Master's Thesis

Western blot characterization of exosomes isolated from rat plasma and evaluation of the efficiency of a precipitation method in exosome isolation

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PREFACE

This Master's Thesis project was conducted in the Epilepsy Research group of Asla Pitkänen, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio. First, I would like to thank my supervisor Jenni Karttunen for excellent guidance to the world of exosomes, and for all the advice and help I received in the laboratory and during the writing process of this thesis. I thank my second supervisor, Sari Mattila, for reading and commenting my thesis. I would like to thank Academy Professor Asla Pitkänen for taking me into her research group and for offering the possibility to continue with the topic also after my Master's Thesis work. Many thanks to my colleague Vicente Navarro Ferrandis for teaching me the use of NanoSight and the importance of doughnut and coffee breaks. Finally, I would like to thank my dearest Tommi Patinen for being my constant peer support both during the Master's Thesis and all the five years of our studies at the University of Jyväskylä.

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Abstract of Master's Thesis

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Abstract:

Exosomes are 40–100 nm membrane-enclosed extracellular vesicles (EVs) secreted outside the cell by the fusion of multivesicular bodies with the plasma membrane. Exosomes play an important role in intercellular signaling by carrying different biomolecules, such as several types of RNA (mRNA, miRNA and other non-coding RNA), lipids and proteins. In the brain, exosomes are involved in neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and traumatic brain injury (TBI). Exosomes can pass the blood-brain barrier, and there has been a growing interest in the therapeutic potential of exosomes. Also, the contents of exosomes carry information about the ongoing processes in their cell of origin, which makes the exosome cargo, for example miRNAs, potential disease biomarkers. To claim the presence of exosomes in the isolate, they are recommended to be characterized according to the guidelines of International Society for Extracellular Vesicles (ISEV).

The aims of this study were 1) to set up a Western blot method for samples from rat plasma 2) to set up a Western blot method for characterization of exosomes according to the ISEV guidelines 3) evaluate the efficiency of the precipitation method in exosome isolation from rat plasma. The set-up of the Western blot method included optimization of the protocol and the antibodies, which were then used in exosome characterization for detection of Alix (EV marker), calnexin (cell contamination marker) and albumin (plasma protein contamination marker). Efficiency of the exosome precipitation was analysed by nanoparticle tracking analysis (NTA), SDS-PAGE and Western blot.

It was succeeded to optimize the anti-Alix, anti-calnexin and anti-albumin primary antibodies. However, finding working conditions for antibodies targeting other exosome marker proteins (CD63 and flotillin-1) failed, possibly because of unfunctional antibodies. In addition, the secondary antibodies were observed to strongly cross-react with the immunoglobulins naturally present in rat plasma, which caused unwanted bands in Western blot. Characterization confirmed the presence of exosomes, however, also the vast amount of contaminating plasma proteins in the exosome isolate. NTA results indicated that the share of larger particles increased during the isolation process, whereas the share of <60 nm particles (proteins and lipoproteins) clearly decreased. The results of the evaluation of the precipitation method efficiency by SDS-PAGE and Western blot indicated that the method removed ~60 % of the contaminating plasma proteins, of which albumin is the most abundant. In conclusion, it was succeeded to establish a Western blot characterization method for rat plasma samples. It was also concluded that the precipitation method enriched exosomes rather than purified them, since a lot of plasma proteins were co-isolated with exosomes. To improve the isolation of exosomes from plasma in future studies, use of alternative isolation methods i.e. size-exclusion chromatography should be investigated.

Keywords: extracellular vesicles, exosomes, characterization, Western blot, plasma, nanoparticle tracking analysis (NTA), precipitation

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Tiivistelmä:

Eksosomit ovat halkaisijaltaan 40-100 nm kokoisia solunulkoisia vesikkeleitä, joita solut erittävät solunsisäisen multivesikulaarisen rakkulan yhdistyessä solukalvoon. Eksosomit ovat tärkeässä roolissa solujen välisessä viestinnässä, sillä ne kuljettavat monenlaisia biomolekyylejä, kuten useita eri RNA-tyyppejä, lipidejä ja proteiineja. Aivoissa eksosomit liittyvät neurodegeneratiivisiin sairauksiin, kuten Parkisonin tautiin, Alzheimerin tautiin ja aivovammaan. Eksosomit kykenevät läpäisemään veri-aivoesteen, ja tutkijat ovatkin esittäneet paljon kiinnostusta eksosomien mahdollisuuksiin uutena hoitokeinona. Lisäksi eksosomien kuljettamat molekyylit sisältävät tietoa eksosomit erittäneen solun toiminnoista, minkä ansiosta ne voisivat mahdollisesti toimia biomarkkereina eri sairauksille. Eksosomeja voidaan puhdistaa usealla tavalla, esimerkiksi saostamalla, ultrafuugaamalla ja pylväskromatografialla. Puhdistuksen onnistumisen toteamiseksi suositellaan eksosomien karakterisointia ISEV:n (International Society for Extracellular Vesicles) asettamien suuntaviivojen mukaisesti.

Tämän tutkimuksen tavoitteet olivat 1) Western blot -menetelmän pystyttäminen rotan plasmanäytteille 2) Western blot menetelmän pystyttäminen eksosomien karakterisointia varten 3) saostusmenetelmän tehokkuuden arviointi eksosomipuhdistuksessa rotan plasmasta. Western blot -menetelmän pystyttämiseen sisältyi protokollan ja karakterisoinnissa käytettävien vasta-aineiden optimointi. Vasta-aineilla tunnistettavat proteiinit olivat Alix (vesikkelimarkkeri), kalneksiini (solukontaminaatiomarkkeri) ja albumiini (plasmaproteiinien kontaminaatiomarkkeri). Saostusmenetelmän tehokkuus arvioitiin usealla menetelmällä (NTA, SDS-PAGE, Western blot).

Anti-Alix, anti-kalneksiini ja anti-albumiini vasta-aineiden optimointi onnistui, mutta eksosomimarkkeriproteiini CD63:n ja flotilliini-1:n tunnistavia vasta-aineita ei saatu toimimaan. Anti-CD63 ja anti-flotilliini-1 eivät luultavasti toimineet ollenkaan, sillä niillä ei saatu signaalia edes positiivikontrollista. Tutkimuksessa huomattiin, että sekundaarivasta-aineet reagoivat vahvasti rotan plasmassa esiintyvien immunoglobuliinien kanssa, mikä aiheutti ylimääräisiä vyöhykkeitä Western blotissa. Eksosomien karakterisointi paljasti, että eristystuote sisälsi eksosomeja, mutta myös paljon albumiinia. NTA:n tulokset näyttivät, että suurempien partikkelien osuus kasvoi, kun taas 60 nanometriä pienempien partikkelien (proteiinit ja lipoproteiinit) osuus pieneni selkeästi eristysprosessin aikana. Arvioinnin muut tulokset osoittivat, että saostusmenetelmä onnistui poistamaan noin 60 % plasmaproteiineista, joista albumiini on kaikista yleisin. Tutkimuksessa onnistuttiin pystyttämään Western blot -menetelmä rotan plasmanäytteiden karakterisoinnille. Lisäksi havaittiin, että saostusmenetelmä ennemmin rikasti kuin puhdisti eksosomeja, sillä eksosomien ohella saostui paljon plasman proteiineja. Tulevaisuudessa olisi kannattavaa yrittää parantaa eksosomien eristystä kokeilemalla jotakin toista puhdistusmenetelmää, esimerkiksi pylväskromatografiaa.

Avainsanat: solunulkoiset vesikkelit, eksosomit, karakterisointi, Western blot, plasma, NTA, saostus

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ABBREVIATIONS

EV	extracellular vesicle
FP	filtered plasma
HDL	high-density lipoprotein
IgG	immunoglobulin G
ISEV	International Society for Extracellular Vesicles
LDL	low-density lipoprotein
MVB	multivesicular body
NTA	nanoparticle tracking analysis
PVDF	polyvinylidene fluoride
SEC	size-exclusion chromatography
TEM	transmission electron microscopy
TP	thrombin-treated plasma

1. INTRODUCTION

1.1 Extracellular vesicles and their role in intercellular signaling

Cells of multicellular organisms use several ways to communicate with each other. Communication can be direct, as in cell-cell contact, or indirect, which means secretion of different signaling molecules. In the 1990s, findings from several studies (Raposo et al., 1996; Zitvogel et al., 1998) suggested that there is also a third mediator of intercellular communication – secretion of extracellular vesicles (EVs).

EVs are lipid bilayer membrane-enclosed vesicles released from the cell. EVs can be classified into three groups – exosomes, microvesicles and apoptotic bodies – based on their biogenesis pathways (see review EL Andaloussi et al., 2013). Exosomes, the smallest type of EVs (40–100 nm), are generated by intraluminal budding of multivesicular bodies (MVBs) and released in the fusion of MVBs with the plasma membrane of the cell (see review Thery et al., 2002). MVBs are endosomes that contain intraluminal vesicles, and they develop from early endosomes (see review Gruenberg and Maxfield, 1995). In contrast to exosomes, microvesicles originate by outward budding of the cell membrane, and their size varies between 50 and 1000 nm (see review EL Andaloussi et al., 2013). Apoptotic bodies are the largest EVs, sized 500–2000 nm, and they are generated by outward blebbing of apoptotic cell membrane. Because of their differing biogenesis pathways, the three EV types have different proteins enriched in them (Turiák et al., 2011), and these proteins can be used as markers to distinguish different EV types from each other. However, the markers are not absolutely specific since the same protein can be found in more than one EV type (Kowal et al., 2016). The overlapping sizes of different EVs cause challenges, especially in isolation and characterization of specific EVs. As the EV nomenclature has not yet permanently settled, in literature EVs are also often referred as microparticles (for review see van der Pol et al., 2016). In addition, even though the EV isolation methods are not able to separate different EV types from each other perfectly, the isolate is often called exosomes. For simplicity, in this thesis the vesicles are called exosomes, however, being aware that they cannot be completely separated from the other EV subtypes.

The role of exosomes in intercellular signaling is based on their contents: the vesicles carry many different biomolecules, such as several types of RNA (mRNA, miRNA and other non-coding RNAs), cytoplasmic and membrane proteins, and lipids (Valadi et al., 2007; Subra et al., 2010; de Jong et al., 2012). Exosomes are able to transfer their content mRNA and miRNA from one cell to another, which has been shown for example by Valadi et al. using mouse and human mast cell lines. Valadi et al. also proved that the exosome- transferred mRNA is functional in the recipient cell, which means it can encode proteins. In addition, the same study identified different miRNAs in exosomes. By delivering their protein and RNA cargo to recipient cells, exosomes participate in the control of biological processes, for example immune response and blood coagulation (Bhatnagar et al., 2007; Srikanthan et al., 2014).

