Isolation Strategies and Proteomic Characterization of Extracellular Vesicles

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To Gizmo,

who barely would have afforded this thesis even a glance (or sniff, as dogs tend to rely more on their olfactory sense) before judging it as an object of clearly inferior quality. He would then, content with the day's work, make his way to the couch, fuss about the best way to lie down on as many pillows as possible, and proceed to fall asleep in a weird position that only grows in absurdity as he drifts deeper into whatever dreams of world domination that he usually has.

"**EFFECT**, *n*. The second of two phenomena which always occur together in the same order. The first, called a Cause, is said to generate the other – which is no more sensible than it would be for one who has never seen a dog except in pursuit of a rabbit to declare the rabbit the cause of the dog."

-Ambrose Bierce, The Devil's Dictionary

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ABSTRACT

"Extracellular vesicles" is the collective term used to describe vesicular entities that are released from cells into the extracellular environment. These vesicles are composed of a delineating lipid membrane and its cargo which can comprise of bioactive molecules such as lipids, RNA, DNA and proteins which can be shuttled between cells and thus function as a means of cell-to-cell communication.

The aims of this thesis were to address how discrepancies in isolation procedure effects the isolate, to distinguish vesicular proteins from co-isolated proteins, to determine the proteome of tissue resident EVs in tumors of colorectal cancer patients and finally to develop a method for high quality vesicle isolates from blood plasma.

We demonstrate that different rotor types will influence not only the yield of isolated vesicles, but also the purity. Furthermore, prolonged ultracentrifugation can up to a point produce higher yields at no apparent cost to purity. Even after purification of vesicles with a density gradient, however, there are proteins in the isolate whose vesicular nature can be questioned as they are susceptible to membrane-impermeable proteolytic digestion. Interestingly, proteolysis of perceived luminal motifs of transmembrane proteins suggests the existence of proteins with unconventional topological orientation within the membrane. We further illustrate that vesicles isolated directly from colorectal tumor tissue greatly differ from vesicles from corresponding healthy tissue in their proteomic makeup. Lastly, we demonstrate the possibility of attaining a highly purified vesicle isolate from blood plasma that is of high enough quality for relevant proteomic evaluation.

In conclusion, we demonstrate how both yield and purity can be optimized in cultured samples as well as in complex biological samples.

Keywords: Colorectal cancer, extracellular vesicles, exosomes, mass spectrometry, plasma, proteomics, ultracentrifugation

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SAMMANFATTNING PÅ SVENSKA

En vesikel är en entitet som avgränsas av ett lipidärt membran med ett vätskefyllt centra. Extracellulära vesiklar är det samlingsnamn som används för att beskriva små vesiklar som frisätts av celler ut i den intercellulära miljön. Dessa vesiklar är submikroskopiska i storleksordning och varierar mellan bara ett tiotal nanometer till ett fåtal mikrometer. Förutom lipidmembranet som utgör den vesikulära kroppen så vet man att vesiklar dessutom kan bestå av ett flertal arter av funktionella molekyler. Dessa inkluderar DNA, RNA och proteiner. Även om termen ”extracellulära vesiklar” omfattar alla de vesiklar som celler frisläpper brukar man oftast tala om endast tre typer av vesiklar i biologin idag. Dessa tre skiljer sig i biogenes men också till viss del i storlek och i komposition. Nästan alla celler som har studerats har visat sig kunna frisätta vesiklar och dessa har man sett kan tas upp av andra celler. De ovannämnda vesikulära beståndsdelarna har dessutom funktionella egenskaper vilket gör att vesiklar kan introducera förändringar i mottagarcellen. Således anser man att vesiklarna utgör en fundamental del av cellers många sätt att kommunicera med varandra. Det faktum att vesiklarna bär på många olika molekyler samt att de på distans kan kommunicera funktionella budskap har lagt grunden för det enorma intresset för extracellulära vesiklar som har skapats de senaste åren, vilket i sin tur har lett till ett växande vetenskapligt fält. Vesiklar har visat sig vara viktiga komponenter både under normalförhållanden men också vid sjukdom. De har visat sig vara aktiva agenter i allt från neurologiska sjukdomar som Alzheimers sjukdom till alla möjliga former av cancer och även som aktiva spelare inom kroppens immunförsvar.

Denna avhandling omfattar ett relativt brett perspektiv vad gäller extracellulära vesiklar. Mycket av arbetet riktar sig mot isolering och karakterisering av vesiklar. Detta är två områden som är tydligt kopplade till varandra då kvaliteten på isoleringen av vesiklar har en direkt effekt på analyser som följer. Mer specifikt utvärderas den isoleringsmetod som kallas differentiell ultracentrifugering i det första delarbetet. Denna metod är vida använd i fältet för att utvinna vesiklar ur biologiska prover. Vår data beskriver att skillnader i instrumentella dimensioner vid ultracentrifugering påverkar såväl avkastning som renhet i det slutliga isolatet. Dessutom påpekar resultaten att man kan uppnå större avkastning utan att isolatets kvalitet påverkas negativt vid längre centrifugeringar, vilket indikerar att konventionella centrifugeringstider som tillämpas vid isolering är otillräckliga för att utvinna majoriteten av vesiklarna ur ett prov. Vidare undersöker vi i det andra delarbetet isolatets proteinkomponenter med masspektrometri och frågar oss huruvida de

isolerade proteinerna är vesikelkomponenter eller om deras närvaro i isolatet är en följd av själva isoleringsprocessen. Till detta ändamål tillämpar vi ett membranimpermeabelt enzym för att bryta ner de proteiner som inte är skyddade av vesiklarnas membran. Våra fynd visar att en andel av isolatets proteiner kan brytas ner på detta sätt vilket innebär att deras närvaro i isolatet kan ifrågasättas. Försöken påvisade dessutom ett fynd som indikerar att vissa membranbundna proteiner i vesiklarna har en okonventionell orientering som skiljer sig från vad som tidigare har rapporterats. Sammantaget säger detta oss att isolaten inte endast innehåller vesikulära komponenter utan möjligen också oönskade proteiner, samt att proteiner i vesikelns membran kan anta orienteringar som tidigare inte rapporterats.

I det tredje delarbetet utvecklar vi en metod för att utvinna vesiklar ur blod, vilket utgör en i särklass svårarbetad kroppsvätska både ur ett vesikelperspektiv men även vad gäller masspektrometriska analyser. Vi etablerar en metod för att separera vesiklar från likartade partiklar i blodet. Detta gör vi genom att i följd tillämpa två isoleringsmetoder. Då var och en av metoderna på egen hand inte uppnår en tillfredsställande separation av vesiklar från övriga blodburna partiklar och proteiner kan de tillsammans producera ett isolat som är tillräckligt rent för masspektrometrisk analys.

Slutligen undersöker vi kolorektalcancer ur ett vesikulärt perspektiv i det fjärde delarbetet. Vi isolerar vesiklar direkt ur tumörvävnad och ur frisk vävnad och undersöker vesiklarnas proteininnehåll. Vesiklarna tagna ur tumörvävnad sågs bära en betydligt annorlunda proteinlast jämfört med vesiklar tagna ur frisk vävnad. Tumörvesiklar visade sig var anrikade på komponenter från det cellulära proteingenerativa maskineriet men var utarmade på komponenter för energiproduktion. Dessutom sågs en starkare närvaro av ett antal enzymer som kan kopplas till utveckling av cancer i tumörvesiklarna, men som också kan komma att fungera som mätbara markörer för sjukdomen.

Sammantaget visar denna avhandling vikten av just isoleringsprocessen och dess inverkan på isolatets komposition, vilket direkt påverkar kvalitén av forskning. Vidare visar den att tumörvesiklar skiljer sig från normala vesiklar och med en robust metod att isolera vesiklar från blod kan detta bana ny väg för upptäckten av sjukdom genom blodburna vesiklar.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles.**
Cvjetkovic A, Lötvall J, Lässer C.
J Extracell Vesicles. 2014 Mar 25;3.
doi:10.3402/jev.v3.23111.

II. **Detailed Analysis of Protein Topology of Extracellular Vesicles-Evidence of Unconventional Membrane Protein Orientation.**
Cvjetkovic A, Jang SC, Konečná B, Höög JL, Sihlbom C, Lässer C, Lötvall
Sci Rep. 2016 Nov 8;6:36338. doi:10.1038/srep36338.

III. **Detailed analysis of the plasma extracellular vesicle proteome after separation from lipoproteins.**
Karimi N, Cvjetkovic A, Jang SC, Crescitelli R, Hosseinpour Feizi MA, Nieuwland R, Lötvall J, Lässer C.
Cell Mol Life Sci. 2018 Aug;75(15):2873-2886.
doi:10.1007/s00018-018-2773-4

IV. **Proteomic profiling of tumor tissue resident EVs of colon cancer patients.**
Cvjetkovic A, Lässer C, Crescitelli R, Thorsell A, Taflin H, Lötvall J
In manuscript.

LIST OF PAPERS NOT INCLUDED IN THE THESIS

Exosomes purified from a single cell type have diverse morphology.
Zabeo D, Cvjetkovic A, Lässer C, Schorb M, Lötvall J, Höög JL
J Extracell Vesicles. 2017 Jun 20;6(1):1329476.
Doi:10.1080/20013078.2017

Extracellular vesicles in motion.
Cvjetkovic A, Crescitelli R, Lässer C, Zabeo D, Widlund P, Nyström T, Höög JL, Lötvall J
Matters (2017) Doi:10.19185/matters.201704000003

CONTENT

ABBREVIATIONS

AB	Apoptotic bodies
CD	Cluster of differentiation
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ESCRT	Endosomal sorting complexes required for transport
EV	Extracellular vesicle
EXO	Exosome
FA	Fixed angle (rotor)
HDL	High-density lipoprotein
ILV	Intraluminal vesicle
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low-density lipoprotein
MS	Mass spectrometry
MV	Microvesicle
NTA	Nanoparticle tracking analysis
PEG	Polyethylene glycol
PK	Proteinase K
RCF	Relative centrifugal field

RNA	Ribonucleic acid
RPS	Resistive pulse sensing
SEC	Size exclusion chromatography
SW	Swinging bucket (rotor)

1 INTRODUCTION

1.1 Basics of EVs

Vesicles were discovered as early as in the mid-20th century when it was shown that small platelet derived particles carrying coagulation capacity could be separated out from plasma through centrifugation (1). Years later, two publications almost simultaneously brought forth the notion of vesicular shedding where the abolition of the transferrin receptor, which was viewed as a part of reticulocyte maturation, was suggested to be facilitated by vesicular expulsion (2, 3). Since then, research on vesicles has gained significant momentum (4). The umbrella term extracellular vesicles (EVs) includes several vesicular subtypes that are often otherwise distinguished according to either their biogenesis and/or their cell of origin. Among the ways to distinguish vesicles, it is by far the former distinction that is most frequently used. Therefore, the discrepancy between apoptotic bodies (ABs), microvesicles (MVs) and exosomes (EXOs) is the one most commonly made. What EVs have in common though is that they are all composed of a lipid bilayer and that they are secreted into the extracellular milieu. To date, almost all cells that have been studied for their ability to secrete vesicles have been found to do so, which speaks of a very fundamental biological role for EVs. Indeed, their biological role seems to become ever more multifaceted as research on these vesicles progresses. As a consequence, they are now thought of as a means of general cellular communication and as a long-distance transmitters of cellular function.

1.1.1 Biogenesis of EVs

There are three basic EV subtypes in terms of biogenesis that are commonly (although not exclusively) taken into account. These are the ABs, MVs and EXOs.

1.1.1.1 Apoptotic Bodies

The ABs, first known as “Councilman”-like bodies and later renamed as “apoptotic bodies” are, as their name implies, formed during the events of cellular apoptosis (5-7). As the cell goes through the orchestrated processes of apoptotic cell death, it starts to form blebs (7-9), and this is sometimes followed

by the formation of protrusions (10, 11). Finally, the blebs join the ranks of EV subtypes by dissociating from what was the main cellular body to form separate units. The mechanism of dissociation is still unclear, although shear forces have been proposed to be a factor (7). The resulting ABs are generally in the size range of 1-5 μm and make up the largest of the subtypes discussed here.

1.1.1.2 Microvesicles

Second in size order are the MVs, which are typically vesicles with a size range of 200 nm to 1 μm. Their biogenesis occurs through outward budding of the cell membrane (12, 13), and this process is arguably the least understood when compared to the generation of ABs and EXOs. The translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, as well as increased calcium influx has been observed to correlate with that of MV budding in platelets (14). Additionally, the arrestin domain-containing protein 1 was shown to be able to recruit TSG101, a component of the endosomal sorting complexes required for transport (ESCRT) machinery, to the plasma membrane, suggesting its involvement in MV formation and budding (15). Finally, the protein ADP-ribosylation factor 6 (ARF6) appears to be a central component when it comes to both loading and shedding of MVs (16). It acts by facilitating the activation of ERK by PDL at the plasma membrane, leading to the activation of the myosin light chain kinase (MLCK) and the subsequent phosphorylation of myosin light chain (MLC) and the contraction of the actomyosin ring, ending in scission and release of the MV (16).

