also in HDL and relatively hydrophilic. It may be displaced from lipoproteins and circulates predominantly as a lipid-free protein. It participates in intestinal lipid absorption and affects various aspects of lipoprotein response to diet. It also modulates the activity of LPL and activates LCAT (Dominiczak and Caslake, 2011).

ApoAV is synthesized in the liver, controls hepatic VLDL synthesis and secretion. The gene coding for apoAV has been strongly related to TG concentration. ApoAV deficiency leads to type V dyslipidemia and reduced post-heparin LPL activity. Its overexpression leads to a reduction in TG concentration.

Apolipoprotein B100 (ApoB100) consists of 4536 amino acids with a molecular weight of 550,000 Da. It contains a globular amino-terminal domain, which reacts with microsomal TG transfer proteins. ApoB gene is found at the short arm of chromosome 2. Copenhagen City Heart Study identified 123 genetic variants in the apoB gene. ApoB100 is synthesized in the liver and degraded within the liver cells when in excess. ApoB is the core apolipoprotein of LDL and chylomicrons (*Apolipoproteins. Apo A1, B, C2. Apolipoproteins information*, 2015). Moreover, it is present in VLDL and LDL particles.

Apolipoprotein B48 (ApoB48) constitutes 2152 amino acids with a molecular mass of 264,000 Da. It is the shortened form of apoB100, and it is produced from the same gene as apo B100. ApoB48 is synthesized in the intestines in humans and secreted by chylomicrons. It is the most appropriate marker for cardiovascular investigation of postprandial chylomicron metabolism. Patsch *et al.* demonstrated that atherosclerosis could be predicted by postprandial lipemia. The plasma concentration of apoB48 corresponded with postprandial lipemia, making apoB48 a biomarker for atherosclerosis (Dominiczak and Caslake, 2011).

Apolipoproteins C is synthesized mostly in the liver and the intestine. It contributes to the assembly of VLDL and chylomicrons. ApoCI is present in TG-rich lipoproteins. ApoCII activates LPL and therefore contributes to VLDL lipolysis. ApoCI and apoCIII inhibit LPL and HTGL. ApoAV may counteract the effects of apoCIII (Dominiczak and Caslake, 2011). Overexpression of apoCIII in humans can lead to atherosclerosis. (Khetarpal *et al.*, 2017)

Apolipoprotein E (ApoE) is a single-chain glycoprotein that consists of 299 amino acids. Its molecular weight is 34,200 Da. It is present in mature VLDL, VLDL remnants, chylomicron remnants, LDL, and HDL. It is associated with a high affinity to the LDL-receptor and the LDL-receptor protein (LRP). ApoE facilitates the clearance of chylomicron and VLDL remnants. Plasma apoE constitutes approximately 60% of HDL, which exchanges it with other lipoproteins. ApoE controls cholesterol outflow from cells together with apoAI. It has antioxidant properties and plays a role in the regulation of inflammatory response (Dominiczak and Caslake, 2011). ApoE occurs in three isoforms: E2, E3, and E4. The differences between the isoforms are a result of amino acid positions 112 and 158. ApoE initiates HTGL, LPL, and LCAT. The affinity of apoE3 and apoE4 to the LDL receptor is similar, while apoE2 has a lower affinity. Overexpression of apoE leads to hypertriglyceridemia through stimulation of VLDL production (Dominiczak and Caslake, 2011). Apolipoprotein E (apoE) is a polymorphic multifunctional protein in humans. It is coded by three alleles (e2, e3, e4) of a

modulator gene. ApoE polymorphism regulates susceptibility to many diseases, including atherosclerotic arterial disorders and neurodegenerative disorders (Davignon, 2005). The e4 allele is related to higher LDL-C, and it initiates atherogenesis whereas, allele e2 has the opposite effect and is associated with lower LDL-C. However, allele e2 may be in correlation with increased plasma triglycerides (Davignon, 2005). Allele and gender-dependent effects on oxidative and reverse cholesterol transport make apoE a prospective biomarker in lipoprotein metabolism and atherogenic diseases.