1.2 Exosomes and neurodegenerative diseases

Exosomes have a role in disease pathogenesis, for example in the development of neurodegenerative diseases (see review Quek and Hill, 2017). The abnormally folded prion protein scrapie has been displayed to be released from the cells in association with exosomes, and the exosome-associated prion protein is also able to transfer infectivity (Fevrier et al., 2004). Exosomes may therefore be involved in the spread of prions from other parts of the body to the brain, which is the manifestation site of prion diseases such as Creutzfeldt-Jakob disease.

In Parkinson's disease, a protein called α-synuclein aggregates in neurons and forms Lewy bodies, which are one of the hallmarks of the disease (see review Antony et al., 2013). Emmanouilidou et al. (2010) presented that cells secrete α-synuclein to the extracellular space in association with exosomes, and α-synuclein-containing cell medium has a negative effect on the viability of the other cells. α-synuclein located in exosomes is more prone to internalization by the cells compared to free extracellular α-synuclein, suggesting a role for exosomes in the spread of toxic aggregates during the development of Parkinson's disease (Danzer et al., 2012).

Alzheimer's disease is characterized by the formation of amyloid-β (Aβ) peptide plaques and tau tangles which leads to the death of neurons (see review Bloom, 2014). A small fraction (<1%) of Aβ has been found to be released from the cell in association with exosomes (Rajendran et al., 2006). However, neuron-derived exosomes have been demonstrated to

promote uptake of Aβ by microglia, which suggests involvement of exosomes in Aβ clearance (Yuyama et al., 2012). Disturbance in the balance of secretion and clearance could possible drive the system towards disease pathogenesis (Yuyama et al., 2012).

Traumatic brain injury (TBI) is an alteration in brain function caused by an external force (Menon et al., 2010). Every year, 2.5 million people suffer TBI in Europe, and it is the leading cause of death and disability in young adults (CENTER-TBI, https://www.center-tbi.eu/, accessed May 18, 2017). TBI causes various secondary pathological conditions, for example neurodegenerative diseases and epilepsy (see review Bramlett and Dietrich, 2015). Exosomes may play a role in the intercellular signaling of the damaged brain: EVs isolated from the cerebrospinal fluid have been presented to carry miRNAs associated with regulation of neuronal functions (Patz et al., 2013), and there are changes in the miRNA profile of EVs after TBI (Harrison et al., 2016).

1.3 Methods for isolation of exosomes

There are several methods for exosome isolation, but the field is still missing a "golden standard" method that could be used in all cases. At the moment, the optimal isolation method depends on the downstream applications and the goals of the study. For example, if the aim is to acquire exosomes of highest purity, it is better to choose an isolation method that minimizes the amount co-isolating proteins. Correspondingly, if subsequent downstream applications require a large volume of exosome preparation, the yield may weigh more than quality. Precipitation of membrane particles, ultracentrifugation, antibody-coated microbeads and size-exclusion chromatography (SEC) are the major exosome isolation methods (see review Szatanek et al., 2015; Gardiner et al., 2016). Nowadays, there are several commercial exosome purification kits available based on different methods.

In exosome precipitation, which was the isolation method used in this thesis, membrane structures are precipitated and pelleted to separate the exosome-containing pellet from the supernatant. The precipitation method can provide high recovery of exosomes; however, many contaminating proteins may get co-precipitated. There are several commercially produced

precipitation kits offered by companies such as Thermo Fisher and Exiqon, and kits are often specified for exosome isolation either from cell culture or biofluids, e.g. plasma.

In the ultracentrifugation method, exosomes are separated from other particles, such as cell debris and larger vesicles, based on their size by multiple centrifugation steps, as the centrifugal force is increased at each step and a subgroup of particles gets pelleted out. The method was first used in the exosome field by Raposo et al. (1996) for purification of exosomes from cell culture media of B lymphocytes. Also size-exclusion chromatography separates particles by size: the porous material in the chromatography column slows down the progress of smaller particles (proteins), as larger particles (exosomes) that do not fit in the pores pass the column faster (Boing et al., 2014).

Compared to methods which aim to isolate the total population of exosomes, the use of antibody-coated microbeads provides a method for specific capture of exosomes from different origins (Hong et al., 2014). Specific isolation of exosomes of interest is an essential tool when one desires to study exosomes secreted by specific cells outside a cell culture. To discover biomarkers for detection of developing disease, it may be important to isolate only the exosomes derived from the cells involved in disease pathogenesis. However, even the exosomes derived from the same cell type form subpopulations with different protein compositions and biological properties (Willms et al., 2016), which makes it difficult to isolate specific exosomes from many subpopulations.

1.4 Requirements for definition of exosomes and their function

The exosome research field has grown rapidly during the past 10–15 years, but it still lacks common standards that would help to compare the exosome preparations between different laboratories. International Society for Extracellular Vesicles (ISEV) has proposed minimal experimental requirements for definition of extracellular vesicles and their function (Lötvall et al., 2014). The criteria set guidelines for characterization of EVs, including exosomes. For example, the guidelines recommend that to claim the presence of EVs/exosomes in preparations, three or more proteins should be investigated in at least semi-quantitative manner, and the investigated proteins should belong to different categories, which are i) transmembrane or lipid-

bound extracellular proteins ii) cytosolic proteins iii) intracellular proteins and iv) extracellular proteins. The reasoning behind the investigation of several proteins is that since one absolute marker protein for exosomes does not exist, it is important for reliability to verify more than one protein that is expected to be enriched in exosomes. Furthermore, the guidelines highly recommend that the presence of proteins that are not expected to be enriched in exosomes is investigated as well. These proteins belong to the groups 3 and 4, and their purpose is to function as indicators of possible contamination from other types of exosomes, such as proteins associated with other membrane compartments, for example calnexin, which is found in endoplasmic reticulum (group 3), or extracellular proteins that can co-isolate with exosomes, such as albumin (group 4).

General characterization of exosomes is often conducted by Western blotting or fluorescence-activated cell sorting flow cytometry. For characterization of single exosomes, ISEV guidelines recommend use of at least two different technologies. Characterization of single exosomes is often performed by microscopy, for example transmission electron microscopy (TEM) or atomic force microscopy, which provide information about the size and morphology of the vesicles (Wu et al., 2015; Höög and Lötvall, 2015). Nanoparticle tracking analysis (NTA) is a method used to measure the concentration and size distribution of particles, and it is often used in exosome characterization to analyse the size of large number of vesicles (Soo et al., 2012).

1.5 Exosome marker proteins

Proteomic studies on the protein content of EVs has showed that the proteomic compositions of exosomes, microvesicles and apoptotic bodies greatly overlap with each other (Turiák et al., 2011; Kowal et al., 2016), which is why reliable identification of EVs originating from different intracellular compartments is challenging. Therefore, the following marker proteins should not be interpreted as exosome-specific, but rather as exosome-enriched proteins (see review Lötvall et al., 2014).

Tetraspanins are transmembrane proteins involved in functions of the plasma membrane. They consist of four transmembrane domains, two extracellular regions and three intracellular regions (see review Charrin et al., 2014). Some tetraspanins, especially CD9, CD63 and CD81, are

commonly used as EV marker proteins, for they have been shown to be selectively enriched in EVs (Raposo et al., 1996; Escola et al., 1998; Théry et al., 1999). The ISEV guidelines classify tetraspanins in the group of membrane-bound exosome marker proteins (Lötvall et al., 2014).

Flotillins (flotillin-1 and flotillin-2) are membrane-associated proteins that form microdomains on the cytosolic side of the plasma membrane, where they are involved in cellular functions such as endocytosis, cell signaling and cytoskeletal regulation (see review Otto and Nichols, 2011). Flotillin-1 and flotillin-2 have been detected in exosomes derived from different cell types, e.g. reticulocytes and cortical neurons, and several cancer cells, i.e. neuroblastoma and melanoma cells (de Gassart et al., 2003; Lazar et al., 2015; Keerthikumar et al., 2015). Therefore, especially flotillin-1 is commonly used as an exosome marker protein. In the ISEV guidelines, it belongs to the group of cytosolic exosome-associated proteins (Lötvall et al., 2014).

Alix (also known as PDCD6IP) is a protein involved in the biogenesis of exosomes (Baietti et al., 2012). Alix was initially discovered in apoptotic signaling, where it interacts with a calcium-binding protein called ALG-2 (Missotten et al., 1999). Years later, Alix was found to be involved with the endosomal sorting complexes required for transport machinery in formation of multivesicular bodies (Matsuo et al., 2004). In the study of Baietti et al., Alix was observed to co-accumulate in exosomes with the proteins syndecan and syntenin. The study showed that syntenin connects syndecans to Alix, and together they regulate the budding of endosomal membranes and therefore exosome formation. More information on the role of Alix was acquired when the study of Iavello et al. (2016) implicated that Alix is involved in the packaging of miRNAs into exosomes released from human liver-like stem cells. As exosome marker, Alix belongs to the group of cytosolic proteins enriched in exosomes (Lötvall et al., 2014), and according to the exosome database ExoCarta (http://www.exocarta.org/), it is one of the most commonly found proteins in exosomes.

1.6 Future perspectives of exosome studies

Recently, there has been an emerging interest in exosomes as therapeutic agents or biomarkers for different diseases. This thesis is a part of a larger project that aims to reveal the role of exosomes in the post-TBI functions of the brain, especially in the development of post-traumatic

epilepsy (for review see Pitkänen and Immonen, 2014). At the moment, there are no treatments to improve recovery after TBI. The ability of exosomes to pass the blood-brain barrier (Alvarez-Erviti et al., 2011) make them an attractive research subject for developing treatments for repairing damage in the central nervous system, such as TBI. Mesenchymal stem cell derived exosomes have already been demonstrated to improve functional recovery after TBI in rats (Zhang et al., 2015).

The contents of exosomes carry information about the ongoing processes in the cell of origin, which makes the exosome cargo a potential research subject in the search for new biomarkers for disease diagnostics (for review see Leung et al., 2015). The purpose of this thesis was to prepare the methods required in the future studies of the Epilepsy Research group on plasma-isolated exosomes. The long-term aim is to discover exosome content-based biomarkers for the detection of developing post-traumatic epilepsy from patient blood samples.

2. AIMS OF THE STUDY

Set up a reliable and repeatable method for characterization of plasma-isolated exosomes by Western blot, including:

- Setting up a Western blot method for rat plasma samples
- Testing and optimisation of antibodies used in Western blotting for detection of EVs according to ISEV guidelines, to set up a method for exosome characterization
- Evaluation of the efficiency of the currently used precipitation method in exosome isolation by analysing sample purity (Western blot) and particle number and size distribution (nanoparticle tracking analysis) at different phases of the protocol

3. MATERIALS AND METHODS

3.1 SDS-PAGE and Western blot

The plasma used in this study was collected earlier from naïve male adult Sprague-Dawley rats according to the standard protocol used in the laboratory and stored in -70 ºC. Exosome isolation from platelet-free rat plasma was conducted using a commercial miRCURY Exosome Isolation Kit – Serum and Plasma (Exiqon, Vedbaek, Denmark). Protein concentration was quantified by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were mixed with 4x Laemmli Sample Buffer (10% β-mercaptoethanol, Bio-Rad, Hercules, CA, USA) and diluted with PBS to acquire equal amounts of protein for Western blotting, and then boiled for 10 min at 95 ºC. From 5 µg to 20 µg of protein samples were run in 12 % acrylamide SDS-PAGE. Coomassie blue was used for staining the gels. For Western blot analysis, samples run in SDS-PAGE were blotted onto PVDF membranes (Amersham Hybond P 0.45, GE Healthcare). After blotting, membranes were blocked in 1xTEN 1% Tween-20 (see Appendix 1) for 2 hours in room temperature or overnight at 4 ºC. Membranes were then incubated with a primary antibody for 1.5 h in room temperature or overnight at 4 ºC. After six five-minute washes in 1xTEN 0.1% Tween-20, membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature, after which washes were repeated. Finally, membranes were subjected to HRP-detecting SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and imaged with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) using Image Lab v. 5.2.1 software (Bio-Rad).