1.1.1.3 Exosomes

The biogenesis of EXOs is closely tied to the endosomal machinery. Endocytosis is the action by which the cell internalizes substances by buddying inwards, engulfing extracellularly located components and plasma membrane (17). This internalized compartment goes on to become the early endosome. Components in the early endosome can either be recycled to the plasma membrane or remain with the early endosome through its maturation into the late endosome. As the endosome matures, it starts budding inward, forming small luminal compartments that are called intraluminal vesicles (ILVs), and as they accumulate the endosomal compartment is rebranded as a multivesicular body (MVB). The fate of the MVB can either be that of

degradation, upon which it will eventually fuse with the lysosome for recycling biomass, or it can fuse with the plasma membrane. It is through the fusion with the plasma membrane that its luminal contents, the ILVs, are released into the extracellular space. These ILVs are now termed as exosomes (13). The molecular mechanisms responsible for EXO biogenesis and release are perhaps the most readily studied in relation to the biogenesis of the aforementioned vesicular subtypes. The ESCRT machinery is a major players in the generation of EXOs, and their loading. It consists of several protein complexes, ranging from ESCRT-0 to ESCRT-III, that accumulate at the endosome and facilitate the formation of ILVs. ESCRT-0 comprises of two proteins, Hrs and STAM1/2. The recruitment of ESCRT-0 to the endosome is through Hrs and its binding to phosphatidylinositol3-phosphate (PtdIns3P) which is enriched on endosomes (18). The two components of ESCRT-0 also bind ubiquitin and have been proposed to facilitate the loading of mono-ubiquitinated proteins (19). Importantly, the ESCRT-0 complex recruits the ESCRT-I complex to the endosome, which in itself doesn't possess a strong affinity for the membrane (19). The ESCRT-I complex consists of TSG101, MVB12, VPS28 and VPS37. It is through the interaction of Hrs of ESCRT-0 with TSG101 of ESCRT-I that the ESCRT-I complex is recruited (20, 21). ESCRT-I in turn can itself interact with ESCRT-II through the interaction of VSP28 with that of EAP45 on ESCRT-II (22, 23), which consists of EAP45, EAP30 and EAP20. ESCRT-II can also bind to PtdIns3P (similar to ESCRT-0) via its subunit EAP45 (23). Finally, ESCRT-III consists of CHMP2, CHMP3, CHMP4 and CHMP6. However, unlike the other ESCRT complexes, these subunits do not form a stable complex that is then recruited to the membrane but are instead recruited one by one (19). ESCRT-III appears to harbor the mechanisms responsible for the formation of the bud itself. The sequence of events are as follows. EAP20 of ESCRT-II binds to the CHMP6 monomer of ESCRT-III thus activating the subunit, enabling its binding to the membrane, and initiating the recruitment of the complex to the membrane (24). CHMP6 in turn initiates the recruitment of CHMP4, which then undergoes oligomerization at the membrane to form a filament (25, 26). It is this filament oligomerization that is thought to actually give form to the bud itself. The termination of filament elongation is facilitated by the binding of CHMP3, which caps the filament. CHMP3 in turn binds CHMP2, which recruits the accessory protein VPS4 that can finally disassemble the filament. This sequence of events describes the assembly and disassembly of the ESCRT machinery leading up to bud formation. What is still unclear is how the machinery can facilitate membrane fission and the subsequent release of an ILV into the lumen. One intriguing model proposes that selective removal of SNF7 monomers from the filament by VPS4 might constrict the neck of the bud to such an extent that a fission is forced to occur (27). Numerous ubiquitin binding sites are present among the subunits of the

ESCRT machinery that facilitate the loading of ubiquitinated proteins into the ILVs (19). ESCRT-0, I and II are mainly the complexes that facilitate the recruiting and loading of cargo, while ESCRT-III, through its accessory molecules facilitates deubiquitination of the cargo (19). Many of the ESCRT subunits have been investigated in vesicle biogenesis through their silencing by shRNA and have been found to influence various aspects of vesicle secretion and composition (28). However well studied the ESCRT machinery is, it does not seem to be the sole factor that facilitates MVB formation (29), and the proposed mechanisms also include roles for lipid components such as ceramide, cholesterol, and phosphatidic acid playing a role in ILV formation (29-31).

Release of EXOs not only require ILVs to form in the MVB, but also the transport of the MVB to the membrane and their subsequent fusion. Rab proteins, a branch of the Ras superfamily of G-proteins, orchestrate many of the trafficking events that take place in cells and thus are also involved in the events that lead to the MVB fusing with the plasma membrane (29, 32). Knock-down studies have shown that efficient release of EXOs is dependent on several Rab proteins (29). Rab27a and Rab27b seem to be relevant for the docking of the MVB to the plasma membrane and their depletion impairs EXO production (33). Similarly, Rab5A, Rab9A, Rab2B, Rab11 and Rab35 inhibition has also been found to impair EXO release (29, 33-35). It is evident that the trafficking events and the molecules that govern them are part of of a multifactorial and complicated molecular machine. While the delivery of the MVB to the plasma membrane is largely left to the machinations of the Rab proteins, the fusion of the two membranes is facilitated by the SNARE proteins (29). As an example, the R-SNARE VAMP7 was shown to be important for exosome release because loss of functional VAMP7 led to decreased EXO production and an increase in MVB size (36). Other SNARE proteins such as YKT6, syntaxin-1A and syntaxin-5 have also been shown to affect EXO release (37-40). Figure 1 illustrates a highly simplified conceptual overview of EV biogenesis.

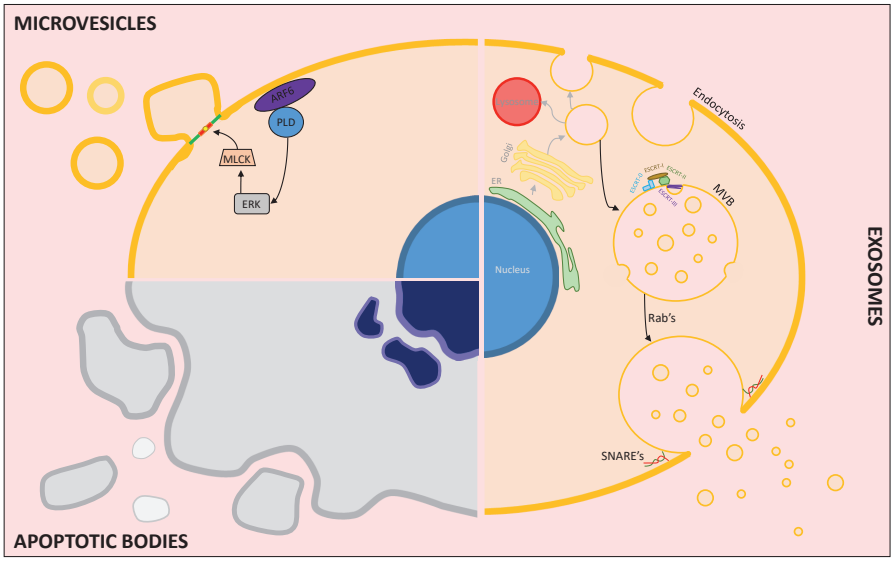

Figure 1. Schematic representation of extracellular vesicle biogenesis including Apoptotic bodies, Microvesicles and Exosomes.

1.1.2 EV Composition

The molecular makeup of EVs has been extensively researched. This is a complex topic since the cargo of EVs is plastic to say the least, being influenced by factors such as cell of origin, the state of the cell (such as stress), and route of biogenesis, to name a few. The trouble of separating EVs from contaminating molecules during EV isolation and the difficulty of attaining isolates of only one particular subtype of EVs further tarnishes our knowledge of specific cargo. Four basic components largely make up the composition of EVs, namely lipids, proteins, RNA and DNA.

1.1.2.1 Lipids

The EVs contain lipids which are present as a lipid bilayer, which gives form to the body of the vesicle itself. Unfortunately, characterization of the lipid contents of EVs has not received nearly as much attention as that of protein cargo characterization. It has been shown, though, that the lipid contents of vesicles is not merely a copy of the cells own plasma membrane, but rather that the composition differs from that of the cell (41). Different enrichments of

lipids in EVs can also be seen when the cell of origin differs (42). Mainly cholesterol, sphingomyelin, phosphatidylserine, and hexocylceramide seem to be enriched in EXOs as compared to cells (30, 41, 43). Differences in lipid composition have also been reported between subtypes of EVs. Ceramides, cholesterol esters, and sphingomyelins were reported to be enriched in MVs, whereas glycolipids, free fatty acids, and phosphatidylserine were enriched in EXOs (44). Apart from functioning as vector to carry other components such as proteins and RNA, the lipid composition also has functional properties. As an example, phosphatidylserine, which normally resides in the inner leaflet, acts as an "eat me" signal when in the outer leaflet and thus exposed to the extracellular environment. This is the case with ABs, where the whole process of apoptosis and blebbing is meant to generate neat "packages" for consumption by maintenance cells such as macrophages. The same can be seen in MVs, which carry their phosphatidylserine on the outer leaflet (45, 46). The localization of phosphatidylserine on EXOs is still seemingly unclear (47).

1.1.2.2 RNA

One of the more thoroughly explored EV cargo molecules are the RNAs. It has been shown that EVs carry a plethora of different RNA species from coding messenger RNA (mRNA) to non-coding species such as long noncoding RNA (lncRNA), circular RNA (cRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), transfer RNA (tRNA), ribosomal RNA (rRNA) and piwi-interacting RNA (piRNA) (48, 49). EVs appear to be able to ferry these RNA molecules between cells as a means of cellular communication. Many thousands of mRNAs have been found to be carried by EVs, and some of these can also be translated into functioning proteins in recipient cells (50, 51). This shows that EVs can transfer functionality to recipient cells in the form of protein blueprints. MicroRNAs have also been of interest in vesicle research. The main role of microRNA is that of attenuator, where the small microRNA binds to complementary sequences of mRNAs and impedes their translation (52). What makes the microRNAs especially interesting is the potential functional "punch" that they can deliver. It has been postulated that on average a microRNA can interact with 200 target mRNAs (53) and it has been shown that the RNA profiles generally differ between different EV subtypes (54).

1.1.2.3 DNA

It is only recently that the DNA content of EVs has been brought up, other than its presence in ABs (which has been known for long) (55). In recent years though, other vesicular subtypes have been shown to carry and transfer DNA between cells (56, 57), and it has also been shown that different EV subpopulations from the same cell line contain differences in DNA cargo (56). The main interest in DNA as EV cargo seems to be the potential biomarker value it carries (in the form of mutations).

1.1.2.4 Proteins

The protein cargo of EVs by far receives most attention as a subject of EV research. Many thousands of proteins have been identified on EVs, and many of these are reported and stored in databases such as EVpedia, Exocarta and Vesiclepedia (along with lipid and RNA data) (58-60). Some kind of proteomic evaluation of vesicles, most often western blot, is included in almost all scientific papers on EVs as the protein content is the most readily used source of EV markers. These markers are usually proteins enriched (but not necessarily unique) in EVs as compared to the cells that produced them. The tetraspanins CD9, CD63 and CD81 are classic examples of such markers and are often seen in western blots among EV papers (61). TSG101 and Alix are two other favorites that are often presented together with the aforementioned tetraspanins. These are components of the ESCRT machinery and are usually found in EV isolates and are used to strengthen the claim of a successful and meaningful isolation (19). Other proteins commonly found in EV isolates are GAPDH, actin (and ezrin, cofilin, and profilin), myosin, tubulin, ICAM-1, enolase-1, heatshock proteins such as HSC70 and HSP90, Rab7A (and other Rabs), syntenin-1, flotillin-1/2, and many others that EVpedia neatly lists in a top 100 list (58). Some of these appear to be relevant to EVs since their presence in the isolates is at least theoretically justified. Flotillin-1 for example, is a marker for lipid rafts, and since a certain EV subtype is thought to be enriched in raft structures this can very well function as an indicator of a successful isolation (62). With other proteins such as actin, the vesicle association becomes muddled because it is a soluble protein that can associate with the membrane via accessory proteins (such as ezrin) and because it is abundant in cells. Some components of the vesicular proteome are more or less always found in EV isolates no matter the source (such as the previously mentioned tetraspanins), while others are more dependent on the cell from which the vesicles originate EVs isolated from seminal plasma were compared to EVs isolated from milk, and a number of proteins were found to be enriched

in one sample as compared to the other (63). The proteins CXCL5 and KLK6, for example, were uniquely found in EVs isolated from milk. In a very early (and important) study, B-cells were shown to release MHCII antigen-containing vesicles (64).

Not only does the protein cargo vary between vesicles from different cell types, but a difference can also be seen between healthy and diseased cells. As an example, EGFRvIII was found on EVs from tumor cells (65). The proteome contains further discrepancies when taking vesicular subtypes into account. These discrepancies were addressed in an excellent study by Thery et al (66). Here they isolated EVs through contemporary methodological means (including a density floatation step) and performed a thorough evaluation of protein components in vesicular subtypes (subtypes based on isolation procedure). They detected an enrichment of factors in different subsets of vesicles, such as the enrichment of actin in large EVs, the enrichment of TSG101 and CD81 in small low-density EVs as well as flotillin, which was similarly present on all EVs, to name a few. Taken together, these results highlight the complexity of the EV proteome. The inconsistencies in pre-analytical factors such as EV isolation protocols further ads confusion regarding the collective pool of what is considered EV proteins, which is highlighted by the study discussed above.

1.2 Isolation of EVs

One of the major factors that limits the progress in the EV field is the limitations of the isolation procedures that are commonly used. Many protocols fail to produce a sufficiently pure sample for downstream analysis due to the difficulty of separating EVs from contaminating factors. Then there is the inability of many protocols to separate vesicular subtypes completely. Of course, once isolated, it is very challenging to determine which biogenesis pathway produced the vesicle. The EV field has in the past couple of years shifted its preferred nomenclature and instead of reading of “exosomes” and “microvesicles” we now see the term “extracellular vesicle” in their place (67). When EV subtypes are addressed in studies, we now see what was previously, rather incorrectly, called “microvesicles” and “exosomes” (as an example) rebranded as “large EVs” or “high-density EVs” and the likes (66, 68). The nomenclature is now more descriptive of the isolation procedure that yielded the isolates rather than reflective a biological background that is usually only assumed in face of the lack of hard evidence.