### 1.2.2 Atherosclerosis and apolipoproteins

The term “atherosclerosis” is defined as the narrowing of the arteries due to fatty deposit (plaque) build-up that can reduce blood supply to the heart and other vital organs such as the brain and intestines. Plaques mainly consist of low-density lipoproteins cholesterol (LDL-C), fibrous tissue, and sometimes calcium deposits. As plaque builds up, it might tear away from the blood vessel and form a thrombus that blocks further vessels. Due to the lessening of blood vessel open areas with plaque build-up, blood clots may also develop. Both thrombi, as well as formed clots, can block downstream arteries, thereby starving vital organs of oxygen and blood. As a result, the organs can be severely damaged or die. Atherosclerosis may lead to several life-threatening cardiovascular diseases. These include ischemic heart disease, coronary heart disease, peripheral blood vessel disease, and chronic kidney disease.

# 2 THE AIMS OF THE THESIS

It has been shown that extracellular vesicles (EVs) are cell-derived nanovesicles found in almost all forms of bodily fluids, with emerging techniques for isolating, detecting, and characterizing being developed in response to the growing clinical interest in their function (Sunkara, Woo and Cho, 2016).

We aimed to:

- Use different purification methods for extracellular vesicle isolation.
- Analyze the number of extracellular vesicles present in the purified samples.
- Detect the prevalence of Apo A and Apo E in differently purified EVs
- Compare our results with data (apoA, apoB, HDL-, LDL, and total cholesterol) we got from a routine clinical laboratory

# 3 EXPERIMENTAL PART

## 3.1 MATERIALS AND METHODS

### 3.1.1 Blood serum and conditions

Blood was collected 08.10.2020 from healthy volunteers in the Institute of Technology, University of Tartu as a part of the Sars-CoV2 antibody study. 4 ml of blood was collected into BD Vacuette SSTII Advanced serum separator tubes. After collection, blood was allowed set for a minimum of 30 min and then centrifuged at 2000 g for 10 minutes. After centrifuging, 1.5 ml of serum was collected, labeled adequately with a TYTI code, and stored at -20° C until further analysis. Table 1. shows the blood samples obtained from the volunteers.

For clinical cholesterol analysis, 500 µl of serum was sent to Tartu University Central laboratory (for LDL-, HDL-, and total cholesterol) and North Estonian Hospital Central laboratory (for apo A and apoB100) measurements on Roche Cobas analyzer.

**Table 1**. Blood samples were obtained from healthy volunteers. Three male and three female adult samples were collected for the experiment.

| TYTI/Samples | Sex | Age |
|---|---|---|
| 8 | male | 31 |
| 10 | female | 56 |
| 14 | female | 25 |
| 38 | male | 59 |
| 78 | female | 30 |
| 105 | male | 54 |

### 3.1.2 Sample Preparation

All six (6) blood samples obtained from healthy volunteers in Tartu University Technology Institute were prepared in three different ways.

The first purification method used was ultracentrifugation through PBS(UC/PBS). 200µl of serum was put into new 1.5ml Eppendorf® tubes, and 800 µl of PBS was added to the containing sample in the tubes. Next, the solution was centrifuged (1200 x g for 30 min). 1ml supernatant was collected and cell pellets discarded. (The supernatant could be stored at -20 ℃ before it can be used later). The process was continued by the addition of 3 ml of PBS into ultracentrifugation tubes. The supernatant was added and mixed thoroughly. The ultracentrifuge tubes are weighed to make sure they contain the same amount of mixture with a difference of +/- 0.02 g. The tubes were then placed in the Beckman Coultier SW55Ti rotor and ultracentrifuged for 4 hours at 32,000 rpm (120,000 g). The supernatant was discarded and allowed to dry upside down for 5 - 10 min. 200 µl of PBS was added to the tubes and allowed to sit for 10 min. After that, cells were resuspended 10 – 20 times to collect all EVs. 200 µl of resuspended samples are then collected into 1.5 ml Eppendorf® tubes and labeled with sample code (UC/PBS) and sample number.