3.2 Antibodies

Primary antibody and secondary antibody dilutions were made in 1xTEN 1 % Tween-20 and 1xTEN 0.1 % Tween-20, respectively. The used primary antibodies and secondary antibodies are listed in Table 1 and Table 2. For each antibody, several different dilutions were tested to optimize the protocol. The tested dilutions ranged from 1:500 to 1:10 000.

Table 1. **List of primary antibodies.**

Antibody target	Host	Code	Company	Marker type
Albumin	goat	sc-46293	Santa Cruz Biotechnology	Plasma protein
Alix	rabbit	ab186429	Abcam	EV marker (intravesicular)
Calnexin	rabbit	sc-11397	Santa Cruz Biotechnology	Cell contamination (ER)
CD63	goat	sc-31214	Santa Cruz Biotechnology	EV marker (transmembrane)
CD63	mouse	sc-5275	Santa Cruz Biotechnology	EV marker (transmembrane)
Flotillin-1	rabbit	ab133497	Abcam	EV marker (membrane protein)

Table 2. **List of secondary antibodies.**

Antibody	Code	Company
goat anti-rabbit, preadsorbed	ab7090	Abcam
goat anti-rabbit	ab6721	Abcam
goat anti-rabbit	656120	Invitrogen
rabbit anti-goat	61-1620	Thermo Fisher
rabbit anti-goat	sc-2922	Santa Cruz Biotechnology
mouse-IgGκ BP-HRP	sc-516102	Santa Cruz Biotechnology

3.3 Collection of exosome isolation step samples

Exosome isolation was performed with starting volume of 300 µl rat plasma using miRCURY Exosome Isolation Kit – Serum and Plasma. The isolation was conducted following the protocol of the kit with an additional filtering step before addition of the precipitation buffer. During the isolation process, three 2 x 10 µl plasma samples were collected at different steps of the isolation protocol (Figure 1): before starting the isolation (plasma), after adding thrombin (thrombin-treated plasma) and after centrifugation and filtering through 0.22 µm PVDF filter (Millex-GV, Merck Millipore) but before precipitation (filtered plasma). Finally, the supernatant and purified exosomes were collected. Samples were stored at -70 ºC until analysis by SDS-PAGE, Western blotting (Alix and albumin) and nanoparticle tracking analysis.

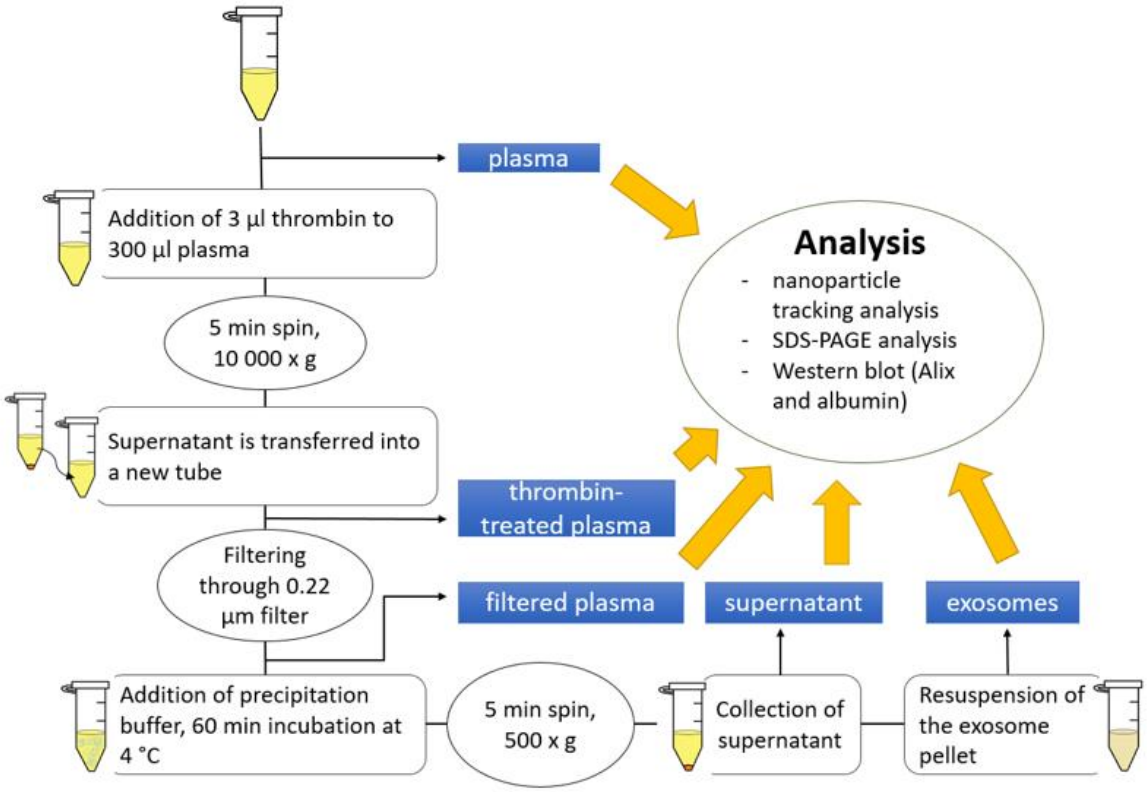

Figure 1. **Flowchart of exosome isolation from plasma with an exosome precipitation kit.** Isolation was conducted using miRCURY Exosome Isolation Kit – Serum and Plasma (Exigon, Vedbaek, Denmark), with additional filtering step through 0.22 µm PVDF filter. The collected samples were analysed by NTA, SDS-PAGE and Western blot to evaluate the progress and efficiency of the isolation protocol.

3.4 Nanoparticle tracking analysis

Size distribution and concentration of particles in EV isolation step samples were measured with NanoSight NS300 instrument (Malvern, Worcestershire, UK) and analysed with NTA v. 3.2 software. Samples were diluted in PBS (0.22 µm filtered) as followed: plasma 1:5000, thrombin-treated and filtered plasma 1:10 000, supernatant 1:300 and exosomes 1:10 000. Syringe pump system was used to introduce the sample, and its speed was set to 40. Recordings were performed during 60 seconds at 25 frames per second, with camera level 13, and the measurement was conducted three times to each sample. For the analysis, the detection threshold was set to 5, and the blur size and the max jump distance were set to automatic.

4. RESULTS

4.1 Optimization of the Western blot protocol and primary antibodies

4.1.1 The effect of blocking and primary antibody incubation time on blot quality

The main challenge in setting up the Western blot method for exosome characterization was finding the working conditions for antibodies used in the immunodetection of the investigated proteins. The aim was to minimize the background and maximize the signal strength of the correct band. Conditions were altered by changing the duration and temperature of blocking and primary antibody incubation, and by preparing several antibody dilutions. The antibody dilutions included the dilution recommended by the manufacturer and a couple of other dilutions slightly higher and lower than that. In addition to the TEN 1 % Tween-20 buffer, also milk-containing buffers were tested in blocking and primary antibody incubation. Nonspecific binding of the primary antibody was a major problem with many blots, however, in these cases, even milk blocking failed to prevent the false bands caused by nonspecific binding. Moreover, milk tended to cause cloudy smudge on the membranes (data not shown). Overnight blocking at 4 ºC and 1.5-hour primary antibody incubation at RT were chosen as default protocol, but also 2-hour blocking at RT followed by overnight primary antibody incubation at 4 ºC were tested with new antibodies. Even though the protocol was not observed to affect the blot result with most of the antibodies, it was found out that with the anti-Alix primary antibody, 2-hour blocking at RT followed by overnight primary antibody incubation clearly reduced the background produced by nonspecific binding of the primary antibody (Figure 2).

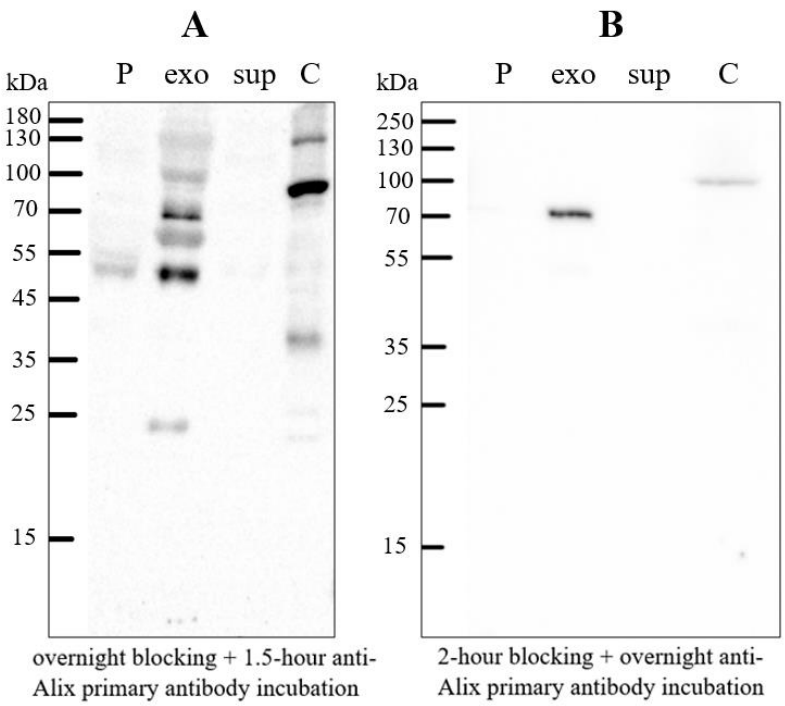

Figure 2. **The effect of blocking and primary antibody incubation time on the blot background.** (A) Blocking overnight at 4 ºC followed by 1.5 hours at RT anti-Alix (1:2000 dilution) primary antibody incubation. (B) Blocking 2 hours at RT followed by overnight incubation in anti-Alix (1:2000 dilution) primary antibody. Both membranes were finally incubated in 1:2000 secondary antibody for 1 hour.

4.1.2 Optimal conditions for anti-Alix primary antibody

The best-working dilution for anti-Alix primary antibody (rabbit, ab186429) was 1:2000, followed by secondary antibody incubation in 1:2000 preadsorbed goat anti-rabbit from Abcam. The best protocol was 2-hour blocking at RT followed by overnight primary antibody incubation at 4 ºC (Fig 2).