The isolation of EVs aims at separating them from unwanted components in the sample. What constitutes an unwanted component varies in accordance with the research question. Usually cells, cell debris, and soluble proteins and lipoprotein particles are such undesirable contaminants as well as ABs that rarely fit within the scope of research as they are a product of apoptosis. Isolation can further aim at collecting subtypes of vesicles and dividing them into separate isolates to study their differences.

1.2.1 Ultracentrifugation

The most commonly used method for the isolation of vesicles has ben and still is ultracentrifugation (69, 70). Two techniques are predominantly used for EV research. These are differential ultracentrifugation and density gradient centrifugations. Differential ultracentrifugation is mainly used to separate components of varying sedimentation rates, which roughly translates to variations in size and density. In general, larger particles sediment faster than smaller particles and will pellet at an earlier time, and thus less force for a shorter duration is typically required in order to pellet large particles compared to smaller particles. Normally a couple of centrifugations are done in succession starting with a shorter lower-speed run to pellet large particles and ending with longer high-speed centrifugations to pellet small particles (71). The supernatant is carried over from one run to the next and the pellet, consisting of an enrichment of increasingly smaller particles for each consecutive run, is either discarded or kept. The method is inherently crude, however, and due to the complexity of samples and the heterogeneity of vesicular subtypes rarely produces isolates of satisfactory purity (72).

Differential centrifugation protocols are increasingly being supplemented with complementary methods to increase the purity of the isolates. A commonly applied method is that of density gradient centrifugations (or density cushions) (69, 71). Whereas differential ultracentrifugation separates particles mainly based on their rate of sedimentation, the use of a density gradient allows particles to be separated based on their difference in density. This is referred to as an isopycnic centrifugation, wherein the centrifugation tube is filled with layers of media of different densities to form a discontinuous gradient. Alternatively, a continuous gradient can be made with a smooth transition between densities rather than a stepwise one. Samples are deposited either on top of the gradient or at the bottom, and the particles are allowed to migrate to their corresponding densities within the gradient where they stay as a result of the mutually cancelling forces of gravity and buoyancy acting on the particle.

Because EVs differ in density from soluble proteins, these two entities can be separated with good efficiency (EVs ≈ 1.12 g/ml, proteins ≈ 1.35 g/ml) (66, 73). As opposed to isopycnic centrifugations, another type, namely rate-zonal centrifugations, aims to separate particles based on sedimentation rate. In this case, a density gradient is used but instead of allowing particles to reach their isopycnic point, the run is terminated beforehand. Particles separate ideally into individual bands positioned in the tube according to their rate of sedimentation, or more generally, according to size. These two methods can be loosely likened to the two separation principles used in 2D protein blots where separation is done according to isoelectric point (proteins are allowed to migrate to a "final" destination) and according to size (regular protein electrophoresis where run is terminated before proteins run out of gel). Interestingly, the two methods could be used in succession for a separation based both on size and density (74).

A simpler form of a gradient is the density cushion, which generally consists of fewer density steps. The cushion is constructed so that a sharp density cut-off is formed between two layers of different densities where the density of the lower layer is too high for the particles of interest (such as EVs) to pass, while at the same time being low enough for contaminants of higher density (such as soluble proteins) to pass freely. Hence it forms an impassible barrier for low-density particles, but does not pose a hindrance for higher-density particles.

Many compounds can be used as a density medium, but two compounds have dominated the EV field. These are sucrose and iodixanol (sold as Optiprep), although sucrose is being gradually replaced by Optiprep. The benefits of Optiprep over sucrose is partly its ease of use but mainly its osmolarity, which is compatible with biological samples (https://www.axis-shield-density-gradient-media.com/, Jan 2019), whereas the high osmaolarity of high-density sucrose fractions raises a concern regarding its effect on vesicular structures. Optiprep further has the property of being able to self-form stable density gradients but is rarely used as such.

Apart from the choice of isolation procedure (differential centrifugation, density gradient, etc) there is also the choice of rotor for the task. The most commonly used rotors are the fixed-angle (FA) rotors and the swinging bucket (SW) rotors. For the task of differential centrifugation, it seems that many researchers tend to utilize what is available to them and there is not always the luxury of choosing what is most suitable. Both FA rotors as well as SW rotors are used for pelleting during differential ultracentrifugation and they both arguably do a good job at it. In general, though, the FA rotor is better suited for the task owing to its design that result in a shorter path length and tolerance

for higher speed. When it comes to density gradient centrifugation, SW rotors are almost exclusively used. The reason for this appears to be the fact that the direction of forces acting on the sample while standing still and during centrifugation are both toward the bottom of the tube. The gradient thus never shifts inside the tube. This does, however, not necessarily make the SW rotor inherently better for gradient separation work. Without arguing for or against either of the rotor types, the FA rotor could arguably also be applied for the job and the shift in sample orientation could work as an advantage. Because there is a reorientation of sample between run and stand still, it has the effect of constricting bands located in the lower part of the rotor and expand those higher up, which could have a positive effect on resolution of separation (75). The shorter path length could also be advantageous because high-density particles would be pelleted and ruled out of the gradient faster than would be the case if a SW rotor were to be used. Finally, there is a case to be made for fixed-angle rotors with a very steep angle, the near-vertical or vertical rotors. These are ideally used with density mediums such as Optiprep in the formation of self-generating stable continuous gradients (76). Such a technique could potentially be developed to suit EV isolation and reduce the complicity of sample prep.

1.2.2 Size Exclusion Chromatography

An old method made relatively new in the EV field after the diligent work of Böing et al, is a cheap and simple method for EV isolation through size exclusion chromatography (SEC) (77). This method of preparative size separating chromatography utilizes porous beads as a stationary phase through and around which the mobile phase can pass. Retention of particles in the samples depends on the particles' accessibility to the bead-pores. Particles with no pore access do not experience retention and elute at the same rate as the void volume (liquid that runs around the beads). Particles with partial access to the pores will elute in size order from larger particles (with more limited access) to smaller particles. The smallest components in the sample, such as salts, which have full access to pores will elute last, approximately after all of the liquid in the column present at the start of the run has been replaced. The method is most suitable for smaller sample volumes and is sometimes preceded by other methods in order to reduce sample volume (78, 79). The method can also be used in combination with other methods in order to achieve increased purity of EV isolates. SEC in combination with density gradient ultracentrifugation managed to achieve adequately pure EV isolates from

plasma for mass spectrometric evaluation where either method alone was insufficient (78, 80).

1.2.3 Affinity capture

Because the molecular composition of EVs is somewhat known, methods to isolate them through affinity capturing against these molecules has been developed. This is frequently done through antibodies immobilized on a fixed surface such a magnetic beads. The target for this capture methods can be (but is not limited to) the classic tetraspanins CD9, CD63 and CD81 which are commonly decorating EVs (66, 81). Others have targeted more tissue or cell-specific markers such as Ep-CAM or HLA-DP/DQ/DR (82, 83). Affinity capturing is not limited to the use of antibodies, though, nor is it limited to proteins as a target. As an example, exposed phosphatidylserine has also been targeted by capture with annexin 5 and Tim4 (84, 85). Whichever epitope is targeted, some preparative steps need to be taken before capturing, such as the elimination of cells, and in some cases the sample needs to be concentrated. One disadvantage of this method is that only a subpopulation of vesicles are targeted, that is, the subpopulation that carries the epitopes for which the antibodies are specific for. Thus whatever downstream analysis are made will only be true for the captured EV subtype and not on a global level. A most intriguing idea, however, has recently been presented to the EV community (86), namely, capturing of EVs based on their most basic property, their highly curved membranes. This very definition of a vesicle should, in the authors mind at least, be an excellent target to focus on. By utilizing peptides that can bind to specifically curved membrane structures it could be possible not only to selectively bind vesicles, but also choose which size of vesicle that is to be preferentially bound (87, 88).

1.2.4 Filtration

This method capitalizes on the size difference of the components in a complex sample. Similar to differential ultracentrifugation, this method can be set up so that a complex sample is sequentially passed through several filters with successively smaller pore sizes (89). Larger particles (such as cell debris and ABs) are captured early on in filters with larger pore size while smaller particles (such as exosomes) are captured later on. Very small particles, such as contaminating soluble proteins, flow through the last filter if the pore size is large enough. The efficiency of this method is contested, however, partly by

claims that contaminants still occur, that yield is below par, and that forcing the samples through pores can be detrimental (90-92). On the other hand, filtration offers a quicker and cheaper option compared to many other isolation methods.

1.2.5 Precipitation

By changing the solubility of components in solution, they can be aggregated and thus sediment faster than they normally would in solution. This is at the heart of precipitation methods. The basic idea here is that by the addition of a compound that acts as a "water sponge", the EVs are depraved of sufficient solute to remain in solution and thus start aggregating together, resulting in an increased sedimentation rate and the possibility to pellet these EVs at lower centrifugation speeds. Polyethylene glycol (PEG) is perhaps the most commonly used agent to facilitate precipitation in the EV field (as many commercial kits use it), and it has been shown to be efficient in generating high yields (93). However, the purity of the isolates has been put into question (94, 95). Naturally, as PEG acts by decreasing the availability of solutes, its effect is not only felt on the vesicles in the sample, but on all components, which makes this an inherently crude isolation method. On the other hand, its ease of use and cost effectiveness speaks for its benefit. Precipitation of EVs could also be facilitated by the positively charged molecule protamine, and was shown to be even more efficient in combination with PEG (96).

1.2.6 Microfluidic and on-chip devices

A more recent addition to the EV toolbox is the microfluidic devices that have emerged. Often they come as a miniaturized chamber into which samples are loaded and then subsequently subjected to a form of isolation/separation that is often based on one of the aforementioned principles. Some work on the principle of size-based retention, where larger structures and soluble contaminants can either flow through and/or are prevented from entering, while vesicle sized particles are entrapped (97, 98). Other strategies rely on affinity capture, often utilizing antibodies specific for EV markers such as CD63 (99, 100). Other examples of microfluidic techniques used in EV research include asymmetric flow field-flow fractionation that relies on laminar flow and diffusion rates of particles for separation and the very imaginative acoustic-based separation that relies on the manipulation of particles by sound waves (101-103). These methods are often miniaturized and

can only handle limited sample volumes. However, the “on-chip” nature of these methods makes them excellent candidates for clinical application.

1.2.7 Remarks on EV isolation

The constant strive for new methodology in EV isolation somewhat reflects the inadequacy of the current toolbox we have at our disposal. Generally, a method will excel in some regard but falls short in others. Often quantity is attained at the cost of quality, or there are constraints on sample volumes. Methods are either too expensive, too lengthy and complex, require specialized equipment, isolate only a subpopulation of EVs, are not scalable, are too crude, etc. However, the pitfall of one method can potentially be overcome by supplementing it with a second method. Usually this entails the initial use of a cruder concentrating method that tolerates larger sample volumes, followed by a cleanup step (66, 79, 104, 105). A commendable review was recently published by Konoshenko et al. listing and explaining the many isolation strategies that have emerged in recent years (106).

With the ever-increasing additions of new methods and tailored protocols to fit the myriad of cell cultures and biological specimens that are being evaluated, there is a risk of drifting away from standardization and comparability between studies. The more we learn of the biology of vesicles with regards to their composition, biogenesis, and accompanying biological surrounding, the less need there seems to be for absolute standardization. Recently, an updated “minimal information for studies of extracellular vesicles”, MISEV for short, was released in an effort to provide guidelines in the research on vesicles (107). This, in part, represents an effort of the community in the EV field to systematize field and to ensure a high quality of research. Table 1 provides an overview of some isolation methods and lists some strengths and weaknesses of each.

Table 1. Isolation methods overview

Isolation method	Principle of separation	Strength	Weakness	Use
Differential Ultra-centrifugation	Sedimentation rate of particles is dependent largely on their size. Thus, particles of larger to smaller size are pelleted out of solution sequentially.	Efficiently concentrates EVs. Large volumes can be handled.	Crude method. Contaminants are usually co-pelleted with vesicles. Difficult to pellet vesicular subtypes due to their heterogenic nature. Requires specialized equipment. Time consuming.	Initial step. Concentrates samples and removes large contaminants.
Density centrifugation	Separation either based on differing densities of particles (isopycninc) or their sedimentation rate (rate-zonal).	Efficiently separates EVs from contaminants.	Limited starting volume, samples have to be concentrated prior. Requires specialized equipment. Time consuming.	Purifying step. Separates EVs from many contaminants.
Size exclusion chromatography	Separates based on particle size.	Good at removing contaminants. Requires no specialized equipment.	Requires small sample volumes.	Purifying step. Separates EVs from many contaminants.
Filtration	Separates based on particle size.	Requires no specialized equipment. Relatively cheap and fast.	Can be detrimental to EV stability if forces are applied. Controversy regarding the purity of isolates.	Can be suitable for clinical samples.
Affinity capture	Specific capturing of EV surface epitopes (using antibodies or other molecules)	High specificity.	Isolates only subtypes bearing target epitope. Expensive.	Can be used on crude samples. Can be useful for capturing and analysing EV subtypes. Could be useful for clinical applications.
Precipitation	Forces aggregation and sedimentation of EVs	Fast, easy and cheap. Doesn't require any specialized equipment. High yield.	Crude method. Contaminants are usually co-isolated with vesicles. Limited starting volume.	Initial step. Concentrates samples.
Microfluidic devices	Different methods (size, affinity, diffusion)	Minimal "hands-on" requirement for some of them. Potential for clinical use.	Need to be evaluated more. Small volumes. Some require special skills to use.	Have clinical potential. Mostly suitable for clinical samples. One-step isolation method.