The second preparation method was precipitation with PEG. Up to a 100 µl of each sample was centrifuged at 10,000 g for 10 min at 4°C (Pico 21, Thermo Scientific) to remove unsoluble material. Then 20 µl of 50% PEG MW 6000 (final concentration 10%) and 1.5 µl of 5M NaCl was added per 100 µl of sample and percipitated overnight at 4°C. EVs were centrifuged at 10,000 g for 10 min. and the pellet was resuspended in 200 µl of PBS. 100 µl of the sample (diluted in 4 ml of PBS) was ultracentrifuged at 120,000 g for 4 hours with Beckman-Coultier SW55Ti rotor. Pellet was resuspended in 200 µl of Dulbecco's PBS (DPBS) (Sigma-Aldrich) and labeled with sample code (PEG/UC) and sample number.

The third sample preparation method was size exclusion chromatography (SEC). In this method, the bottom and top of PURE-EVs Size Exclusion Chromatography columns (Hansa-BioMed Life Sciences Ltd, Estonia) were opened, and 1 ml of PBS was pipetted into it. With a new SEC column, it was first needed to add 10 ml of PBS and let it run through to wash out the preserving solution, after which 100 µl of serum sample was added into the column. 1 ml of PBS is constantly added to the mixture to avoid the top layer from drying out. The first 500 ul of the run-through was collected at the bottom of the column into a separate 1.5 ml Eppendorf tube and labeled. Thereafter, 100 µl fractions (about three drops) were collected for 1000 µl / 10 fractions, and the collected samples were named and marked accordingly. Another 500 µl of the run-through is collected into the Eppendorf® tube and labeled as a second run-through with sample code. After the run-through was collected, another 10 ml of PBS was added to wash the column, and the run-through liquid was discarded.

Once the column was washed, 500 ml of 1 x PBS was added, and the bottom and top stoppers were secured in place. All the collected fractions were labeled with sample code and fractions number (SEC fr), as well as the sample number.

### 3.1.3 Gel Electrophoresis and Semi-dry protein transfer

After samples were prepared, they needed to be run on 10% SDS - PAGE gels and transferred to Immobilon®-P PVDF membranes (Carl Roth, Germany). To begin the process, 10% SDS-PAGE gels were prepared with 10% separating gel and 5% stacking gel. Samples were prepared for UC/PBS and PEG/UC by mixing 20 µl of 2x Laemlli + DTT with 20 µl of samples. They were kept on 100 °C heat for 10 min, vortexed and centrifuged for 2 min. 5 µl of PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (Thermo Fischer Scientific, USA) was loaded on the first lane of the gel followed by 10 µl of the prepared UC/PBS samples to the next lanes. Gel electrophoresis was run between 100 – 150 volts. These processes were also repeated for PEG/UC samples. Once the electrophoresis was done, the stacking gel was cut – off, and the size of the separating gel was measured. The gel was placed in a semi-dry transfer buffer. Six (6) Whatman® Blotting Papers (Merck, Germany) and Immobilon®-P PVDF membrane were then cut to identical size to the gel. Three (3) Whatman® Blotting Papers were soaked in semi-dry buffer solution, the Immobilon®-P PVDF membrane was pre-soaked in methanol for 1 min and then later placed in semi-dry buffer solution. The gel was stacked onto the PVDF membrane and then sandwiched together with the Whatman® Blotting Papers. The assembled sandwich was transferred to the semi-dry protein transfer machine and run at 15 volts for 30 min.