4.1.3 Optimal conditions for anti-albumin and anti-calnexin primary antibodies

The primary antibodies against albumin and calnexin had already been confirmed to work before, but they were also tested to optimize the blot quality. For anti-albumin (goat, sc-46293), both tested dilutions (1:500 and 1:2000) worked well, however, 1:2000 dilution was chosen to minimize antibody consumption. The best-working secondary antibody with anti-albumin was rabbit anti-goat from Thermo Fisher Scientific, with a dilution of 1:2000. Anti-calnexin primary antibody (rabbit, sc-11397) displayed good results both with 1:500 and 1:1000 primary antibody dilutions, however, the 1:1000 dilution was chosen to save the reagents. The best result was

obtained with the anti-rabbit secondary antibody from Abcam (ab7090) in 1:3000 dilution (Fig 4).

4.1.4 Anti-CD63 and anti-flotillin-1 primary antibodies failed to produce correct bands

Finding working conditions for anti-CD63 (goat, sc-31214) and anti-flotillin-1 (rabbit, ab133497) primary antibodies failed: Blots with anti-CD63 and anti-flotillin-1 antibodies did not yield any bands of correct size in exosomes, plasma or supernatant with any of the tested immunodetection protocols and antibody dilutions. The antibodies did not present a band even in the positive control (rat cortex lysate). In addition to the goat anti-CD63 antibody, another anti-CD63 primary antibody (mouse, sc-5275) from Santa Cruz Biotechnologies was tested. However, also this antibody failed to produce any bands of correct size.

The tested primary antibodies and their best-working protocols are listed in Table 3.

Table 3. **Tested primary antibodies and their best immunodetection protocols.**

Antibody target	Code	Blocking	1° ab dilution	1° ab incubation	2° ab dilution	2° antibody	2° incubation
Albumin	sc-46293	o/n 4 °C	1:2000	1.5 h RT	1:2000	61-1620	1 h RT
Alix	ab186429	2 h RT	1:2000	o/n 4 °C	1:2000	ab7090	1 h RT
Calnexin	sc-11397	o/n 4 °C	1:1000	1.5 h RT	1:3000	ab6721	1 h RT
CD63	sc-31214	-	-	-	-	-	-
CD63	sc-5275	-	-	-	-	-	-
Flotillin-1	ab133497	-	-	-	-	-	-

4.2 Cross-reactivity of secondary antibodies with plasma IgGs causes unwanted bands

Cross-reactivity of the secondary antibodies with immunoglobulins present in rat plasma caused problems during the optimization process. Control blots (Fig 3A-F) that were incubated only in the secondary antibody revealed that the ~55 kDa band appearing in the blots was produced by binding of the secondary antibody to the heavy chain of immunoglobulin G (IgG), size of which is about 55 kDa. In addition, also a band of ~26 kDa was detected with some antibodies, and it was especially strong with the anti-mouse secondary antibody from Promega (Fig 3C).

The ~26 kDa band was produced by secondary antibody binding to the IgG light chain (25 kDa). From all tested secondary antibodies, the rabbit anti-goat antibody from Santa Cruz Biotechnologies was observed to exhibit least cross-reactivity (Fig 3B). However, subsequent blots revealed that with a longer detection time, the IgG heavy chain band was observed also with this antibody. All in all, cross-reactivity with the plasma IgGs was observed with all the tested secondary antibodies, band intensity depending on the detection time. Importantly, the IgG bands appeared stronger in the exosome fraction than in plasma, which indicated that the exosome isolation method precipitated rat plasma IgGs to the exosome fraction. Since the cross-reactivity issue could not be completely omitted, it was decided to change the exosome marker from the medium-sized proteins CD63 and flotillin-1 to Alix, which is sized 97 kDa and could therefore be distinguished from the IgG bands. The two secondary antibodies that exhibited least cross-reactivity – rabbit anti-goat (Santa Cruz Biotechnologies, Fig 3B) and preadsorbed goat anti-rabbit (Abcam, Fig 3F) – were chosen as the best secondary antibodies to be used in future. All tested secondary antibodies are presented in Table 4.

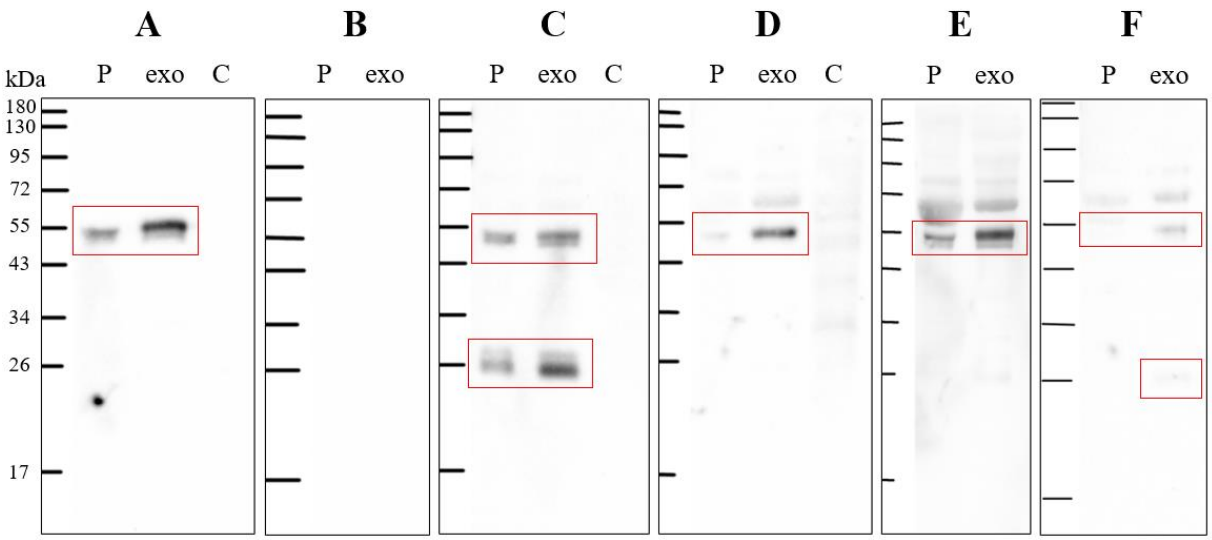

Figure 3. **Cross-reactivity of the secondary antibodies with plasma immunoglobulin G (IgG).** The image shows Western blots incubated only in the tested secondary antibodies, which are (A) rabbit anti-goat (Thermo Scientific) (B) rabbit anti-goat (Santa Cruz Biotech.) (C) anti-mouse (Promega) (D) goat anti-rabbit (Invitrogen) (E) goat anti-rabbit (Abcam) (F) goat anti-rabbit preadsorbed (Abcam) Automatic detection time was used in imaging. The 55-kDa band is caused by cross-reactivity of the secondary antibody with the heavy chain of plasma IgGs. The 25-kDa band that was especially strong with the anti-mouse secondary antibody (panel C) comes from the IgG light chain. IgG bands are marked by red boxes. From all the tested antibodies, the rabbit anti-goat from Santa Cruz Biotechnologies (panel B) exhibited least cross-reactivity. However, with longer detection time, the IgG heavy chain band was observed also with this antibody. P = plasma, exo = exosomes, C = rat cortex lysate

Table 4. **Tested secondary antibodies.**

Antibody	Code	Company	Cross-reactivity with plasma IgGs
goat anti-rabbit, preadsorbed	ab7090	Abcam	+ +
goat anti-rabbit	ab6721	Abcam	+ + +
goat anti-rabbit	656120	Invitrogen	+ +
rabbit anti-goat	61-1620	Thermo Fisher	+ + +
rabbit anti-goat	sc-2922	Santa Cruz Biotech.	+
m-IgGk Bp-HRP	sc-516102	Santa Cruz Biotech.	+ + +

4.3 Method for general Western blot characterization of exosomes

The minimal experimental requirements set by ISEV demand investigation of certain proteins to evaluate the purity of the exosome isolate. Alix is an exosome marker protein with the predicted size of 97 kDa. In the guidelines of ISEV, it belongs to the group of cytosolic EV marker proteins. Western blot with anti-Alix antibody presented an intense band in the exosome fraction near the 70 kDa marker, and a fainter, about 100 kDa band in the rat cortex lysate, which was the positive control (Fig 4A). The lower band matches the size of Alix without its C-terminal domain. No bands were detected in the plasma and the supernatant.

Calnexin is used as a positive control to detect contamination by non-exosomal vesicles originating from other membrane compartments when cells are broken. Immunodetection of calnexin produced a band in the positive control (rat cortex lysate) near the 100 kDa marker, which corresponds to the predicted size of 90 kDa (Fig 4B). The absence of calnexin in the other lanes indicated that plasma, exosomes and supernatant did not contain cell contaminants.

Albumin is used as a marker for protein contamination in the exosome isolate. Immunodetection of albumin resulted in thick and intense bands below the 72 kDa mark in plasma, exosomes and supernatant (Fig 4C). The predicted size of serum albumin is 66 kDa, which matches the observed band. The results indicated abundance of contaminating plasma proteins also in the exosome fraction, however, the amount of proteins appeared to be smaller than in plasma and supernatant.

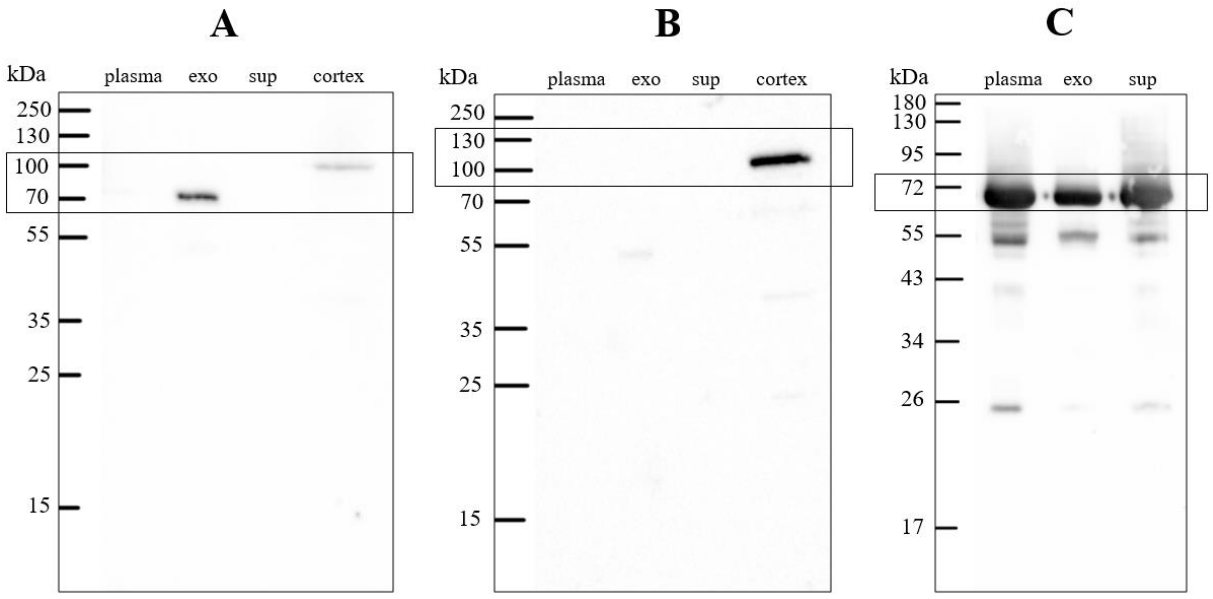

Figure 4. **General characterization of the isolated exosomes by Western blot.** Precipitation kit-purified rat plasma exosomes were characterized by investigating the presence of (A) EV marker protein Alix, (B) cell contamination marker protein calnexin and (C) plasma protein contamination marker albumin. The correct bands are marked by black boxes. Exo = exosome isolate, sup = supernatant, cortex = rat cortex lysate (positive control).