1.3 Detection and analysis of EVs

As described, even though current isolation techniques have their strengths and their weaknesses, the isolation of EVs is far from a perfected art form. This is directly reflected in the isolate and as such can complicate downstream analysis. When isolates have been acquired, they are generally analyzed for three characteristics – quality, quantity and morphology (107). Quality is usually measured by the examination of a number of factors, such as the presence of enriched vesicular proteins and the absence of non-vesicle proteins. Quantity measurements are usually performed either with total protein estimation (or sometimes RNA estimation) or by particle counting with nanoparticle measurement technology. The morphology can be assessed also by particle measurements as with particle counting since these technologies usually provide data on both concentration and size distribution. Electron microscopy is often used as well as sometimes light microscopy. Taken together, these metrics give the researcher a general idea of the components in the isolates (108).

1.3.1 Cargo

The molecular composition of EVs is constantly being unraveled, and as a result the distinction between isolated EVs and co-isolated contaminants can be made all the clearer. When it comes to cargo measurements, the go to method is usually western blotting. Certain proteins, although not necessarily being unique to EVs, are at the very least enriched in them. The tetraspanins CD9, CD63 and CD81 are three examples of these (109, 110). Furthermore, the presence of proteins involved in the biogenesis of EVs such as Alix and TSG101 are also commonly used as markers, as are certain heat shock proteins which have been commonly found in EVs (82). Additional proteins have been used to illustrate the presence of EVs such as the lipid raft-associated protein flotillin-1, the phospholipid-binding annexin 2A, or proteins involved in vesicular trafficking such as Rab-5b (110). The analysis including the aforementioned components is normally performed on the isolate in relation to their cells of origin. Thus, a cell lysate is included compared to which the vesicular proteins (CD9 and CD81 as an example) should be enriched. Other than showing the presence of positive markers, the inclusion of a negative marker further emphasizes the successful enrichment of EVs in the isolates over other components. To this end, proteins that should be specifically located in other cellular compartments than those involved in EV-biogenesis are chosen and could include calnexin (an ER marker), cytochrome C (a

mitochondrial marker), and/or GM130 (a Golgi marker) amongst others (111). Taken together, an analysis as described above should provide valuable information about the composition of the isolates and the enrichment of vesicular components over other cellular components (107).

Similarly, flow cytometry can be used to illustrate the presence of EVs in isolates. Because EVs are too small for the efficient detection in a normal flow cytometer, antibody-coated beads are used to immobilize vesicles based on binding to CD63 as an example (112-114). Although other surface epitopes will do as well as other molecules to facilitate capturing (as discussed in other chapters). This is usually followed by incubation with a second fluorochrome-conjugated antibody for detection against either the same epitope as the bead-bound or another epitope. However, new technological advancements seems to be rendering bead-dependence obsolete with the promise of single-vesicle analysis by the nano-flow cytometry (115). Furthermore, other antibody-based methods such as ELISA has been utilized for the detection of vesicles (116). A sandwich ELISA targeting two membrane proteins, one by capturing and the other for detection, further strengthens the vesicular nature of the isolate since such detection should only be possible if the two epitopes are on the same membrane entity. Similarly, a microscopy-based system operating on the principle of vesicle capturing onto surface-immobilized antibodies and a sandwich system with fluorescent antibodies can be used (117).

Although less common, RNA is also occasionally used to verify vesicular isolations. The RNA peak profile generated by a bioanalyzer (chip-based automated electrophoresis instrument) normally produces profiles that differ between vesicles and cells. Most prominently, the reduction or absence of ribosomal RNA peaks is observed in MVs and EXOs while they are very prominent in whole cell RNA extracts (118, 119).

1.3.2 Particles and morphology

Apart from the compositional analysis of vesicles, a highly valued proof of vesicle presence in the isolates is a visual appraise of the sample. Owing to their small size, regular light microscopy is inadequate to visualize vesicles. The most widely applied method is that of electron microscopy (EM). Instead of beaming light onto the sample, this method uses electrons to achieve a shorter wavelength than that of visible light and thus greater resolution. Different techniques for electron microscopy exists, but the most widely used are the scanning (SEM), transmission (TEM) and cryo-transmission- (Cryo-

TEM) electron microscopy. In short, SEM operates by systematically scanning over the sample with a focused electron beam that scatters upon impact. The scattered signal is then translated into an image of the sample surface. TEM on the other hand transmits the beam through the sample. After passing, the projected beam is altered and the resulting signal can be translated into an image. Cryo-TEM works on the similar principles as TEM, but sample preparations in this method do not rely on fixation and contrast staining but rather on freezing (120). Thus this method produces the most detailed images of the three where even membrane bilayers can be easily visualized (121). The visualization of vesicles by one of these three methods (or equivalent ones) is a most desired component of most papers (107). This method often serves as a definitive sign that isolates contain EVs (but by no means proves their abundance nor purity). EM has further been used to illustrate the heterogeneity of vesicles in isolates (121-123). The technique can also be combined with antibody labeling to visualize surface epitopes (123).

On one side of the spectrum is EM which is low-throughput method used to visualize individual vesicles in great detail. On the other are the high throughput instrumentations for particle measurements. These include nanoparticle tracking analysis (NTA), resistive pulse sensing (RPS) and dynamic light scattering (DLS) to name a few. These methods do not necessarily distinguish vesicles from other components in the sample, but instead detect the general "particle" that is in their detection range. NTA generally consists of a light source to illuminate the particles in a solution as well as optics connected to a camera to record the illuminated particles. Particles of varying sizes undergo what is known as Brownian motion, which describes their stochastic movement resulting from their interaction with the solute. Smaller particles would thus experience more motion than larger ones. Hence, by visualizing the particles by light scattering, they can be tracked and both the size and concentration of particles can be measured (124). Another method, DLS, which also operates on the basis of light scattering and Brownian motion, measures the scattered light of particles in solution. As they undergo Brownian motion, a shift in the amount of scattered light can be measured and from it the size and concentration of particles can be deduced (125). RPS, rather than relying on light scattering for detection and Brownian motion for size estimation, measures resistance in electrical current caused by the transition of particles through a pore that separates two chambers. Particle size can thus be extrapolated from the resistance that a passing particle confers, larger ones resulting in higher resistance than smaller ones by blocking a larger portion of the pore, while concentration is proportional to the frequency of measured instances of resistance (126). These techniques, although they have their advantages and definitely are useful, suffer from some disadvantages as

well. It has been postulated that DLS, although being able to measure a wide size-range of samples, unfortunately underperforms when such a size range is present during the same measurement due to the signal from larger particles masking that of the smaller ones (127). The same can be said for NTA, which is perhaps the more used method of the two. Furthermore, there are issues when it comes to the detection of very small vesicles because the smallest detectable size is in the vicinity of about 50 nm (112). Thus, a couple of concerns have been raised and among these techniques, the most pressing one from a personal opinion is taht raised by Maas et al. where they highlight the need for understanding of the technical aspects of these methods and plead for understanding regarding instrument setup and the effect it has on measurements (124, 127, 128)

1.3.3 Proteomics

Instead of describing both genomics and transcriptomics, to which both EVs have been subjected, this thesis will focus mainly on proteomics as this is the most relevant in relation to the work presented herein. Out of the three, proteomics is likely the most commonly applied to EV research. Not to omit the others completely though, and to highlight their relevance, at least some studies should be brought forward. With the rise of next generation methods for sequencing, and the reduction in cost, more and more studies are readily conducted, which is promising in the hunt for biomarkers and functional components carried by EVs. Thus a few examples to highlight the importance of these types of analyses are warranted. Deep sequencing by Nolte-‘t Hoen et al. suggested that small RNA species were enriched in vesicles as compared to cells (49). Selmaj et al. were early to conduct a global characterization of circulating serum vesicles from relapsing-remitting multiple sclerosis patients where they could identify different RNA profiles in the vesicles of these patients (129). Bellingham et al. identified a panel of miRNA that were specifically enriched in prion-infected neuronal cells, also highlighting the potential of these as biomarkers of disease (130). Thakur et al. examined different tumor-derived exosomes and found through sequencing that the whole genome was represented (131). As genetic abnormalities have been found on vesicles before, this represents yet another source to be probed for biomarkers (131-133).

Mass spectrometry, as is the case with next generation sequencing, is a method that in later years has become quite affordable, with the price of analysis

nearing that of the purchase of an antibody, if not slightly cheaper even. This will surely lead to an increased number of proteomic studies in the EV field.

In general terms, a mass spectrometer is, as the name somewhat implies, an instrument for the measurement of masses of molecules (or atoms). The approach generally employed when a proteome is studied is a bottom-up method usually referred to as shotgun proteomics. This requires protein extraction from the sample followed by its digestion with a proteolytic enzyme. Trypsin is ordinarily applied to this end. Samples are then subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. First they are fractionated through an on-line reverse phase chromatography column from which peptides successively elute and are ionized by electrospray ionization before being injected into the mass analyzer. In a data-dependent acquisition mode, first the peptides are analyzed in MS1, where the top peaks are selected and isolated for fragmentation by collision with inert gas molecules such as helium for example, breaking them apart preferentially at the peptide bonds through a process known as collision-induced dissociation (CID). The fragments are then analyzed in MS2. The data generated is then searched against a database to ultimately identify proteins in the sample. Due to evolutionary conservation, different proteins can comprise of overlapping sequences. Thus when it comes to protein identification, it can be beneficial to only use unique peptide sequences, those not shared between proteins, to infer protein identity. The same sequence conservation problem exists across species and could be a potential problem in EV research because fetal calf serum is a common addition to cell culture medium. Even though depletion steps are performed, there is likely still contamination of fetal vesicles in cell culture EV isolates (134).

A couple of factors will influence the number of identifications in an MS run. Among them is the complexity of the sample as well as its dynamic range. Because analysis time is used up on high-intensity peaks, peptides with lower peak intensity could remain unidentified due to never being selected for fragmentation. As with many other methods, the input will reflect the output, which also holds true for mass spectrometry. The use of crude isolation methods without any purification steps will inevitably increase sample complexity, and as a result lower-abundance EV-proteins stand an even smaller chance of being identified (82). The EV database Vesiclepedia (vesiclepedia.org) comprises a data repository of transcriptomic, genomic and proteomic studies that in many cases includes information of isolation method for the submitted studies (59). After a quick glance at the database, it appears that much of the submitted proteomic studies could very well contain misleading data due to the lack of appropriate purification steps (Figure 2). If

crudely grouping the isolation methods into levels of perceived purity (see figure for explanation), it appears that more than half of the studies that reported isolation method did not purify their isolates and thus introduce non-EV proteins in the data that they generated. Naturally, such purification steps are not always an option, and different EV sources require different methods of purification. Additionally, validation steps can in many cases be made after proteomics to secure the validity of claims pertaining to identified proteins and their vesicular nature. Regardless, the foresight of the database creators to include annotation on isolation method is fortunate indeed since this is an important parameter when considering the data itself.

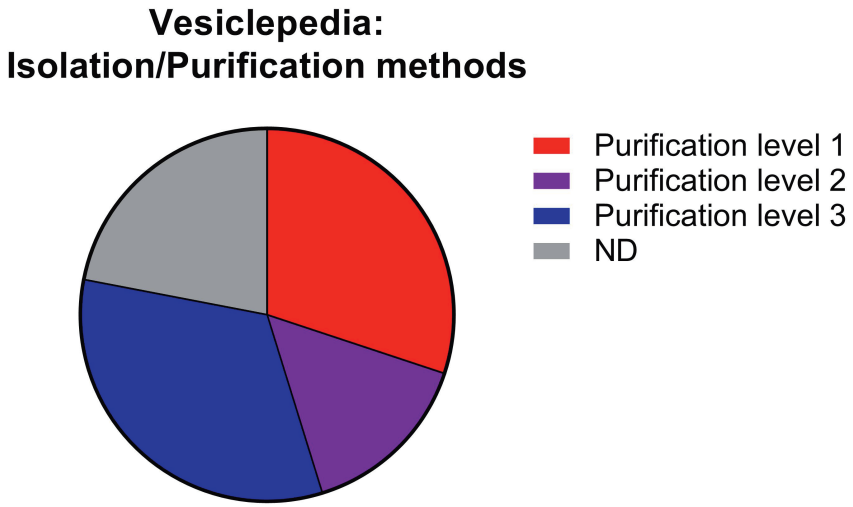

Figure 2. Approximation of isolate purity based on isolation methods for proteomic studies from Vesiclepedia. Metrics on proteomic data from Vesiclepedia.org were evaluated for isolation methods. These were then roughly grouped based on the level of purity that they normally produce. The data were filtered for studies conducting proteomics by mass spectrometry. Isolation strategies were grouped into "Purification levels" ranging from 1 to 3 with 1 considered containing the least pure isolates and 3 containing the most pure isolates. Purity level 2 is considered either to be equivalent to 1 or of intermediate purity. The isolation strategy grouping is as follows. Purification level 1 = isolation by either ultracentrifugation, precipitation, or both. Purification level 2 = isolation by ultracentrifugation, precipitation or both but with the added requirement of filtration or isolation only with filtration. Purification level 3 = Isolation with any of the aforementioned methods but with the added requirement of either density centrifugation, SEC, or affinity capture, alternatively isolation by only one or more of these last three. ND = No method reported.