**Table 2**. Ultracentrifugation samples prepared for gel electrophoresis

| **Samples (UC/PBS, PEG/UC)** | **Loading volume** |
|---|---|
| PageRuler™ Prestained Protein Ladder | 5 µl |
| TYTI 8 | 10 µl |
| TYTI 10 | 10 µl |
| TYTI 14 | 10 µl |
| TYTI 38 | 10 µl |

| | |
|---|---|
| TYTI 78 | 10 µl |
| TYTI 10 | 10 µl |

### 3.1.4 Western Blot

After transfer of proteins to the membranes was completed, the membranes were blocked for 1 hour at room temperature of 25 °C in 2% non -fat dried (NFDM) milk prepared in WB–washing buffer. Membranes were subsequently transferred and incubated in primary antibody solution (2% NFDM in WB- Washing buffer) against the target protein (apo A1, apo E) for 1 hour at room temperature. The blot was rinsed 3x for 10 min with WB- Washing buffer and incubated in HRP- conjugated secondary antibody solution (2% NFDM in WB-Washing buffer) for 45 min with the corresponding primary antibody. Table 3. shows the overview of the sample purification methods and the concentration of antibodies used in the process. The blot rinsing was repeated (3x for 10 min). The imaging of proteins of interest was performed by applying a chemiluminescence substrate to the blot according to manufacturer recommendation, and signals were captured using an X-ray film.

**Table 3**. Purification methods and corresponding antibodies concentrations used in Western blotting

| | **Samples** | **Primary antibody** | **Secondary antibody** |
|---|---|---|---|
| Western Blots | UC/PBS samples | Apo A1, 26 kDa 1:1000 | Goat anti-mouse (GAM) 1:10000 |
| | | Apo E, 36 kDa 1:2000 | Goat anti-rabbit (GAR) 1:10000 |
| | PEG/UC samples | Apo A1, 26 kDa 1:1000 | Goat anti-mouse (GAM) 1:10000 |
| | | Apo E, 36 kDa 1:2000 | Goat anti-rabbit (GAR) 1:10000 |
| | SEC fraction samples | Apo A1, 26 kDa 1:1000 | Goat anti-mouse (GAM) 1:10000 |
| | | Apo E, 36 kDa 1:2000 | Goat anti-rabbit (GAR) 1:10000 |

## 3.2 RESULTS

### 3.2.1 Clinical laboratory analysis

Clinical data show that LDL – cholesterol and total cholesterol of samples 10 and 105 were the above normal value (marked in red). At the same time, HDL was relatively higher in all samples except sample 38.

**Table 4.** This is a clinical lab result of Apo A1, ApoB100, and cholesterol values from the 6 individuals.

| Samples | S,P-Chol (mmol/L) | S,P-HDL-Chol (mmol/L) | S,P-LDL-Chol (mmol/L) | S,P-non-HDL-Chol (mmol/L) | Apo A1 | Apo B100 | Sex | Age |
|---|---|---|---|---|---|---|---|---|
| **Reference value →** | **<5,0 mmol/l** | **>1,0 mmol/l** | **<3,0 mmol/l** | **<3,9 mmol/l** | **>43 µmol/l for men; >50 µmol/l for women** | **>1,95 µmol/l** | | |
| **8** | 3.8 | 1.16 | 2.11 | 2.64 | 46 | 1.3 | male | 31 |
| **10** | 6.5 | 1.44 | 3.86 | 5.06 | 64 | 2.28 | female | 56 |
| **105** | 6.6 | 1.89 | 3.74 | 4.71 | 62 | 1.05 | male | 54 |
| **38** | 2.5 | 0.85 | 1.17 | 1.65 | 62 | 1.62 | male | 59 |
| **14** | 3.7 | 1.57 | 1.47 | 2.13 | 63 | 1.26 | female | 25 |
| **78** | 4.3 | 1.79 | 2.07 | 2.51 | 54 | 1.64 | female | 30 |