4.4 Evaluation of the efficiency of the precipitation method in exosome isolation

4.4.1 Particle concentration and size-distribution at different steps of the exosome isolation

In order to analyse the progress of exosome isolation, the particle concentration and size distribution were measured from samples collected at five different steps of the exosome isolation protocol. Nanoparticle tracking analysis revealed that the particle concentration of plasma was 3.10×10^{12} particles/ml (Fig 5A). The particle concentration of thrombin-treated plasma (TP) and filtered plasma (FP) were 5.46×10^{12} and 5.33×10^{12} particles/ml, respectively. Particle concentration of the exosome isolate was 4.67×10^{12} particles/ml. Compared to the other samples, the particle concentration of the supernatant was over 100 times lower, only 2.77×10^{10} particles/ml.

Organization of particles in groups based on their size revealed the particle size distribution for each sample (Figure 5B-F). In all samples, particles sized 60–80 nm formed the largest group, and their concentration grew when proceeding from plasma to TP and finally to FP, where their concentration was the highest. Although particles <60 nm were the second largest group in plasma, TP and FP, their share was very small in the exosome sample, where the second largest

group were particles sized 81–100 nm. While plasma, TP and FP all had quite similar size distribution profiles except the 60–80 nm peak in FP, the share of larger particles, especially 101–120 nm, 121–140 nm and 161–180 nm groups, was relatively larger in the exosome fraction than in the other samples.

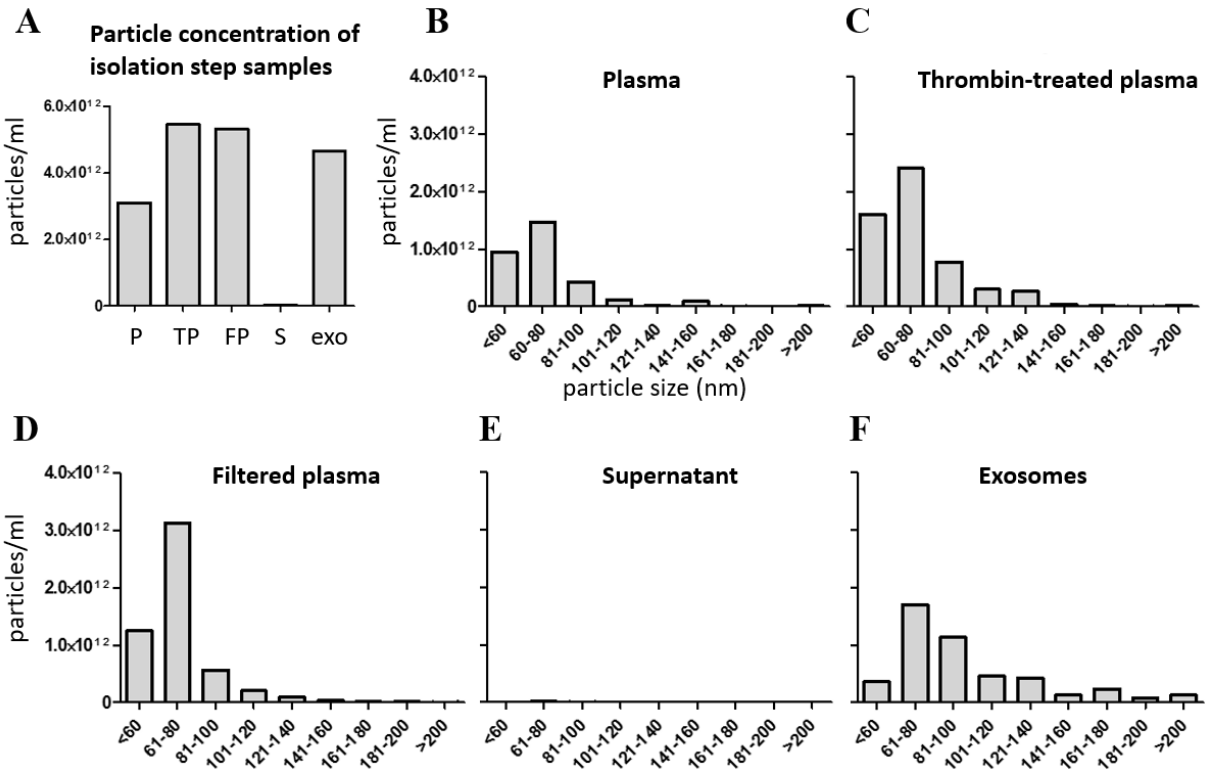

Figure 5. **Particle concentration and size distribution of exosome isolation step samples.** Samples were collected at five different steps of the exosome precipitation protocol. Particle concentration and size distribution were measured by nanoparticle tracking analysis (NTA). (A) Measured particle concentrations (particles/ml) were $3.10x10^{12}$ (plasma), $5.46x10^{12}$ (thrombin-treated plasma), $5.33x10^{12}$ (filtered plasma), $2.77x10^{10}$ (supernatant) and $4.67x10^{12}$ (exosomes). (B-F) Particle size distribution.

4.4.2 SDS-PAGE and Western blot analysis of the exosome isolation process

The first aim of the SDS-PAGE analysis was to compare the lane profiles of exosome isolation step samples to detect which proteins are enriched into the exosome fraction during the isolation. It was observed that some bands appeared stronger in the exosome fraction compared to the P, TP and FP samples, which indicates that these proteins are enriched by the precipitation protocol (Fig 6A).

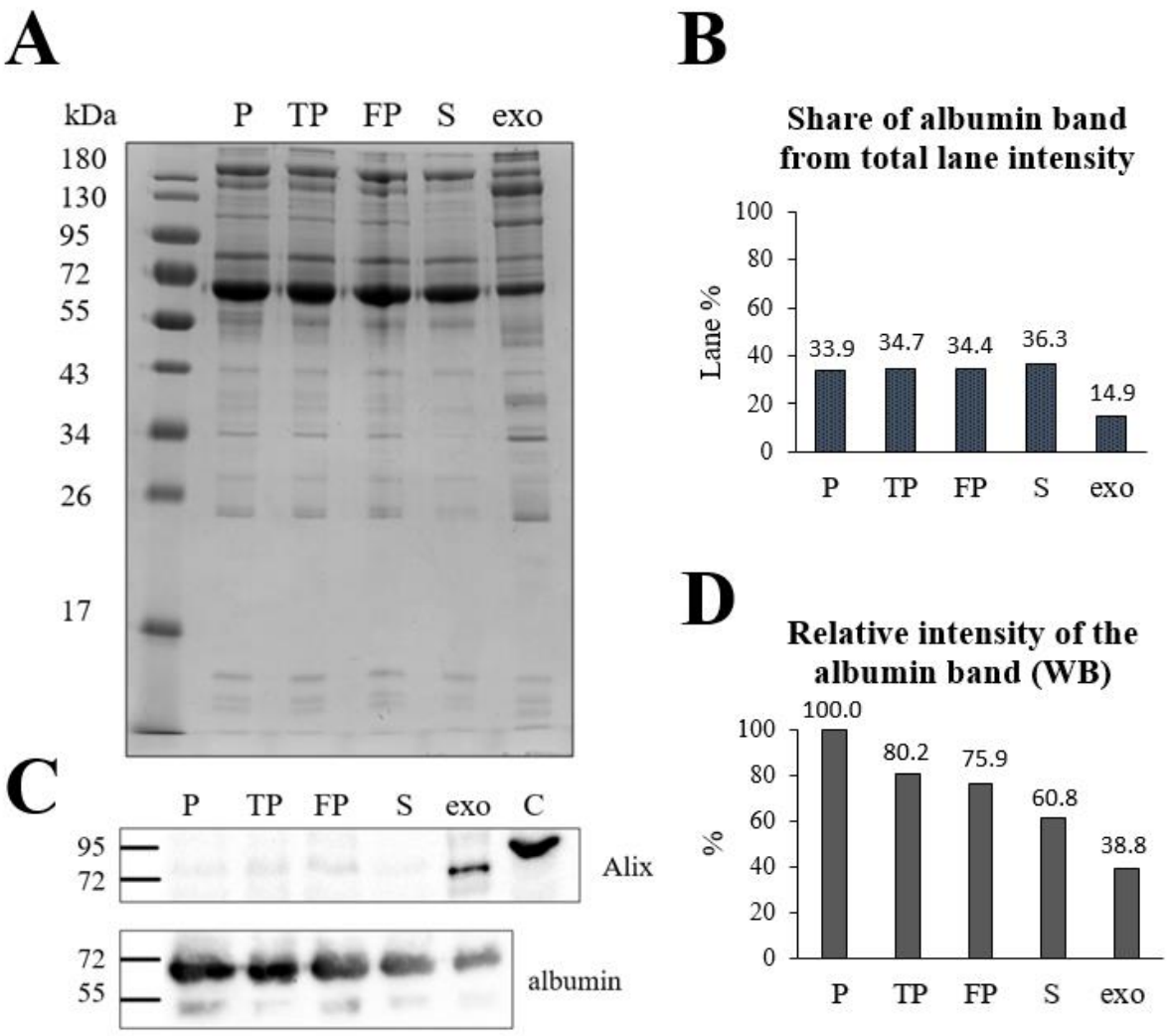

Figure 6. **Analysis of the exosome isolation process by SDS-PAGE and Western blot.** (A) SDS-PAGE profile of the samples taken at different steps of the exosome precipitation kit protocol. (B) Intensity of the albumin band measured from SDS-PAGE compared to the total lane intensity presents the share of albumin from all protein content of the sample. (C) Investigation of the presence of EV marker Alix and the plasma protein contamination marker albumin by Western blot. (D) Intensity of the albumin band in exosome isolation step samples compared to starting point (plasma). Intensities were measured from Western blot, and divided by the intensity of the albumin band in the plasma sample. P = plasma, TP = thrombin-treated plasma, FP = filtered plasma, S = supernatant, exo = exosome isolate, WB = Western blot.