Other steps can be taken to increase identification and potentially identify proteins of rarer nature. Off-line sample fractionation, for example, can reduce the complexity to such a degree that significantly more proteins are identified. This can be in the form of sample fractionation by SDS-PAGE or by chromatographic methods among others. In the case of EVs, one might even consider that mass spectrometry could be preceded by vesicle sub-fractionation to reduce sample complexity, either through different ultracentrifugation methods, gel filtration, or affinity capturing, to name a few. In effect though, this would separate the samples into vesicular subtypes based on either density, size or, surface epitope amongst others depending on the method used.

Furthermore, mass spectrometry can be used as a semi-quantitative method in discovery proteomics. This can be done through labeling methods and through label-free methods. In simple terms, in label-free methods samples are usually run separately. Retention time and m/z measurements are used to align the corresponding peptides across samples, and signal intensity is used to deduce relative quantities either on precursor ions or through spectral counting, which also relies on the MS2 spectra. Labeling methods come in two general categories – metabolic labeling and chemical labeling. In metabolic labeling, such as the SILAC method (Stable Isotope Labeling by/with Amino acids in Cell culture), amino acids labeled with heavy isotope are added to cell cultures. Cells grown in separate cultures are fed either labeled or regular amino acid supplements and as such incorporate these during protein synthesis. Samples are then combined after harvest and sample preparation and mass spectrometric analysis is carried out with the different samples as one. This effectively negates the variations otherwise introduced during sample preparation when samples are kept separate, as in say label-free quantification. Because this method requires metabolic incorporation of labeled substances, it is unsuitable for work with most clinical samples.

Chemical labeling on the other hand takes place farther down the line, usually at the peptide level after tryptic digestion. Two similar commercially available reagents are normally used, ITRAQ (Isobaric tags for relative and absolute quantitation) and TMT (Tandem mass tags), and these both utilize amine-reactive chemistry to label peptides. The tags can functionally be divided into three parts. The amine-reactive part facilitates the covalent binding to peptides. The reporter part uses tags that can differ in mass through the use of different atom isotopes. Between them is the mass-normalizing part, which is designed so that different tags all maintain the same mass throughout the run. Between the mass normalizing part and the reporter is a cleavable linker. By applying specific energy during CID, the reporter is separated from the rest of the molecule and thus the reporters will be detectable with varying m/z and their

intensities can be used for relative quantification. Generally, labeling methods are more expensive than label-free methods due to reagent costs. as Also, because label-free quantification does not have a limit of samples that can be compared, labeling methods are restricted by the limitation of isotope variations that can be produced. The combination of samples during labeling methods, other than being efficient in negating some of sample-prep variations, also makes it possible for more extensive off-line sample fractionation and reduced machine time as compared to an equivalent label-free experiment.

Mass spectrometry has been a very valuable tool with which to characterize the vesicular proteome and has been used both to understand the basic biology of vesicles and to characterize vesicular discrepancies in health and disease (66, 135, 136). It has been a tool to investigate the proteome of EVs from numerous biological fluids such as plasma, urine, and cerebrospinal fluid to name a few (137-139)

1.4 EVs in pathology

The main reason why EVs have been awarded as much attention as they have in the past decade is largely thanks to their potential role in different diseases. EVs are a cell communication medium able to transfer a wide range of functional molecules between cells (140). Additionally, this molecular cargo can vary depending on the cell of origin as well as which state the cell is in. It thus follows that the vesicular phenotype changes with that of its parental cell. The implications of this puts the EVs in a position to participate in the disease mechanism and also makes them candidates as both treatment targets and as carriers of disease markers (141, 142).

1.4.1 Role of EV in disease

The role of EVs in disease has been found to be both detrimental in some cases and beneficial in others. Just as they seem to find their place in most bodily processes, so they seem to find a role in the immune system. For instance, macrophages infected with bacteria can release pro-inflammatory EVs carrying bacterial coat proteins that in turn can activate other immune cells and seem to be able to release EVs that play a role in tuberculosis infection (143, 144). Vesicles released from natural killer (NK) cells have been shown to carry FasL and that the EVs exert cytotoxicity toward tumor cells (145). Mast cells

have been proposed to release EVs that promote a fibrotic phenotype in hepatic stellate cells, likely through the transfer of KIT (146), and dendritic cell-derived EVs are able to elicit T cell responses (147-149). This just names a few instances that show the involvement of EVs in the immune system, which assigns them an important role in most diseases that involve the host defense mechanism in any way (140, 150, 151). Elevated levels of EVs with altered cargo were found in the circulation of diabetes patients, suggesting a role of EVs in type 2 diabetes (152), and in obese patients EVs shed from adipose tissue carry miRNA cargo that can influence signaling events in recipient cells (153). Vesicles have also been described as facilitators of the spread of prions (154, 155). Furthermore, in neurodegenerative diseases such as Alzheimer disease, EVs have been shown to facilitate the spread of Tau proteins and amyloid-beta (156, 157). These are just some of the many pathologies in which EVs have been implicated (151).

1.4.2 EVs in cancer

A large part of disease-related EV research is focused on cancer. Granted, as the term “cancer” encompasses a vast heterogeneity of conditions, this might not come as a surprise. Cancers cells, just like all other cells in a multicellular organism, exist in a context in which they interact with the environment around them, and the propagation of the disease is thus also dependent on these interactions. Vesicles, as they are a means of cell-to-cell communication, participate in these signaling events and have been found to perform numerous tasks. Almost a decade ago Hanahan et al. described a set of physiological parameters that cancerous cells have acquired and termed them the hallmarks of cancer (158). Later these were revisited by Meehan et al. who provided a comprehensive review with a vesicular perspective, clearly pointing to the multifaceted role of EVs and their importance in cancer biology (159).

One of the main traits of tumor cells is their sustained and uncontrolled proliferation. The vesicular involvement in this process has been shown on numerous occasions with several different kinds of cancers. Glioblastoma EVs carrying the truncated mutant EGFRvIII can induce proliferation in glioma cells (51, 65), and EVs from gastric cancer have been shown to induce proliferation in tumor cells through the activation of the PI3K/AKT and MAPK/ERK pathways (160). Similarly, circulating hepatocellular carcinoma EVs carrying miR665 can induce proliferation supposedly through the MAPK/ERK pathway (161). It has been shown that the integrin b4 carried on vesicles can facilitate proliferation in osteosarcoma and that in glioma EVs

carrying miR-148a can do the same by acting on CADM1 (162, 163). In line with proliferation, tumors have a certain ability to resist cell death, which presents yet another process in which EVs have found their place. EVs from bladder cancer cells can inhibit apoptosis in recipient tumor cells, and this treatment was shown to up-regulate Bcl-2 and cyclin D1 but down-regulate Bax and caspase-3 (164). Vesicles can further carry anti-apoptotic factors, like the anti-apoptotic protein survivin, among other inhibitors of apoptosis family members, as described by Khan et al. (165-167). When it comes to resisting cell death, tumors also have to find a way to deal with the threat that the immune system poses. As an example of the EVs' involvement in this process, vesicles released in hepatocellular carcinoma carrying HMGB1 have been proposed to regulate tumor immune responses through regulatory B cells (168). Tumor-derived EVs have also been proposed to promote the formation of regulatory T cells and to simultaneously impair cytotoxic T cells (169). Furthermore, tumor cell-derived EVs carrying TGFB1 and NKG2D-ligand were shown to attenuate both NK cells and cytotoxic T cells (170). Another process that is crucial for the development and progression of the tumor is the formation of blood-vessels through angiogenesis. Numerous studies have shown the ability of tumor-derived EVs to promote angiogenesis in endothelial cells (171). A subset of EVs from renal carcinoma cancer stem cells carrying pro-angiogenic mRNAs for VEGF, FGF, angiopoietin 1, ephrin A3 and, MMP2/9 were shown to be able to induce an angiogenic phenotype in endothelial cells (172). The vesicular transfer of EGFR between cancer cells and endothelial cells was shown to induce MAPK/AKT signaling in recipient endothelial cells as well as VEGF expression and accompanied autocrine signaling through VEGF-receptor 2 and thus promote angiogenesis (173).

The progression of tumors often leads them to colonize other sites and organs through metastases. An initiating part of this process is the adoption of a more migratory phenotype in tumor cells through the epithelial-to-mesenchymal transition (EMT). It has been shown that EVs carrying HIF1a and LMP1 can induce cellular changes that lead to EMT (174). Similarly, EVs from cancer cells grown under hypoxic conditions, circumstances, which are associated with increased tumor aggressiveness, were also shown to carry factors tied to EMT and could induce a more migratory and invasive phenotype (175). A role for EVs in pre-metastatic niche formation has also been proposed (176). Melanoma EVs were shown to facilitate metastases of cancer cells in regional lymph nodes likely by preparing the site for such a colonization (177). Vesicles have also been shown to carry varying species of integrins, and the composition of these adhesion molecules have been proposed to be of predictive value as to where the future site of metastases will occur (178). Additionally, EVs derived from pancreatic ductal adenocarcinoma were shown

to facilitate niche formation in the liver through a mechanism likely dependent on EV-borne MIF (179).

These examples of vesicular involvement in the many processes of cancer biology highlight both the importance of understand their role more intimately and brings to light their potential as biomarkers. A study by Allenson et al. could detect mutant *KRAS* DNA in circulating EXOs and found that vesicle-borne *KRAS* mutations were better suited to predict pancreatic ductal adenocarcinoma status than cell free DNA (180). Work by Cazzoli et al., looking into circulating EV-carried microRNA, found a panel of four microRNAs that could distinguish patients with lung adenocarcinoma from healthy and another panel of six microRNAs that could distinguish patients with adenocarcinoma from patients with granuloma (181). The protein MIF was found on EVs in circulation and was found to be predictive of metastases formation in patients with pancreatic ductal adenocarcinoma (179). As is illustrated by these few examples, the biomarker-potential of vesicles is elevated in accord with the variety of molecules which they carry.

1.4.3 EVs in colorectal cancer

Colorectal cancer is one of the most common cancers worldwide. However, its prevalence is not equally distributed across the globe. Both incidence and mortality are higher in countries with a high human development index (HDI). However, in countries with the highest HDI, mortality has plateaued or even decreased, likely owing to better screening and treatment options (182). Many genetic alterations have been recognized to contribute to the formation of colorectal cancer, and some commonly seen alterations include alterations in *APC*, *CTNNB1*, *KRAS*, *BRAF*, *SMAD4*, *TGFBR2*, *TP53*, *PIK3CA*, *ARID1A*, *SOX9*, *FAM123B* and *ERBB2* along with excessive CpG island methylation and microsatellite instability (183, 184).

Just as with other cancers, so has the role of EVs been studied in the context of colorectal cancer. Their contribution to disease development is exemplified in a number of studies. For example, vesicles isolated from a *KRAS* mutant cell line exhibited a proteome different from that of EVs from wild-type cells, including a higher amount of mutant KRAS, EGFR and several integrin amongst others (185). What's more, these vesicles managed to induce growth in recipient wild-type cells. Using the same cell lines, it was demonstrated that miRNA loading was altered and that miR-100 was found at elevated levels on EVs of the mutant cell line, which could subsequently be taken up by recipient

cells where the functionality was also demonstrated through target silencing (186). This illustrates the potential of EVs to spread the cancer phenotype to seemingly healthy cells without direct cell-to-cell contact. EVs from colorectal cancer cells grown under hypoxia, so as to mirror the hypoxic conditions that might exist in tissue, induce an angiogenic phenotype in endothelial cells through the Wnt/B-catenin pathway (187). EVs from colorectal cancer cells have also been shown to effect mesenchymal stromal cells, increasing their proliferation, migration, and invasion, thus suggesting that they are important in progression of the disease (188). Both V-ATPase and carcinoembryonic antigen (CEA) were observed to increase in EV-treated stromal cells, and both of these are important in colon cancer with the latter being one of the few commonly used markers for disease (189, 190). Moreover, EVs carrying miR-210 were suggested to facilitate EMT in other tumor cells, converting them to suspended cells. This however only lasted for as long as the cells received EVs as the cells reverted to an adherent phenotype in their absence (191). This is interesting from a metastases point of view because for it to occur cells need to detach from the primary tumor site, but also need to become adherent later on at the site of metastases.

EVs have not only received attention due to their functional participation in diseases, and their potential as biomarkers has also drawn attention. Their escape from the tumor microenvironment and subsequent circulation in the blood make them especially attractive as biomarker candidates because this could provide a good opportunity to conduct non-invasive liquid biopsies. Furthermore, the abundance of EVs in circulation is higher in patients carrying tumors, which in itself has been suggested to function as a marker for disease (192, 193). Thus, it is no wonder that significant efforts have been made to find biomarker candidates among the EVs in circulation. The currently used biomarker CEA is mostly used in the diagnosis of recurrence but its ability is debated due to low sensitivity and specificity (194). Thus, there is a need for better markers that allow early detection of disease and thus to allow earlier treatment and better patient prognosis. The long non-coding RNA (lnRNA) CRNDE-h was found in EVs from blood and is proposedly to function as a biomarker for colorectal cancer (195). Higher levels of CRNDE-h was found in patients with colorectal cancer compared to healthy controls or those with hyperplastic polyps, inflammatory bowel disease, or adenoma, and the CRND-h levels were associated with poor clinical outcome. Xiao et al. proposed three circulating markers for colorectal cancer (196). Starting out with cell cultures and successively working their way through mouse xenografts to patient blood samples, they came to propose CK19 as a marker for colorectal tissue vesicles, TAG72 as a marker for treatment-resistant (5-FU) disease phenotype, and CA125 as a metastases marker. Shiromizu et al. in their impressive work took

another approach relying heavily on mass spectrometry (197). Biomarker candidates were selected through a combination of literature searches and shotgun proteomics in cell culture EVs and patient serum EVs. These candidates were verified by selected reaction monitoring (SRM) and further validated by the same method in a separate cohort. The authors proposed a total of 33 peptides to be useful for the early detection of colorectal cancer as well as four members of the annexin family to be highly sensitive biomarker candidates. What the authors showed is how useful the application of SRM can be in the screening for biomarkers and how multiple peptides can be used in combination to improve the potential of detection. The authors proposed that targeted proteomics in a clinical setting could replace current screening methods. This could in effect bring biomarker discovery closer to clinical application because similar tools would be used in both cases.