### 3.2.2 Purification and analysis of extracellular vesicles

After SEC fractions were done, one sample, TYTI 38, was used to determine the number of proteins purified in all SEC collected fractions. For this, sample fractions of TYTI 38 were

selected and run on 10% SDS-PAGE gel with 5µl PageRuler™ Prestained Protein Ladder marker on the first lane followed by the fractions 3 to 10. The gel was put in Page Blue protein stain overnight after electrophoresis was completed. The gel was rinsed in distilled water for about 5 to 6 hours, and the gel was scanned. From the picture in (Figure 2), we can choose which SEC purified fractions should be used for Western blotting and also determine the fractions which contain EVs. The same SEC fraction samples of TYTI 38 were sent to Particle metrix laboratory for Zetaview Nanoparticle tracking analysis to determine the concentration of EVs in the samples. The results obtained can be seen in the Figure 3 graph. Page Blue staining image representation is compared to the graph in Figure 2, where some parallels between the images can be found (Figure 3).

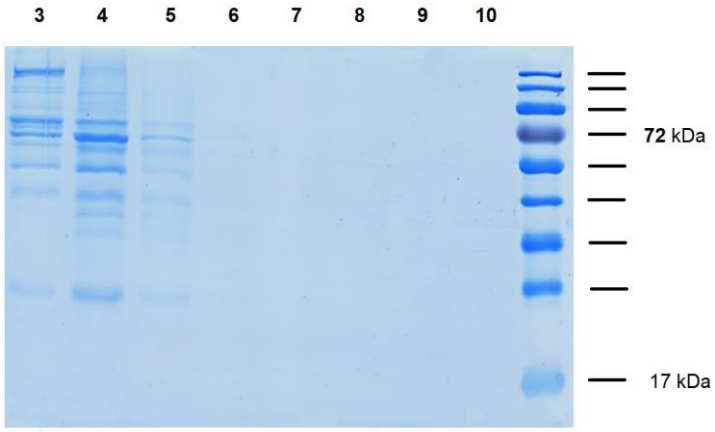


Figure 2. **Page Blue protein staining** of SEC fractions of sample TYTI 38 showing the various bands of proteins sizes from fraction 3 to 10. As shown in the diagram above, fraction 4 contains the most significant number of EVs.

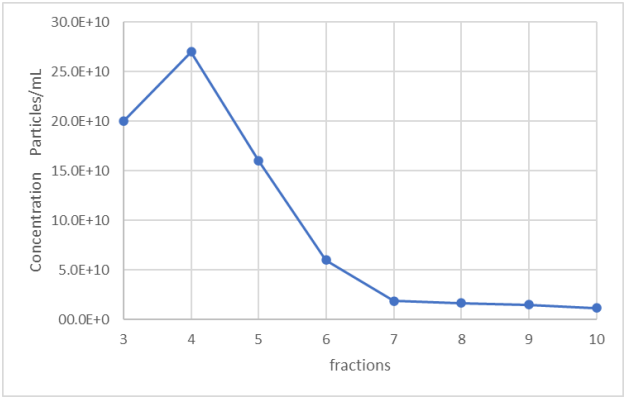


Figure 3. **Zetaview Nanoparticle tracking analysis** graph obtained from using SEC fraction of TYTI 38. The diagram illustrates the concentration of detected particles per milliliter

(mL) in corresponding fractions and shows the number of EVs in the fraction samples. For example, fraction 4 has the highest peak (27 x $10^{10}$ Particle/mL), according to the above graph in figure 2.

### 3.2.3 Western Blot results

Six TYTI samples were used for Western blot analysis. The Western blot analysis was done using UC/PBS and PEG/UC (Figure 4, Figure 5). Samples where secondary antibodies, GAM and GAR, were used against primary antibodies apo A1 and apo E respectively to confirm apolipoproteins in the samples. Apo A1 and apo E were detected at 26 kDa and 46 kDa, respectively, in UC/PBS samples. PEG/UC samples with Western blotting showed apo A1 and apo E were 26 kDa and 36 kDa, respectively. The final analysis was done using SEC fractions (Figure 6). The primary antibody used was apo E with secondary antibody GAR. Apo E was detected at 36 kDa. The result obtained from Western blotting is a confirmation of apolipoproteins in the samples using different purification methods.