The second aim was to analyse the efficacy of the method in removing contaminating proteins when isolating exosomes from plasma. Albumin was chosen as the marker for protein contamination since it is the most abundant protein in plasma. The intensity of the albumin band in Western blot was measured and compared to the total intensity of the lane (= total protein content). The relative intensity of the 66-kDa albumin band from total lane intensity were 33.9 %,

34.7 % and 34.4 % in plasma, TP and FP, respectively (Fig 6B). The share of the albumin band intensity was largest in the supernatant, 36.3 %, and smallest in exosomes, where the band intensity contributed to only 14.9 % of the total intensity. The results indicated that the isolation method succeeded to remove a large quantity of contaminating plasma proteins.

In addition to the SDS-PAGE analysis, a Western blot analysis was conducted to verify the presence of exosomes and albumin in the samples, and to investigate whether the Western blot analysis would yield similar results on the progression of the isolation process. The presence of exosomes in the isolate was analysed with the antibody against the EV marker protein Alix (Fig 6C). Rat cortex lysate was used as a positive control. Immunodetection of Alix yielded a strong band both in the exosome isolate and the positive control. In plasma, TP and FP, very faint Alix bands of ~72 kDa were detected. The Western blot analysis with anti-albumin antibody confirmed that the ~66 kDa-bands observed in SDS-PAGE were indeed albumin (Fig 6C). To compare the abundance of contaminating proteins in the beginning and in the end of the isolation process, the albumin band intensity in TP, FP and exosome isolate were compared to the intensity in the starting plasma sample (Fig 6D). The analysis displayed that the amount of albumin clearly decreased as the exosome isolation progressed. Compared to the starting plasma sample, the intensity of the albumin band in the exosome fraction decreased to 38.8 %. In other words, the precipitation method removed ~60 % of the contaminating plasma proteins.

5. DISCUSSION

5.1 Challenges of Western blot and setting up the method

The aims of the study were to set up a Western blot method for the analysis of rat plasma samples, and to test and optimize the antibodies used in exosome characterization by Western blotting. Western blot is regularly used in exosome studies to confirm the presence of vesicles in the sample (Lötvall et al., 2014). Therefore, establishment of the Western blot method was extremely important for the conduction of further studies. The optimization was performed by testing several antibody dilutions and different blocking and antibody incubation conditions with each antibody. As observed with the anti-Alix primary antibody, a change in one condition in the protocol could have a dramatic effect on the result (Fig 2), which is why it was important

to test the conditions systematically. The best-working protocols are presented in Table 3, but it is possible to alter the protocols to some extent and still obtain good results. The antibodies functioned in several different dilutions, but the optimization of the used antibody dilutions saves resources when the minimal volume of antibody is used.

Apart from Alix, the detection of other exosome marker proteins by Western blot turned out to be a challenging task. The antibodies against the commonly used exosome markers CD63 and flotillin-1 did not manage to detect their targets despite many tries and changes in the conditions of the protocol. On the contrary, the antibodies against albumin and calnexin, which were used to evaluate the purity of isolated exosomes, worked in several conditions and produced good quality blots (Fig 4). The results indicate that the Western blot method worked in general, which suggests that the detection problems of exosome marker proteins likely arise from the quality of the sample rather than other factors, for example the used reagents.

90 % of plasma is water and about 8 % proteins, of which albumin is the most predominant with the share of approximately half of the total protein content. Also IgGs are very abundant in plasma. Consequently, exosome marker proteins form only a tiny fraction of all proteins in plasma, which makes their detection by Western blot quite challenging. The abundance of plasma proteins, especially albumin, which can be seen in Western blot in Figure 3, may cover the presence of proteins that appear in plasma in remarkably smaller quantities. Co-isolated proteins seem to be a struggle especially with precipitation methods, and not only when working with plasma. In the study of Alvarez et al. (2012), the commercial ExoQuick (System Biosciences Inc.) precipitation method conducted with urine resulted in lower purity of exosome proteins compared to ultracentrifugation methods, which was observed as absence or weaker bands of Alix and another EV marker protein, Tsg101, in Western blot. In addition, the results from another isolation method-comparing study (Lobb et al., 2015) suggested that the co-isolation of contaminating proteins during the precipitation method results in poorer performance in Western blot. There are also contradictory findings: Caradec et al. (2014) found serum protein contamination problem to be more severe with ultracentrifugation than with the precipitation method.

One major issue encountered with CD63 and flotillin-1 was their molecular weight: they are expected to migrate in SDS-PAGE the distance corresponding to 30–60 kDa (CD63) and 47 kDa (flotillin-1). During the study, it was found out that a large quantity of plasma IgGs get co-precipitated along with exosomes. Western blots where primary antibody was omitted from the protocol revealed that nearly all the tested secondary antibodies cross-reacted with the rat immunoglobulins (Fig 3), causing a IgG heavy chain band at the 55-kDa mark (Anderson and Anderson, 1977). In some blots (Fig 3C and F), there was also a 25-kDa band, which corresponds to IgG light chain. Since the IgG heavy chain is sized 55 kDa, it can be confused with CD63, flotillin-1 and other proteins falling in the same size region, for the secondary antibodies bind not only to primary antibodies, but also to the IgGs derived from plasma. Because of the cross-reactivity issue, it was decided to choose Alix as a new exosome marker protein, since it is sized 97 kDa and could therefore be clearly distinguished from the bands caused by the plasma IgGs. Another possible solution to the cross-reactivity problem could be to use secondary antibodies that specifically recognize native form IgGs, such as TrueBlot antibodies from Rockland Immunochemicals Inc. SDS-PAGE sample preparation denatures the proteins, which means that only the IgGs added in immunoblotting are in their native form, so the native form-recognizing secondary antibody would bind to them but not to the denatured plasma-derived IgGs.

Many exosome marker proteins, including CD63 and flotillin-1, are membrane proteins, which complicates their detection by Western blotting. There is evidence that the standard Western blot protocol may require additional denaturing steps when working with membrane proteins (Kaur and Bachhawat, 2009), and in addition, membrane proteins have been noticed to have difficulties in transfer from the SDS-PAGE gel to the membrane (Abeyrathne and Lam, 2007). However, in above mentioned experiments the membrane was made of nitrocellulose, whereas in this thesis, PVDF membrane was used. Nevertheless, it is possible that some of the difficulties associated with membrane proteins may also apply when using PVDF membranes. In this thesis, the success of transfer was monitored by staining the SDS-PAGE after transfer to see that proteins had moved out of the gel, and no striking problems were observed. However, it may be necessary to also optimize the transfer part in the future to ensure the best possible outcome.

5.2 Reliability of the Western blot characterization of exosomes

Even though Western blot is a good technique, there are some pitfalls that should be kept in mind to avoid trouble and false interpretations (see review Gorr and Vogel, 2015). For example, artefactual chemical modifications or diversity of proteins, which means e.g. post-translational modifications, may result in extra bands or a band of different size than expected. Interestingly, in the Western blot (Fig 4A) the Alix band given by the exosome fraction was about 70 kDa, while cortex – which was used as a positive control – gave a band of about 95 kDa as expected. One plausible explanation is that the lower band came from Alix protein that lacked the C-terminal domain, as it matches the observed size (Pires, 2008). On the other hand, one should not exclude the possibility of nonspecific antibody binding, which is another common reason for extra bands (Gorr and Vogel, 2015). The blots conducted only with the secondary antibodies (Fig 3) did not show bands that would match the bands observed with the anti-Alix antibody, which indicated that the 70-kDa band did not come from the secondary antibody. Instead, it could be possible that the primary antibody had affinity also to some other protein than Alix, but the investigation of this option would have required further validation of the antibody (see review Uhlen et al., 2016), which was not possible under this study due to limited time and resources.

Western blot detection problems of exosome marker proteins are a common struggle in the exosome research field and usually only the successful blots find their way to publications. It is important to look at Western blot results with a critical eye, as the method has its shortcomings. As mentioned in earlier, the guidelines suggested by ISEV (Lötvall et al., 2014) recommended the investigation of minimum one protein from each of the four categories to claim the presence of extracellular vesicles. The Western blot method for the characterization of exosomes from plasma failed to completely fulfil the requirements, as the membrane-associated exosome marker CD63 could not be detected. Detection of CD63 and flotillin-1 would have increased the reliability of the results, for in the literature especially CD63 is often used as a marker for exosomes (Caby et al., 2005; Andreu and Yáñez-Mó, 2014; Lobb et al., 2015). However, also other studies have presented poor detection of exosome marker proteins when isolating exosomes from plasma by precipitation method (Lobb et al., 2015). Also, it should be noted that

the antibodies against CD63 and flotillin-1 did not produce a signal even in the positive control, despite a large sample volume and a long detection time, which suggests that the negative result was caused by unfunctional antibodies rather than by the absence of exosomes.

5.3 Success of exosome isolation from plasma

The chemical composition of biofluids is more complicated than that of cell culture medium, which sets many challenges for isolation of exosomes from plasma. As mentioned above, plasma is abundant with proteins such as albumin, which cause contamination when they co-isolate with extracellular vesicles. What is even more important, plasma contains lipoproteins, for example HDL and LDL, which function in cholesterol transfer (see review Feingold and Grunfeld, 2000).

The exosome precipitation methods are based on the capture of water molecules that in suspension form a hydrate envelope of particles (see review Li et al., 2017). Without the envelope exosomes become insoluble and precipitate, where after they can be separated from the rest of the solution by pelleting them out of the solution. Western blot for detection of the exosome marker protein Alix indicated that the precipitation succeeded in isolation of exosomes from plasma: there was a strong signal from the exosome fraction, whereas there was no signal from supernatant (Fig 6C).

There are some previous estimations of number of particles in plasma, such as $1–5x10^{12}$ or $3x10^{11}$ particles/ml, which were acquired by NTA (Gardiner et al., 2013; Mork et al., 2016). However, the number includes all other particles found in plasma that fall in the exosome size range, such as lipoproteins. NTA gave plasma particle concentration of $3.10x10^{12}$ particles/ml, which is quite similar to the reference values. NTA confirmed that particles were enriched to the exosome fraction, as the particle concentration of the exosome fraction was over 100-fold higher compared to the supernatant (Fig 5A). NTA also displayed change in particle size distribution during the isolation process: the shares of larger particles were clearly increased in the exosome fraction compared to the starting point, which indicates that the precipitation kit managed to enrich large particles and remove at least a part of the smaller ones (< 60 nm), which probably consist of proteins and lipoproteins (Yuana et al., 2014; Sódar et al., 2016).

The precipitation kit was not able to completely purify exosomes from contaminating plasma proteins, for SDS-PAGE and Western blot results from the samples taken at different steps of the isolation protocol showed that a lot of albumin was co-precipitated along with exosomes (Fig 6). On the other hand, the amount of albumin in the exosome fraction was noticeable smaller than before isolation protocol: the SDS-PAGE analysis showed that the share of albumin was only ~15 % in the exosome fraction, whereas in plasma it contributed to about 1/3 of all proteins loaded in the gel. Western blot showed similar effect: intensity of the albumin band decreased by 60 % during the isolation process (Fig 6D). The precipitation method isolated exosomes from plasma quite efficiently despite the complicated nature of the starting material, but as a large amount of contaminating proteins was still present in the isolate, the process should be called exosome-enrichment rather than purification.