2 AIM

Research on EVs is still relatively young, and as such the methodological aspects in this field are as of yet unperfected and are still being refined. This inevitably reflects on the results that a study produces and forces us to interpret them cautiously. A better understanding of the methodological impact on isolates and deeper insight into vesicular biology will thus allow us to interpret results in a more correct manner. These aspects are touched upon in the work presented here. If viewed conceptually, the different papers herein touch upon the idea of understanding isolation method (paper I), understanding basic EV biology (paper II), understanding the EV environment/context (paper III), and finally understanding EVs' role in disease (paper IV). It can also be seen as an ascension through the steps of isolation, characterization and finally application.

Specific aims for each paper:

1. Paper I

The aim was to determine how different centrifuge rotors impact the yield and purity of EVs isolated with ultracentrifugation protocols.

2. Paper II

The aim was to characterize the EV proteome in isolates and to distinguish vesicular proteins from co-isolated proteins that could be contaminants.

3. Paper III

The aim was to produce a viable method for the isolation of EVs from blood plasma sufficiently free from contaminating plasma proteins and lipoproteins.

4. Paper IV

The aim was to isolate EVs directly from the tumor tissue of colorectal cancer patients in order to analyze their proteomic cargo and gain insight into the tumor EV secretome.

3 MATERIAL AND METHODS

3.1 Patient material collection and processing

Papers III and IV

For Paper III, blood was collected from healthy volunteers after overnight fasting. Blood meant for plasma samples was collected in K2 EDTA tubes while blood meant for serum was collected in clot activator tubes. Serum samples were allowed to rest at room temperature to facilitate blood clotting. Both plasma and serum were centrifuged at 1880 × g for 10 minutes at room temperature (RT) followed by a 2500 × g centrifugation for 10 minutes of the supernatantat RT. Samples were then subjected to EV isolation (as described below).

For paper IV, samples were collected from patients who had given written informed consent, and both study design and execution were approved by the Regional Ethics Committee in Gothenburg. Tissue specimen were excised during patient surgery where both tumor tissue and macroscopically normal-appearing mucosal tissue 10 centimeters from the tumor (non-tumor tissue) were sampled. In total, 20 tissue samples, one each of tumor tissue and non-tumor tissue from 10 patients, were collected. Once collected, tissue samples were weighed and then divided into 0.2 g pieces, and each piece was separately submerged in 2 mL of RPMI medium (Paper IV, Figure 1). They were then gently minced into smaller pieces of approximately 1 mm^3 and had DNase 1 (Roche) and Collagenase D (Roche) added to them at final concentration of 40 U/mL and 2 mg/mL, respectively. Enzymatic digestion was carried out at 37°C for 30 min under gentle agitation after which samples were filtered through 70 µm filters to separate vesicle-containing supernatant from tissue pieces, at which stage samples were combined to make a tumor sample pool and a non-tumor sample pool. Samples were then immediately subjected to EV isolation (as described below).

3.2 Cell cultures

Papers I and II

The human mast cell line HMC-1 (provided by Dr. Joseph Butterfield, Mayo Clinic, Rochester, MN, USA) was used as a model cell line in papers I and II. Fetal bovine serum (FBS) used for cell cultures was depleted of EVs through an 18 hours ultracentrifugation at 118,000 × g_{avg} (Type 45 Ti rotor, k-factor

178.6) at 4°C. Cells were grown in a humidified incubator at 37°C, with 5% CO_2 on IMDM culture media supplemented with 2 mM L-glutamine (HyClone), 100 units/ml penicillin, 100 µg/ml streptomycin (HyClone), and 1.2 U/ml 1-thioglycerol (Sigma Aldrich), and10% EV-depleted FBS (Sigma Aldrich). Seeding density was 0.5×10^6 cells/ml and EVs were harvested either three or four days later, after which cells were re-seeded for consecutive isolations. Culture viability was evaluated microscopically with Trypan blue staining before each EV harvest and was not allowed to drop below 95% viability.

3.3 EV isolation

Density media preparation

Density media was prepared using a stock solution of 60% Iodixanol (Optiprep, Sigma Aldrich) and different concentrations were prepared by combining Optiprep with buffers so that they all finally contained a final concentration 0.25 M sucrose, 10 mM Tris, and 1 mM EDTA in water.

Size exclusion column preparation

Sepharose CL-2B (GE Healthcare) was used for column preparation. Buffer was first exchanged by letting the Sepharose sediment after which stock liquid was aspired and replaced with 0.2 µm filtered PBS. This was repeated three times after which the washed Sepharose was packed into a Telos SPE column (Kinesis) to a final volume of 10 mL and a polyethylene frit was fixed on top. The column was equilibrated with 0.2 µm filtered PBS.

Paper I, II and IV

All samples except blood samples for paper III were subjected to an initial centrifugation at 300 × g for 10 minutes to deplete samples of cells. Supernatants were then carried over for subsequent isolation steps as follows below.

Paper I

All cell-free supernatants were subjected to a 20 min ultracentrifugation using a fixed angle rotor at 16,500 × g_{avg} at 4°C (Type 70 Ti (FA); k-factor: 950.6, Beckman Coulter) in order to deplete the supernatant of ABs, MVs, and cell debris.

For the rotor comparison part of paper I, AB, MV, and debris-depleted supernatants were filtered through 0.2 µm filters (Sarstedt) before further centrifugation. After filtration, samples were split from the main pool and centrifuged either for 70 minutes with a Type 70 Ti rotor at 118,000 × g_{avg} (k-factor: 133.7), 70 minutes with a SW 32 Ti rotor at 118,000 × g_{avg} (k-factor: 217.6) or 114 minutes with a SW 32 Ti rotor at 118,000 × g_{avg} (k-factor: 217.6) (Paper I, Table 1). All centrifugations were carried out at 4°C.

For the duration comparison part, only the FA rotor was used as described above except that the use of a 0.22 µm filter was omitted. For the final pelleting step, samples were centrifuged for either 70 minutes, 155 minutes, 4 hours, 11 hours or 37 hours (see Paper I for further details).

Paper II

Cell free supernatants were centrifuged at 16,500 × g_{avg} (Type 45 Ti, k-factor 1275.1) for 20 min to remove ABs and larger particles. Supernatants were carried over to fresh ultracentrifuge tubes and pelleted with a 118,000 × g_{avg} (Type 45 Ti, k-factor 178.3) centrifugation for 3.5 h. The resulting pellet was resuspended in PBS. Pellet EVs were then purified through a density gradient separation by mixing 1 ml of EVs with 3 ml of Optiprep stock solution and deposited on the bottom of a tube. A discontinuous density gradient was stacked on top of the samples by layering 1 ml each of 35%, 30%, 28%, 26%, 24%, 22%, 22% again, and 20% Optiprep solution, in that order. The gradient was centrifuged at 178,000 × g_{avg} (SW 41 Ti, k-factor 143.9) for 16 h at 4°C. After centrifugation, fractions of 1 ml each were collected from the top down and analyzed for vesicle presence through western blots. Fractions 2 and 3 were then pooled and diluted with PBS, and washed through ultrancetrifugation at 18,000 × g_{avg} (Type 45 Ti) for 3.5 h. The pellet was resuspended in PBS. All isolation procedures were performed in one go so as to avoid freezing of EVs.

Paper III

Three isolation strategies were employed – SEC only, density gradient with SEC and a combination of ultracentrifugation, density gradient, and SEC.

SEC only isolation: 1 ml of fresh plasma or serum was deposited on top of the premade column, and 30 fractions of 0.5 ml each were collected with filtered PBS as the elution buffer.

Density gradient with SEC isolation: 6 ml of plasma prepared as described above was on top of a layer of Optiprep cushion consisting of 2 ml 50%, 2 ml 30%, and 2 ml 10% Optiprep in that ascending order. The cushion was

centrifuged at 178,000 × g_{avg} (SW 41 Ti rotor, k-factor 143.9, Beckman Coulter, Brea, CA, USA) for 2 h at 4°C. Two visible bands formed, one at the intersection of 10% and 30% (the high-density band) and one floating on the very top of the tube (the low-density band). Both bands were separately collected as 1 ml with a pipette. They were then run on separate SEC columns as described above.

Ultracentrifugation, density gradient and SEC isolation: 40–80 ml of plasma pooled from several individuals was diluted in PBS. Larger EVs such as MVs were pelleted at 16,500 × g_{avg} (Type 70 Ti, *k*-factor 950.6) for 20 min and resuspended in PBS. The supernatant was further centrifuged at 118,000 × g_{avg} (Type 70 Ti, *k*-factor 133.7) for 2.5 h to pellet smaller EVs such as EXOs. The pellet was resuspended in PBS, and the two pellets were then pooled and diluted to a final volume of 6 ml. Density gradient and SEC was then preformed as described above.

Paper IV

Cell free supernatants were centrifuged at 2,000 × g for 20 min to remove any cell debris large EVs such as ABs. Supernatants were then centrifuged at 16,500 × g_{avg} for 6 min (TLA 100.3, k-factor: 404.5, Beckman Coulter) in order to pellet intermediate-sized vesicles. Supernatants were again centrifuged at 120,000 x g_{avg} for 65 min (TLA 100.3, k- actor: 55.5) to pellet smaller vesicles. Intermediate-sized and small vesicles were both resuspended in PBS and pooled. The pooled vesicles were bottom-loaded by mixing 1 ml sample with 3 ml of 60% Optiprep placed at the bottom of an ultracentrifuge tube. On top of this, 4 ml of 30% Optiprep and 4 ml of 10% Optiprep were carefully layered on top. The samples were then centrifuged at 97,000 × g_{avg} for 2 h (SW 41 Ti, k-factor: 265.1) at 4°C. A visible band floated at the intersection of 30% and 10% Optiprep and was collected as 1 ml.

3.4 Protein estimation

Paper I, II and III

Samples were lysed with 20 mM Tris-HCL and 1% SDS and then sonicated three times for five minutes each with vortexing in between. The total protein amount was estimated using a BCA protein assay kit (Thermo Scientific Pierce) as per the manufacturer's instructions.

Paper IV

Protein was estimated with the Qubit assay system (Thermo Fischer Scientific) as per manufacturer's instructions.

3.5 Nanoparticle measurements

Paper II, and III

Nanoparticle measurements were performed using a ZetaView PMX 110 (Particle Metrix), and data were analyzed using the ZetaView analysis software version 8.2.30.1. Minimum size was set to 5 and maximum size to 1000 with a minimum brightness of 20. For Paper II, the camera sensitivity was set to 70 and for Paper III it was set to 80. Two positions were measured three consecutive times for the samples in Paper II, while in Paper III 11 positions were measured.

3.6 Fluorescent microscopy

Paper II

EVs were deposited on a superfrost+ microscopy slide and allowed to adhere to the surface overnight at 4°C. Slides were washed with PBS and then blocked with 1% BSA in PBS for 30 minutes. Samples that were permeabilized were done so by the addition of 0.1% Tween-20 in PBS for 5 minutes followed by washing with PBS and then blocked with 0.1% Tween-20 in PBS. Primary antibodies against STX4, SCAMP3 or beta-actin were diluted in PBS containing 1% BSA and then incubated with samples for 1 h at RT. Samples were then washed and incubated with secondary antibodies also diluted in PBS containing 1% BSA. After incubation and washing, EVs were dyed with PKH225 Red Fluorescent Cell Linker Kit (Sigma Aldrich) and after washing samples were mounted and imaged with an Axio Observer microscope (Zeiss). The exposure time for the two channels evaluated was constant for all the samples. Computational analysis was done using the ZEN Blue and ImageJ software.

3.7 Electron microscopy

Paper I, II, III and IV

For details regarding electron microscopy, see material and method sections in each paper. Generally, for TEM a sample volume corresponding to 10 or 15 µg of protein was loaded onto formvar carbon-coated grids (Ted Pella Inc). Samples were fixed in either 2% paraformaldehyde and 2.5% glutaraldehyde or just in 2.5% glutaraldehyde and stained using 2% uranyl acetate. Electron micrograms were obtained using a digitized LEO 912AB Omega electron microscope (Carl Zeiss SMT, Mainz, Germany). For Cryo-TEM, EVs were plunge frozen using a Vitrobot Mk2 (FEI). Images were acquired using the TVIPS EMMENU 3.0 software and a TVIPS TemCam F224 camera on a FEI CM200 microscope.