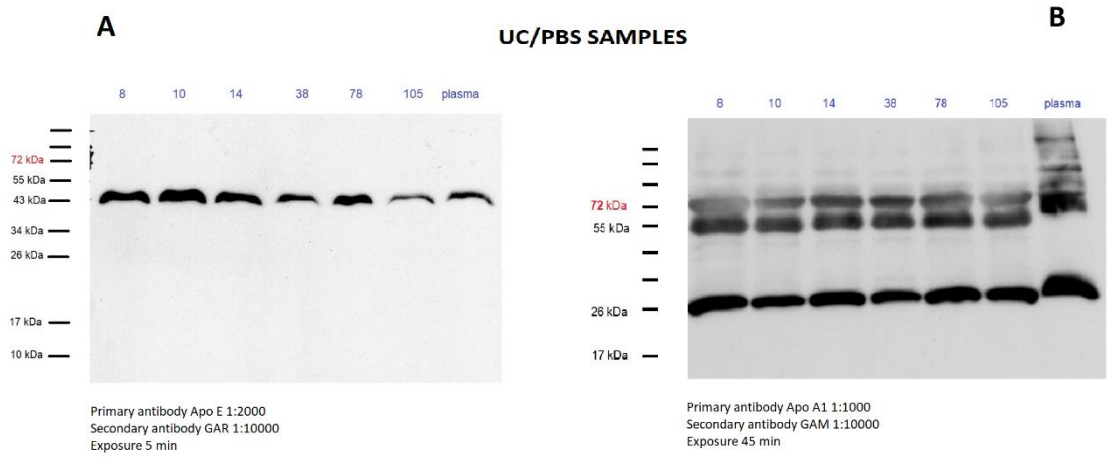


**Figure 4.** Analysis of Apo A1 and Apo E of UC/PBS purification method. **A:** Detection of Apo E in UC/PBS samples. The expected protein size is 36 kDa. **B:** Detection of Apo A1 in UC/PBS samples. The expected protein size is 26 kDa. The greater the intensity of the bands, the greater the number of target proteins present in the samples. UC/PBS demonstrated a remarkable process for apolipoprotein co-purification.

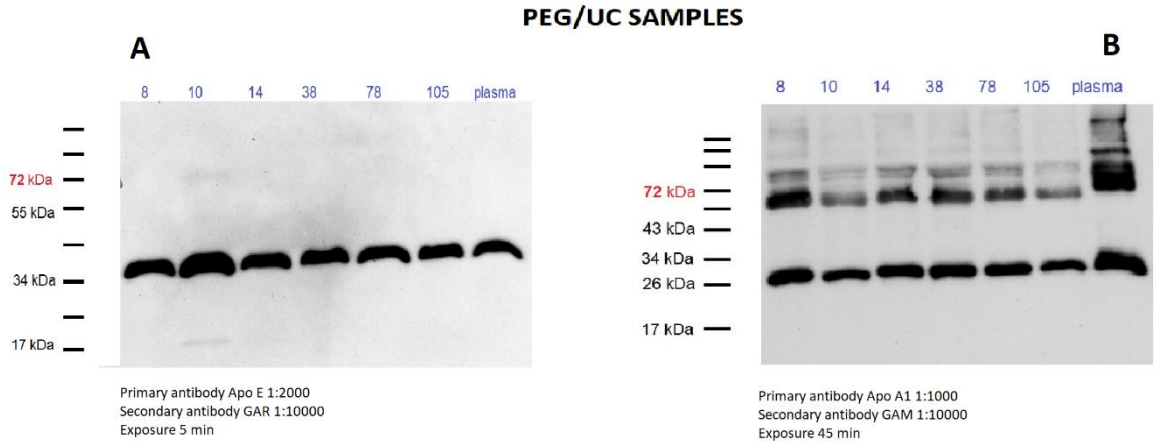


**Figure 5.** Analysis of confirmation of protein sizes of Apo A1 and Apo E using PEG/UC method for apolipoprotein detection. **A:** Detection of Apo E in PEG/UC samples with secondary antibody, GAR. The expected proteins size is 36 kDa. **B:** Detection of Apo A1 in PEG/UC samples with secondary antibody, GAM. The expected protein size is 26 kDa. Furthermore, strong bands indicate the number of target proteins in the samples. PEG/UC have also shown an outstanding method for detecting apolipoproteins.