In addition to large number of contaminating proteins, plasma possesses another challenge to isolation of exosomes – the presence of lipoproteins. Sódar et al. (2016) found out that LDL co-purifies with exosomes from plasma and cannot be separated from them with current techniques. Their results implicated that LDL can form aggregates that overlap with the size of exosomes, which may cause overestimation of exosome number in NTA. Sódar et al. also showed that the commercial LDL attached to exosomes *in vitro,* which suggest that the association may also occur in blood. Furthermore, also HDL has been shown to co-isolate to the same fraction with exosomes in density gradient ultracentrifugation (Yuana et al., 2014). What is important, in addition to causing error in NTA, attached to the surface of exosomes lipoproteins may hinder the detection of surface proteins by antibodies, which may cause difficulties for example in the analysis of exosome protein composition or in immunoprecipitation. Even though the above-mentioned experiments used the ultracentrifugation method, it is likely that lipoprotein co-isolation occurs also in the precipitation method, which means that possibility of lipoprotein contamination in the acquired exosomes cannot be excluded.

5.4 Methods to improve the exosome isolation process

Results from the efficiency evaluation of the precipitation method in exosome isolation indicated that co-isolation of contaminating plasma proteins was the major drawback of the method (Fig 6). Because of the abundance of proteins in plasma, precipitation appears to be a

method too crude when working with plasma samples. The purity of the exosome isolate could be improved by changing to a method that separates exosomes from plasma based on their size, for example size-exclusion chromatography (see review Li et al., 2017).

Boing et al. (2014) introduced a simple SEC method for isolation of exosomes from human platelet-free supernatant of platelet concentrates or human plasma, and the method seems to be able to resolve some of the problems associated with centrifugation methods, such as generation of protein aggregates and co-isolation of lipoproteins of same density as exosomes of interest. In the study of Gámez-Valero et al. (2016) the SEC column described by Boing et al. produced better quality exosomes compared to precipitations methods, which displayed notable plasma protein contamination. There are also commercial SEC columns for EV isolation, such as qEV from IZON. In the study of Lobb et al. (2015), the qEV column outperformed the other tested methods (precipitation and spin column) in isolating exosomes from plasma, as it produced exosomes with the highest particle concentration/protein concentration ratio. In conclusion, self-made or commercial SEC columns are a feasible alternative for the precipitation method in the future studies.

In the development of plasma exosome-based biomarkers, one challenge is the minute number of exosomes coming from the cells of interest. More than 50 % of the EVs in blood plasma are derived from erythrocytes and platelets (Arraud et al., 2014), which means that for example brain-derived exosomes form only a tiny fraction of all the vesicles in the isolate. This complicates the downstream analysis of neuron-derived exosomes and the search for epilepsy or TBI markers, as most of the material for e.g. RNA-sequencing comes from exosomes derived somewhere else than the brain. Therefore, it would be beneficial to move towards more specific exosome isolation methods, such as antibody-coated microbeads (Clayton et al., 2001; Hong et al., 2014). Specific capture of exosomes of interest would provide the possibility for more precise analysis of their contents.

5.5 Conclusion

In this work, it was succeeded to establish a Western blot method for characterization of exosomes isolated from rat plasma. The Western blot characterization of the exosome isolate displayed the presence of exosome marker protein Alix. The other tested exosome markers CD63 and flotillin-1 were not detected neither from the isolate nor the positive control, which suggests that the primary antibodies did not function properly. Based on the results from Western blot and NTA, the precipitation kit succeeded to collect exosomes from the plasma sample to the exosome fraction. However, the isolate still contained a notable amount of contaminating plasma proteins such as albumin and immunoglobulins, and the presence of LDL and HDL cannot be excluded. In conclusion, the precipitation method enriches exosomes rather than purifies them, and alternative isolation methods should be tested to improve the purity of the exosomes.

REFERENCES

Abeyrathne, P.D. and J.S. Lam. 2007. Conditions that allow for effective transfer of membrane proteins onto nitrocellulose membrane in Western blots. *Can J Microbiol.* 53:526-532.

Alvarez, M.L., M. Khosroheidari, R. Kanchi Ravi and J.K. DiStefano. 2012. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. *Kidney Int.* 82:1024-1032.

Alvarez-Erviti, L., Y. Seow, H. Yin, C. Betts, S. Lakhal and M.J.A. Wood. 2011. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol.* 29:341-345. doi: 10.1038/nbt.1807.

Anderson, L. and N.G. Anderson. 1977. High resolution two-dimensional electrophoresis of human plasma proteins. *Proc Natl Acad Sci U S A.* 5421-5425.

Andreu, Z. and M. Yáñez-Mó. 2014. Tetraspanins in extracellular vesicle formation and function. *Front Immunol.* 5:442. doi: 10.3389/fimmu.2014.00442.

Antony, P.M., N.J. Diederich, R. Kruger and R. Balling. 2013. The hallmarks of Parkinson's disease. *Febs J.* 280:5981-5993.

Arraud, N., R. Linares, S. Tan, C. Gounou, J.M. Pasquet, S. Mornet and A.R. Brisson. 2014. Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. *J Thromb Haemost.* 12:614-627.

Baietti, M.F., Z. Zhang, E. Mortier, A. Melchior, G. Degeest, A. Geeraerts, Y. Ivarsson, F. Depoortere, C. Coomans, E. Vermeiren, P. Zimmermann and G. David. 2012. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat Cell Biol.* 14:677. doi: 10.1038/ncb2502.

Bhatnagar, S., K. Shinagawa, F.J. Castellino and J.S. Schorey. 2007. Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. *Blood.* 110:3234-3244.

Bloom, G.S. 2014. Amyloid-beta and tau: the trigger and bullet in Alzheimer disease pathogenesis. *JAMA Neurol.* 71:505-508.

Boing, A.N., E. van der Pol, A.E. Grootemaat, F.A. Coumans, A. Sturk and R. Nieuwland. 2014. Single-step isolation of extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles.* 3:10.3402/jev.v3.23430. eCollection 2014.

Bramlett, H.M. and W.D. Dietrich. 2015. Long-Term Consequences of Traumatic Brain Injury: Current Status of Potential Mechanisms of Injury and Neurological Outcomes. *J Neurotrauma.* 32:1834-1848.

Caby, M.P., D. Lankar, C. Vincendeau-Scherrer, G. Raposo and C. Bonnerot. 2005. Exosomal-like vesicles are present in human blood plasma. *Int Immunol.* 17:879-887.

Caradec, J., G. Kharmate, E. Hosseini-Beheshti, H. Adomat, M. Gleave and E. Guns. 2014. Reproducibility and efficiency of serum-derived exosome extraction methods. *Clin Biochem.* 47:1286-1292.

Charrin, S., S. Jouannet, C. Boucheix and E. Rubinstein. 2014. Tetraspanins at a glance. *J Cell Sci.* 127:3641-3648. doi: 10.1242/jcs.154906.

Clayton, A., J. Court, H. Navabi, M. Adams, M.D. Mason, J.A. Hobot, G.R. Newman and B. Jasani. 2001. Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. *J Immunol Methods.* 247:163-174. doi: 10.1016/S0022-1759(00)00321-5.

Danzer, K.M., L.R. Kranich, W.P. Ruf, O. Cagsal-Getkin, A.R. Winslow, L. Zhu, C.R. Vanderburg and P.J. McLean. 2012. Exosomal cell-to-cell transmission of alpha synuclein oligomers. *Mol Neurodegener.* 7:42.

de Gassart, A., C. Geminard, B. Fevrier, G. Raposo and M. Vidal. 2003. Lipid raft-associated protein sorting in exosomes. *Blood.* 102:4336-4344. doi: 10.1182/blood-2003-03-0871.

de Jong, O.G., M.C. Verhaar, Y. Chen, P. Vader, H. Gremmels, G. Posthuma, R.M. Schiffelers, M. Gucek and B.W. van Balkom. 2012. Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. *J Extracell Vesicles.* 1:10.3402/jev.v1i0.18396.

EL Andaloussi, S., I. Mager, X.O. Breakefield and M.J. Wood. 2013. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov.* 12:347-357.

Emmanouilidou, E., K. Melachroinou, T. Roumeliotis, S.D. Garbis, M. Ntzouni, L.H. Margaritis, L. Stefanis and K. Vekrellis. 2010. Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J Neurosci.* 30:6838-6851. doi: 10.1523/JNEUROSCI.5699-09.2010.

Escola, J., M.J. Kleijmeer, W. Stoorvogel, J.M. Griffith, O. Yoshie and H.J. Geuze. 1998. Selective Enrichment of Tetraspan Proteins on the Internal Vesicles of Multivesicular Endosomes and on Exosomes Secreted by Human B-lymphocytes. *J Biol Chem.* 273:20121-20127. doi: 10.1074/jbc.273.32.20121.

Feingold, K.R., and C. Grunfeld. 2000. Introduction to Lipids and Lipoproteins. Endotext. L.J. De Groot, G. Chrousos, K. Dungan, K.R. Feingold, A. Grossman, J.M. Hershman, C. Koch, M. Korbonits, R. McLachlan, M. New, J. Purnell, R. Rebar, F. Singer and A. Vinik, edit. MDText.com, Inc, South Dartmouth (MA).

Fevrier, B., D. Vilette, F. Archer, D. Loew, W. Faigle, M. Vidal, H. Laude and G. Raposo. 2004. Cells Release Prions in Association with Exosomes. *Pro Natl Acad Sci U S A.* 101:9683-9688. doi: 10.1073/pnas.0308413101.

Gámez-Valero, A., M. Monguió-Tortajada, L. Carreras-Planella, M.l. Franquesa, K. Beyer and F.E. Borràs. 2016. Size-Exclusion Chromatography-based isolation minimally alters Extracellular Vesicles' characteristics compared to precipitating agents. *Sci Rep.* 6:33641. doi: 10.1038/srep33641.

Gardiner, C., Y.J. Ferreira, R.A. Dragovic, C.W. Redman and I.L. Sargent. 2013. Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis. *J Extracell Vesicles.* 2:10.3402/jev.v2i0.19671.

Gardiner, C., D.D. Vizio, S. Sahoo, C. Théry, K.W. Witwer, M. Wauben and A.F. Hill. 2016. Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey. *J Extracell Vesicles.* 5:1-6. doi: 10.3402/jev.v5.32945.

Gorr, T.A. and J. Vogel. 2015. Western blotting revisited: critical perusal of underappreciated technical issues. *Proteomics Clin Appl.* 9:396-405.

Gruenberg, J. and F.R. Maxfield. 1995. Membrane transport in the endocytic pathway. *Curr Opin Cell Biol.* 7:552-563. doi: 10.1016/0955-0674(95)80013-1.