3.8 Western blot

Paper I, II and III

EV protein extracts were separated by SDS-PAGE. Paper I utilized 10% polyacrylamide gels made in-house and were transferred onto nitrocellulose membranes. In Paper II and III, SDS-PAGE was done on commercially available Mini-Protean TGX precast 4–20% gels (Bio-Rad) and transferred onto PVDF membranes using a Trans-Blot Turbo Transfer system (Bio-Rad). All blots were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.05% of tween-20 (TBST). Membranes were then incubated overnight at 4°C with primary antibodies dissolved in 0.25% blocking buffer. The membranes were washed with TBST and then incubated with the secondary antibodies. After washing with TBST, the bands were visualized using either SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) or Amersham ECL Prime Western Blotting Detection Reagent (GE healthcare) using a VersaDoc 4000 MP (Bio-Rad). For antibodies and more detailed information, see individual papers.

3.9 PageBlue protein staining

Paper III

SDS-PAGE was performed as described above. Gels were incubated with PageBlue Protein Staining Solution (Thermo Scientific) for 2 h at RT with gentle agitation and afterwards washed three times with water. Gels were imaged with a VersaDoc 4000 MP imaging system.

3.10 Flow cytometry

Paper II

Antibody-coated magnetic beads were used to for all flow cytometry measurements. The CD63-coated beads were commercially purchased (Life Technologies AS or Thermo Fisher Scientific, essentially the same reagent). Beads coated with antibodies against STX4 and SCAMP3 were generated in-house using a bead conjugation kit (Dynabeads Antibody Coupling Kit, Thermo Fisher Scientific) according to the manufacturer's instructions. Antibody-coated beads and EVs samples corresponding to 15 µg of protein were incubated overnight at 4°C. After washing, the beads were incubated with human IgG antibodies followed by further washing and incubation with PE-labeled antibodies against CD9, CD63, CD81, or isotype control (BD Bioscience). Samples were washed again before analysis with a FACSAria (BD Pharmingen), and the results were analyzed using the FlowJo software (Tri Star).

3.11 ELISA

Paper III

SEC fraction 10, which had the highest particle measurements, was used as a reference sample from which a volume corresponding to 500 ng of protein was taken. The same volume was then collected from each other fraction as well and diluted with PBS to a final volume of 1 ml, of which 100 µl of each diluted fraction was deposited in the wells of a black-walled 96-well plate and incubated at 4°C overnight. The plate was then washed three times with PBS and then blocked with PBS containing 1% BSA at RT for 1 hour. After blocking, primary antibodies against CD9, CD63, and CD81 (Santa Cruz Biotechnology) were added at a 1:200 dilution and left to incubate at RT for 2

hours. Another three washes with PBS containing 1% BSA was performed after which secondary donkey anti-rabbit IgG HRP-linked F(ab′)2 fragment (1:2000 dilution) or sheep anti-mouse IgG HRP-linked F(ab′)2 fragment (1:2000 dilution) (GE Healthcare) was added and incubated at RT for 1 hour. Samples were washed four times with followed by the addition of BM Chemiluminescence ELISA substrate (Roche) and measurement of chemiluminescence on a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific).

3.12 Proteinase K treatment

Paper II

EVs were used at a protein concentration of 860 µg/ml and incubated with 20 µg/mL Proteinase K (PK) (Invitrogen) and 5 mM $CaCl_2$ in PBS for 1 h at 37 °C with gentle vortexing every 15 minutes. Enzymatic activity of PK was inhibited by the addition of 5 mM phenylmethylsulfonyl fluoride for 10 minutes at room temperature.

3.13 Trypsin/Lys-C digestion and biotin labeling

Paper II

EVs were incubated with a mixture of 20 µg/ml trypsin and 10 µg/ml Lys-C for 2 h at 37 °C, after which the sample was incubated with 20 mM EZ-Link Sulfo-NHS-Biotin (Thermo Fischer Scientific) for 30 min at RT. Excess biotin was quenched using 10 mM hydroxylamine. Separation was performed using FASP sample processing, and the samples were desalted with C18 spin columns according to the manufacturer's instructions.

3.14 RNA isolation and quantification

Paper I

RNA isolation was performed with the miRCURY RNA isolation kit Cell and Plant (Exiqon) as per manufacturer's instructions. Briefly, EV isolates were lysed with lysis buffer containing 1% 2-mercaptoethanol after which ethanol was added and samples were vortexed. Samples were transferred to spin

columns after which a sequence of washing steps were performed through the addition of wash buffers and centrifugations. Finally, RNA was eluted from the columns by the addition of elution buffer and was used either immediately or stored in -80°C. RNA quantity and profiling was performed with a Bioanalyser (Agilent Technologies) using an Agilent RNA 6000 Nano chip. See Paper I for details.

Paper III

RNA isolations were performed on both the high-density and low-density bands acquired after density cushion centrifugation as well as on pelleted pooled SEC fractions (see EV isolation section). SEC fractions were pooled six at a time forming the pools F1-6, F7-12, F13–18, F19–24, and F25–30, which were all pelleted at 115,000 × g_{avg} for 1 h (TLA-100.3 rotor, k-factor 52.8). The miRCURY RNA isolation kit-Cell and Plant was used to extract RNA. Of the density cushion bands, 300 µl was collected and a volume of 700 µl lysis buffer was added, while 300 µl buffer was added to the pellets of the pooled fractions. Samples were processed as described above in Paper I, and RNA quantity and profiling were performed with a Bioanalyser (Agilent Technologies) using an Agilent RNA 6000 Pico chip.

3.15 RNase treatment

Paper I

RNase-treated samples and non-treated controls were derived from the same pool and were thus paired. EV sample aliquots were diluted with PBS to a final volume of 95 µl to which 5 µl RNase A (Thermo Fischer Scientific) was added resulting in a final concentration of 0.5 µg/µl and incubated at 37°C for 20 minutes. Water was added to non-treated sample, which was kept on ice. RNA isolation was carried out as described above with the exception that 2-mercaptoethanol was added at a final concentration of 2% instead of 1% in the lysis buffer. Degradation of isolated cellular RNA was used as an efficiency control for the RNase.

3.16 Proteomics

Paper I, II and III

Detailed experimental procedures are presented in the material and methods section of each paper.

Sample preparations were performed using the Filter-Aided Sample Preparation (FASP)(198). In general terms, samples were lysed, reduced, and then placed on a filter (30 kDa MWCO). After alkylation, the samples were digested by two rounds of trypsin addition. Peptides were eluted and analyzed with either an Orbitrap Fusion Tribid (Paper II and IV) or with a Q Exactive mass spectrometer, both with on-line nnano-liquid chromatography systems.

Both Paper II and Paper IV had some general exceptions to the procedure described above. In paper II, trypsin treatment was shorter than in the other papers consisting of a 2 hours incubation followed by an additional 1 hour incubation rather than overnight and 2 hours incubations which were done for the other papers. Additionally, two injections per sample was performed for Paper II. For Paper IV, eluted peptides were labeled with TMT labels, and samples were combined and subjected to off-line pre-fractionation by basic reverse phase liquid chromatography. Paper II and Paper IV were semi-quantitative studies with Paper II relying on label-free quantitation and Paper IV relying on chemical labeling, while Paper III used an identification-only approach. Analytical parameters were set slightly differently in each study and are described in more detail in the methods section of each individual paper.

3.17 Bioinformatics and databases

Paper II, III and IV

Uniprot was extensively used for protein localization data. The Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) was used for gene ontology (GO) data. Venny was used to compare datasets for overlapping proteins (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Protter (http://wlab.ethz.ch/protter/) was used to visualize protein topology. The Qlucore software (Qlucore) was used to generate PCA plots and heatmaps representing correlations among proteins in Paper IV. Networkanalyst (https://www.networkanalyst.ca), the String database (https://string-db.org/) and Cytoscape were used to generate protein-protein interaction (PPI) networks.

4 RESULTS AND DISCUSSION

4.1 Centrifugation parameters influence EV isolation (Paper I)

Much of EV research published to date has used differential ultracentrifugation either as the sole method for isolation or in combination with other methods (69). Therefore a decent understanding of the principles by which ultracentrifugation works, and to be able to foresee what potential effects on the isolate a change in ultracentrifugation protocol brings with it, is of great importance. Detecting a certain lack of regard to the fact that the use of different rotor types prohibits accurate replications of protocols, we set out to demonstrate the difference in isolates between the use of a fixed angle (FA) rotor and swinging bucket (SW) rotor. Three different centrifugation schemes were designed where the FA settings was considered base line to which the SW rotor was compared (Table 2). Two protocols were designed for the SW rotor, one which copies the RCF and duration of centrifugation of the FA protocol and is considered to be unadjusted for the change in rotor, and one which copies the RCF but compensates for the change in rotor parameters through prolonging the centrifugation duration, and was thus considered as adjusted (See Paper I, Figure 1 for conversion).

Table 2. Rotor settings

Isolation setting	Rotor	RCF (average)	RPM	Time (minutes)	k-factor
FA 70 minutes	Type 70 Ti (Fixed angle)	118,000	40,000	70	133.7
SW 70 minutes	SW 32 Ti (Swinging bucket)	118,000	31,000	70	217.6
SW 114 minutes	SW 32 Ti (Swinging bucket)	118,000	31,000	114	217.6

Adapted from Paper I. The three different centrifugation settings used for the comparison of rotors. The FA 70 minutes setting was used as a reference to which the SW 70 minutes setting (which did not compensated for the change in rotor) and the SW 114 minutes setting (which compensated for the change in rotor by prolonged centrifugation duration) were compared to.

As expected, less RNA was isolated by the unadjusted SW protocol, likely due to the fact that the rotor has a higher k-factor and thus lower pelleting efficiency, while the adjusted protocol manages to pellet comparative amounts to that of the FA protocol (Figure 3). However, the unadjusted SW protocol pellets the same amount of proteins as the FA protocol, and the adjusted protocol pelleted significantly more. This is contrary to what a rotor conversion using the k-factor predicts, which highlights the imperfection of such predictions and the idea that they are not completely rooted in reality as there are more dimensions to be considered than purely the geometry of the rotor. Why the results appear as they do is not revealed by these experiments, but one could consider the positioning of the pellet during centrifugation and after, which would be the same for the SW rotor, but as the pellet is pressed to the side of the tube in the FA rotor it will no longer occupy the "gravitational" bottom at the end of the run. Thus impurities might slide away in a different way. More aspects could be considered, as the sample interaction with the tube wall, which might be different in the two rotors. Because proteins are considered to be a measure of total material pelleted and RNA a measure of EVs, looking at the ratio of protein to RNA it appears that the FA rotor produces purer EV isolates.

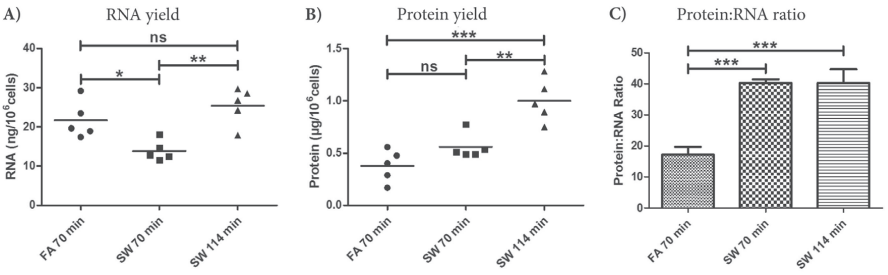

Figure 3. Rotor comparison From Paper I. Comparison of FA and SW rotors with regard to EV yield and purity. Yield is represented by RNA (A) and protein (B). The purity is interpreted by the protein:RNA ratio where a disproportionate increase in protein is interpreted as loss of purity (C).

We further wanted to investigate if the current gold standard of EV isolation, namely differential ultracentrifugation, was optimized for the task, and to some extent further demonstrate the impact of protocol changes on isolates. To that end, the FA rotor was used and isolations were performed with increasing centrifugation durations, which was escalated in half-log increments starting at 70 minutes and ending at 37 hours. As expected, both RNA and protein yields increased in accordance to increase in duration, and purity (as measured

by protein to RNA ratios) decreased (Figure 4). This speaks for the fact that a 70 minute isolation step does not manage to pellet all the components in the media and that one could easily increase the duration to 155 minutes without changing the protein to RNA ratio and thus purity of the isolates. Based on this, there seems to be no reason to keep to a 70 minute centrifugation when more can be gained by prolonging it to an etent with no apparent consequences as far as purity is concerned. When looking at the increase of RNA and protein across all durations, it can be concluded that as RNA yield starts to plateau after 11 hours of centrifugation, the protein yield just keeps rising. Looking at the yield in relation to the time spent centrifuging, one can see that most of the RNA is pelleted early on and that the additional yield gained after the initial 155 minutes is acquired at the cost of considerable increase in duration.