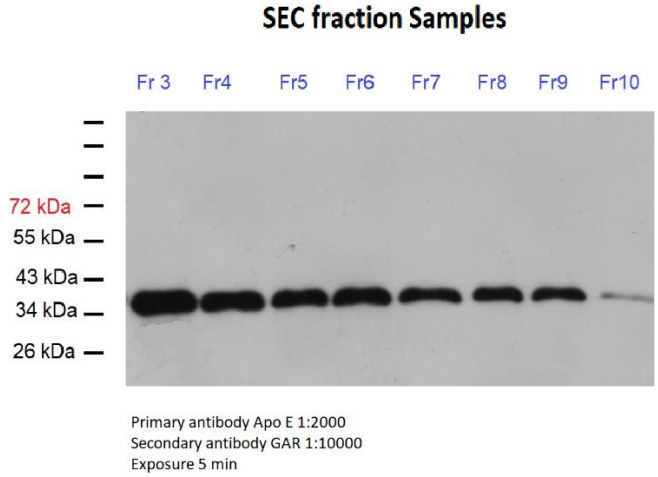


**Figure 6.** Analysis of the Apo E protein after SEC purification method. The intensity of bands decrease with lower fractions (Fr).

## 3.3 DISCUSSION AND CONCLUSION

Metabolism of lipoproteins is based on apolipoproteins. These multifunctional proteins act as templates for the assembly of particles of lipoprotein, preserve their structures, and guide their metabolism by binding them to membrane receptors (Dominiczak and Caslake, 2011). Lipoproteins can co-purify with extracellular vesicles. To detect apolipoproteins in extracellular vesicles from blood samples, as potential biomarkers or immunological studies for prognostic and prediction responses to therapies, they must first be isolated without losses as biologically purified active vesicles. There are currently several commercially established approaches for the enhancement and isolation of EVs (Muller *et al.*, 2014).

The main question here is whether we could detect apolipoproteins in samples of extracellular vesicles. To determine the presence of apolipoprotein in EVs, first three isolation methods were used to purify the EVs, which included ultracentrifugation through PBS, ultracentrifugation through PEG, and by size exclusion chromatography. However, according to the Western blotting results obtained from the above experiments in Figure 4, Figure 5, and Figure 6, it can be shown that apolipoproteins can be detected on all differently purified EVs. Similar research conducted by (Hirowatari *et al.*, 2010) also used sequential ultracentrifugation and chromatography methods for purifying blood serum and then analysed results with Western blotting to confirm the presence of apolipoproteins in the blood samples. They used two goat polyclonal antibodies against apo A1 and apo B and one goat polyclonal antibody against apo E. The results they obtained from the bands of apo A1 and apo E were 27 kDa and 35 kDa, respectively.

The other issue was to compare the connection between the strength of apolipoprotein and the purification procedures. The various purification methods used for the experiment were all able to detect apolipoproteins, but PEG/UC gave outstanding results, as evidenced by comparing the images in Figure 4, Figure **5**, and Figure **6**. Bands are more intense in the images in Figure 5.

Lastly, experimental results can be compared to clinical laboratory results. Apo A1 and Apo E can be compared to the HDL - cholesterol values in the clinical laboratory results. The link between the clinical laboratory results and the experimental work is that a higher clinical result is represented by the higher intensity bands in the western blotting results.

In a word, my research is based on determining the prevalence of apolipoproteins in EVs, if this prevalence is dependent on the method used, and how it could be linked to clinical analysis results done in a routine clinical laboratory where serum is used.

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