Harrison, E.B., C.G. Hochfelder, B.G. Lamberty, B.M. Meays, B.M. Morsey, M.L. Kelso, H.S. Fox and S.V. Yelamanchili. 2016. Traumatic brain injury increases levels of miR-21 in extracellular vesicles: implications for neuroinflammation. *FEBS Open Bio.* 6:835-846. doi: 10.1002/2211-5463.12092.

Hong, C.S., L. Muller, M. Boyiadzis and T.L. Whiteside. 2014. Isolation and characterization of CD34+ blast-derived exosomes in acute myeloid leukemia. *PLoS One.* 9:e103310.

Höög, J.L. and J. Lötvall. 2015. Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. *J Extracell Vesicles.* 4:28680. doi: 10.3402/jev.v4.28680.

Iavello, A., V.S.L. Frech, C. Gai, M.C. Deregibus, P.J. Quesenberry and G. Camussi. 2016. Role of Alix in miRNA packaging during extracellular vesicle biogenesis. *Int J Mol Med.* 37:958-966. doi: 10.3892/ijmm.2016.2488.

Kaur, J. and A.K. Bachhawat. 2009. A modified Western blot protocol for enhanced sensitivity in the detection of a membrane protein. *Anal Biochem.* 384:348-349.

Keerthikumar, S., L. Gangoda, M. Liem, P. Fonseka, I. Atukorala, C. Ozcitti, A. Mechler, C.G. Adda, C.S. Ang and S. Mathivanan. 2015. Proteogenomic analysis reveals exosomes are more oncogenic than ectosomes. *Oncotarget.* 6:15375-15396.

Kowal, J., G. Arras, M. Colombo, M. Jouve, J.P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach and C. Théry. 2016. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Pro Natl Acad Sci.* 113:E977. doi: 10.1073/pnas.1521230113.

Lazar, I., E. Clement, M. Ducoux-Petit, L. Denat, V. Soldan, S. Dauvillier, S. Balor, O. Burlet-Schiltz, L. Larue, C. Muller and L. Nieto. 2015. Proteome characterization of melanoma exosomes reveals a specific signature for metastatic cell lines. *Pigment Cell Melanoma Res.* 28:464-475.

Leung, N.H.L., C. Xu, D.K.M. Ip and B.J. Cowling. 2015. Exosomes: Novel Biomarkers for Clinical Diagnosis. *Epidemiology.* 26:862-872.

Li, P., M. Kaslan, S.H. Lee, J. Yao and Z. Gao. 2017. Progress in Exosome Isolation Techniques. *Theranostics.* 7:789-804.

Lobb, R.J., M. Becker, S.W. Wen, C.S. Wong, A.P. Wiegmans, A. Leimgruber and A. Moller. 2015. Optimized exosome isolation protocol for cell culture supernatant and human plasma. *J Extracell Vesicles.* 4:27031.

Lötvall, J., A.F. Hill, F. Hochberg, E.I. Buzás, D. Di Vizio, C. Gardiner, Y.S. Gho, I.V. Kurochkin, S. Mathivanan, P. Quesenberry, S. Sahoo, H. Tahara, M.H. Wauben, K.W. Witwer and C. Théry. 2014. Minimal experimental requirements for definition of extracellular vesicles and their functions : a position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles.* 3:26913. doi: 10.3402/jev.v3.26913.

Matsuo, H., J. Chevallier, N. Mayran, I. Le Blanc, C. Ferguson, J. Fauré, N.S. Blanc, S. Matile, J. Dubochet, R. Sadoul, R.G. Parton, F. Vilbois and J. Gruenberg. 2004. Role of LBPA and Alix in Multivesicular Liposome Formation and Endosome Organization. *Science.* 303:531-534. doi: 10.1126/science.1092425.

Menon, D.K., K. Schwab, D.W. Wright and A.I. Maas. 2010. Position statement: definition of traumatic brain injury. *Arch Phys Med Rehabil.* 91:1637-1640.

Missotten, M., A. Nichols, K. Rieger and R. Sadoul. 1999. Alix, a novel mouse protein undergoing calcium-dependent interaction with the apoptosis-linked-gene 2 (ALG-2) protein. *Cell Death Differ.* 6:124-129. doi: 10.1038/sj.cdd.4400456.

Mork, M., S. Pedersen, J. Botha, S.M. Lund and S.R. Kristensen. 2016. Preanalytical, analytical, and biological variation of blood plasma submicron particle levels measured with nanoparticle tracking analysis and tunable resistive pulse sensing. *Scand J Clin Lab Invest.* 76:349-360.

Otto, G.P. and B.J. Nichols. 2011. The roles of flotillin microdomains--endocytosis and beyond. *J Cell Sci.* 124:3933-3940. doi: 10.1242/jcs.092015.

Patz, S., C. Trattnig, G. Grunbacher, B. Ebner, C. Gully, A. Novak, B. Rinner, G. Leitinger, M. Absenger, O.A. Tomescu, G.G. Thallinger, U. Fasching, S. Wissa, J. Archelos-Garcia and U. Schafer. 2013. More than cell dust: microparticles isolated from cerebrospinal fluid of brain injured patients are messengers carrying mRNAs, miRNAs, and proteins. *J Neurotrauma.* 30:1232-1242.

Pires, R. 2008. Structural and functional studies of ALIX. *Biomolecules.* Université Joseph-Fourier - Grenoble I.

Pitkänen, A. and R. Immonen. 2014. Epilepsy related to traumatic brain injury. *Neurotherapeutics.* 11:286-296.

Quek, C. and A.F. Hill. 2017. The role of extracellular vesicles in neurodegenerative diseases. *Biochem Biophys Res Commun.* 483:1178-1186.

Rajendran, L., M. Honsho, T.R. Zahn, P. Keller, K.D. Geiger, P. Verkade and K. Simons. 2006. Alzheimer's Disease β-Amyloid Peptides Are Released in Association with Exosomes. *Pro Natl Acad Sci U S A.* 103:11172-11177. doi: 10.1073/pnas.0603838103.

Raposo, G., H.W. Nijman, W. Stoorvoge, R. Leijendekker, C.V. Harding, C.J.M. Melief and H.J. Geuze. 1996. B Lymphocytes Secrete Antigen-presenting Vesicles. *J Exp Med.* 183:1161-1172.

Sódar, B.W., Á Kittel, K. Pálóczi, K.V. Vukman, X. Osteikoetxea, K. Szabó-Taylor, A. Németh, B. Sperlágh, T. Baranyai, Z. Giricz, Z. Wiener, L. Turiák, L. Drahos, É Pállinger, K. Vékey, P. Ferdinandy, A. Falus and E.I. Buzás. 2016. Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection. *Sci Rep.* 6:24316.

Soo, C.Y., Y. Song, Y. Zheng, E.C. Campbell, A.C. Riches, F. Gunn-Moore and S.J. Powis. 2012. Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. *Immunology.* 136:192-197.

Srikanthan, S., W. Li, R.L. Silverstein and T.M. McIntyre. 2014. Exosome poly-ubiquitin inhibits platelet activation, downregulates CD36 and inhibits pro-atherothombotic cellular functions. *J Thromb Haemost.* 12:1906-1917.

Subra, C., D. Grand, K. Laulagnier, A. Stella, G. Lambeau, M. Paillasse, P. De Medina, B. Monsarrat, B. Perret, S. Silvente-Poirot, M. Poirot and M. Record. 2010. Exosomes account for vesicle-mediated transcellular transport of activatable phospholipases and prostaglandins. *J Lipid Res.* 51:2105-2120.

Szatanek, R., J. Baran, M.J. Siedlar and M. Baj-Kryzworzeka. 2015. Isolation of extracellular vesicles: Determining the correct approach. *Int J Mol Med.* 36:11-17. doi: 10.3892/ijmm.2015.2194.

Thery, C., L. Zitvogel and S. Amigorena. 2002. Exosomes: composition, biogenesis and function. *Nat Rev Immunol.* 2:569-579.

Théry, C., A. Regnault, J. Garin, J. Wolfers, L. Zitvogel, P. Ricciardi-Castagnoli, G. Raposo and S. Amigorena. 1999. Molecular Characterization of Dendritic Cell-derived Exosomes: Selective Accumulation of the Heat Shock Protein hsc73. *J Cell Biol.* 147:599-610.

Turiák, L., P. Misják, T.G. Szabó, B. Aradi, K. Pálóczi, O. Ozohanics, L. Drahos, Á Kittel, A. Falus, E.I. Buzás and K. Vékey. 2011. Proteomic characterization of thymocyte-derived microvesicles and apoptotic bodies in BALB/c mice. *J Proteomics.* 74:2025-2033. doi: 10.1016/j.jprot.2011.05.023.

Uhlen, M., A. Bandrowski, S. Carr, A. Edwards, J. Ellenberg, E. Lundberg, D.L. Rimm, H. Rodriguez, T. Hiltke, M. Snyder and T. Yamamoto. 2016. A proposal for validation of antibodies. *Nat Methods.* 13:823-827.

Valadi, H., M. Sjöstrand, A. Bossios, K. Ekström, J.J. Lee and J.O. Lötvall. 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol.* 9:654-659. doi: 10.1038/ncb1596.

van der Pol, E., A.N. Boing, E.L. Gool and R. Nieuwland. 2016. Recent developments in the nomenclature, presence, isolation, detection and clinical impact of extracellular vesicles. *J Thromb Haemost.* 14:48-56.

Willms, E., H.J. Johansson, I. Mager, Y. Lee, K.E. Blomberg, M. Sadik, A. Alaarg, C.I. Smith, J. Lehtio, S. El Andaloussi, M.J. Wood and P. Vader. 2016. Cells release subpopulations of exosomes with distinct molecular and biological properties. *Sci Rep.* 6:22519.

Wu, Y., W. Deng and D.J. Klinke. 2015. Exosomes: improved methods to characterize their morphology, RNA content, and surface protein biomarkers. *Analyst.* 140:6631-6642.

Yuana, Y., J. Levels, A. Grootemaat, A. Sturk and R. Nieuwland. 2014. Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation. *J Extracell Vesicles.* 3:10.3402/jev.v3.23262.

Yuyama, K., H. Sun, S. Mitsutake and Y. Igarashi. 2012. Sphingolipid-modulated exosome secretion promotes clearance of amyloid-beta by microglia. *J Biol Chem.* 287:10977-10989.

Zhang, Y., M. Chopp, Y. Meng, M. Katakowski, H. Xin, A. Mahmood and Y. Xiong. 2015. Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. *J Neurosurg.* 122:856-867. doi: 10.3171/2014.11.JNS14770.

Zitvogel, L., A. Regnault, A. Lozier, J. Wolfers, C. Flament, D. Tenza, P. Ricciardi-Castagnoli, G. Raposo and S. Amigorena. 1998. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med.* 4:594-600.

APPENDICES

Appendix 1

10xTEN:

0.5 M Tris Base
0.05 M EDTA
1.5 M NaCl
pH 7.4

The stock solution was diluted 1:10 for preparation of 1xTEN 1% Tween-20 and 1xTEN 0.1% Tween-20 buffers used in immunodetection.