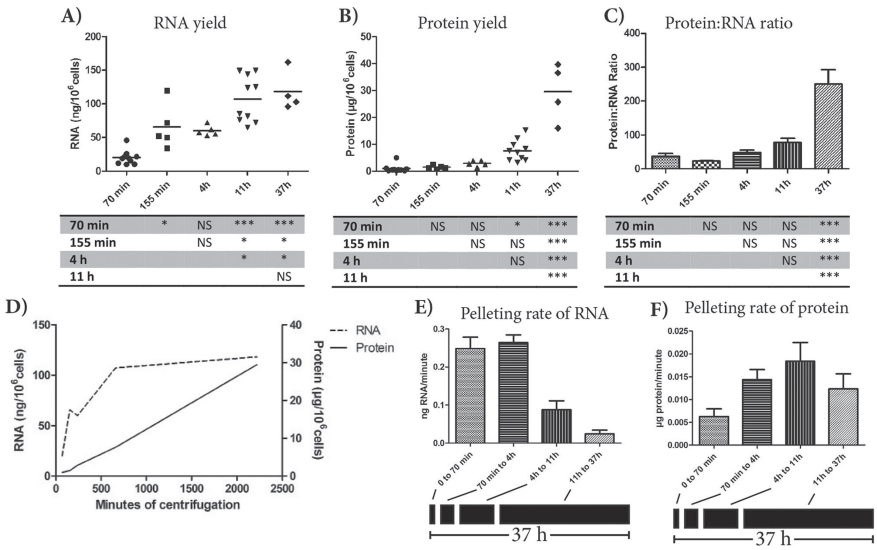

Figure 4. Impact of centrifugation duration. Both RNA (A) and protein (B) increase as longer pelleting protocols are applied. The purity of isolates is maintained for 70 and 155 minutes of centrifugation but decrease with longer durations (C). Protein yield steadily increases while RNA yield plateaus (D). The rate of RNA pelleting (E) decreases after 155 minutes of centrifugation, while the rate of protein pelleting (F) decreases much later on.

The maintained purity in the 155 minute pellet can be illustrated by western blot, showing that band intensities of EV markers are maintained in the 70 and 155 minutes samples when equal protein amounts are loaded. Longer centrifugations do not show the same band intensity and thus it can be assumed that contaminants such as soluble proteins make up a bigger percentage of the total pelleted protein. Although not evaluated, this might be permissible if

followed up with adequate cleaning steps and could speculatively result in higher yield and enrichment of smaller EV species. Taken together, these results highlight in particular some of the parameters that deserve consideration when isolating EVs. Something that on a similar note was demonstrated by Jeppesen et al., although with a slightly different approach (199). This study, and that by Jeppesen, both show the relevance of methodological optimization and that there is merit in re-evaluating methods when, for example, the EV source or centrifugation rotor is changed.

4.2 Distinguishing EV-proteins and non-EV proteins (Paper II)

As was shown in Paper I and has been continuously pointed out, differential ultracentrifugation is a crude isolation method and leaves much to be desired in terms of a pure EV isolate (82, 108, 200). The main contaminant from a proteomic perspective is the co-pelleting of soluble proteins from the medium. However, the density of vesicular structures differs from that of most proteins and this property can be used as the basis for the separation of the two. To this end, the pelleted sample can be loaded onto a density gradient and the solute allowed to migrate to its corresponding buoyant density. Here we seek to take a more detailed view at the floated vesicles that are presumably pure of co-isolated proteins. The strategy employed was two-fold, to either enzymatically digest or label with biotin the proteins not enclosed within a lipid bilayer, as these proteins were acessible to these two treatments. Running mass spectrometric analysis and comparing non-treated samples to either digested or labeled samples should thus give insight into which proteins are vesicular and which are co-isolated (Figure 5).

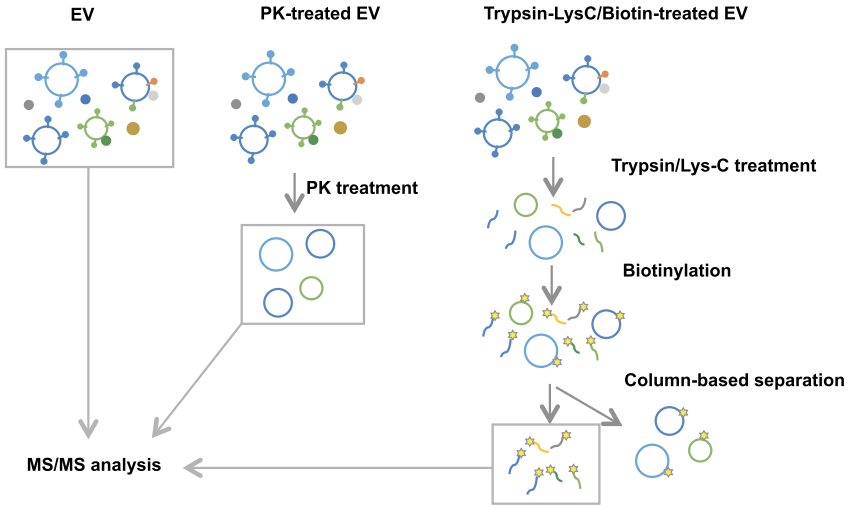

Figure 5. Schematic representation of study. EVs were treated with either proteinase K (PK) or with trypsin/Lys-C followed by biotin labeling with sulfo-LC-biotin. Biotinylated peptides were isolated by column-based separation. Non-treated EVs, PK-treated EVs, and biotinylated peptides were analyzed with LC-MS/MS.

In total, 1,956 and 1,784 proteins were identified in the non-treats and PK-treated samples, respectively, with 1,662 proteins being identified in both sets (Figure 6). Label-free quantification showed that among the overlapping proteins, 450 were more abundant in PK-treated samples, while 464 were more abundant in non-treated samples. The remaining proteins did not have a fold change above 2-fold and were thus considered as being similarly abundant in both samples. The interpretation of this data suggests that the proteins with decreased abundance following PK-treatment are proteins that PK has access to and are thus exposed on the outside of the EVs, while the proteins with increased relative abundance are not accessible and therefore are protected in the EV lumen by a lipid bilayer. The proteins unique for non-treated samples and the proteins unique for the PK-treated samples were automatically considered as being sensitive or protected from PK-treatment, respectively. The proteins were divided into categories that describe them as either being part of the EV proteome or as being part of the surface-accessible proteome (Fig 7D). Proteins that were more abundant in the PK-treated samples were considered to be a part of the EVs as were membrane proteins since these most likely were isolated because of their membrane association to a vesicle, and them being degraded by the PK likely means that they were displayed on the outside of the vesicle. Non-membrane proteins that were less abundant when treated with PK were further cross-referenced to the biotinylated non-membrane proteins. If a protein was found to be both less abundant as a result of PK treatment as well as being tagged with biotin, its positioning outside of

the EV was twice assured and was thus categorized in the group labeled "definite surface-accessible proteome". Those that did not overlap but were indicated to be outside of the EVs were grouped as "potential surface-accessible proteome".

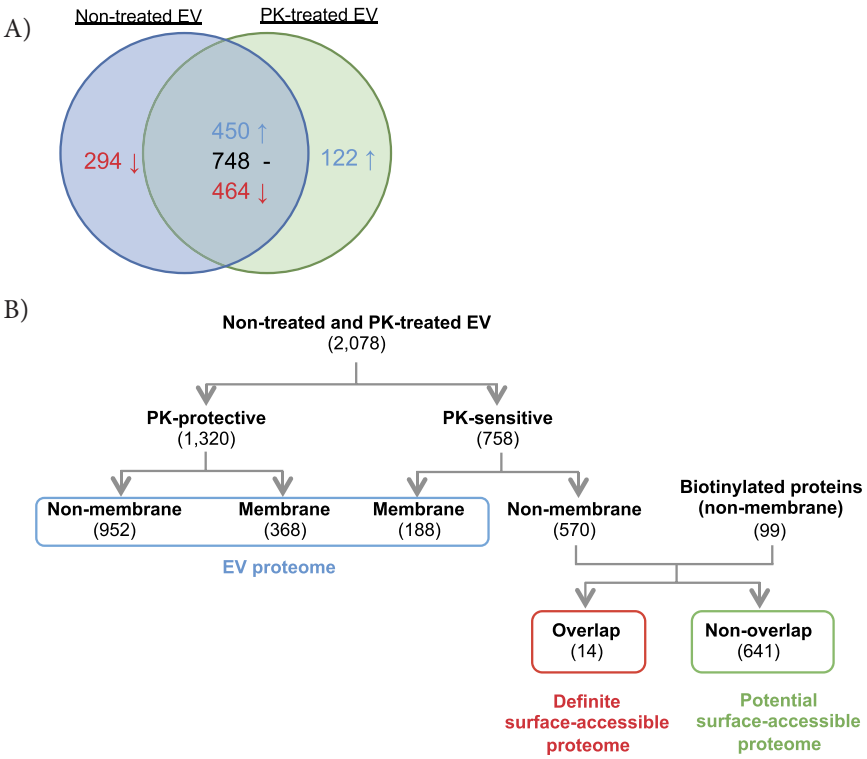

Figure 6. Defining the EV and surface-accessible proteomes. Venn diagram depicting the number of identified proteins in the non-treated and PK-treated samples as well as their overlap and 2-fold up- or down-regulation after PK-treatment (A). Proteins categorized as EV proteome or surface-accessible proteome (B).

Protein localization of the EV and surface-accessible proteins according to Uniprot differed to a degree in that the EV proteome contained more proteins localized to membrane, cytosol, endosome, and endoplasmic reticulum, while the surface-accessible proteome had more proteins localized to the nucleus and secreted proteins. The two groups also differed in gene ontology with regards to biological processes with the EV proteome being more enriched in terms such as protein localization and vesicle-mediated transport, which is more easily tied to EV biogenesis and processes. A couple of protected and surface-accessible proteins were validated with western blot. The proteins flotillin-1 and TSG101 were predicted to be protected, while GAPDH, STUB1, histone

H1, and PCNA were predicted to be surface-accessible. As it appears, the whereabouts of a protein in the isolates is not necessarily dictated by the available data on its cellular localization. Proteins belonging to different cellular compartments as well as cytoplasmic and secreted proteins, which should in theory be either inside or outside of the vesicles, are according to the data found on both sides of the vesicular membrane. Differential ultracentrifugation is a relatively crude method of isolation, and contaminants are to be expected in the pellet. The floatation on a density gradient should clean this up. However, the pelleting could potentially force the formation of aggregates of vesicles and/or proteins (201). Such complexes could potentially float at the EV density and thus explain the surface-accessible proteome present in the isolates. Even though the word "contaminant" is often used to describe these proteins, it is not possible to deduce their nature based on the data presented here as their EV association could be more than experimentally induced (202).

A peculiar pattern presented itself when the data were evaluated on the peptide level as some membrane proteins in the PK-treated sample were represented disproportionally by their extracellular peptides rather than their cytoplasmic peptides. This should theoretically have been the other way around considering which portion of the proteins that PK has access to. This prompted further investigation, which led to the development of a simple scoring system. Proteins that were either transmembrane proteins or were associated with the membrane through a lipid anchor were taken into consideration (Fig 10A). Only peptides with a 2-fold difference in abundance between non-treated and PK-treated samples were scored. If an extracellularly located peptide was more abundant in the PK-treated sample, it was given a score of −1, and if a cytoplasmic peptide was more abundant in PK-treated sample then it was scored with +1. Likewise, a peptide that was more abundant in the non-treated sample was scored with +1 if located extracellularly and −1 if found cytoplasmically. Biotinylation was similarly scored with cytoplasmic biotinylation earning a score of −1 and extracellular biotinylation earning a score of +1. The cytoplasmic and extracellular localization of course corresponding to the lumen and extra luminal space in this case since EVs are the entities being investigated. Scores were added together, and a final positive score was taken as an indication that the protein retained a conventional topology while a negative score indicated a controversial one. All in all, four proteins (SCAMP3, STX4, SLC12A6 and leukosialin) earned a negative score, which was confirmed by both PK treatment and biotinylation (Figure 7). Additionally, 139 proteins earned a negative score from just one of the methods, while 5 proteins received conflicting scores from both methods.

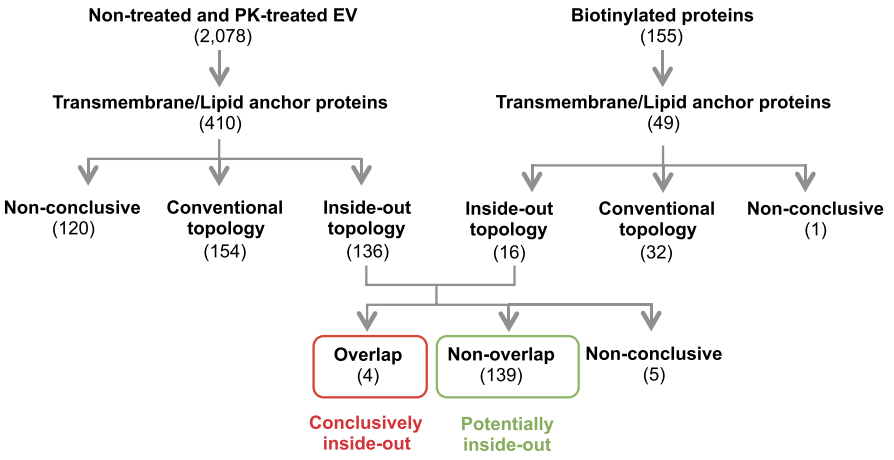

Figure 7. Scheme illustrating the selection criteria for determining topology of membrane proteins. Proteins were categorized as either "conclusively inside-out", "potentially inside-out", or "non-conclusive" according to the scoring system.

One out of those four proteins as well as one, STX4, for which only one approach showed a reversed topology were validated for their reversed topology by flow cytometry using bead capturing with antibody-coated beads specific for the cytoplasmic part of the proteins and a second validation using an ELISA system with the same antibodies.

The proteins displaying unconventional topology is a puzzling discovery to say the least. However, it is not an entirely new prospect that membrane proteins can attain more than one topological conformation, and the case has been made for the existence of dual stable topological insertions as well as dynamic topologies as reviewed by von Heijne (203). What caused this topological anomaly in the vesicles investigated here is as yet unexplored, as are the potential functional implications of such proteins.

Much of the topological information provided by this study defies our preconceptions of the whereabouts of proteins in relation to the vesicles. Thus it is worth keeping in mind that the wealth of information we have on cellular proteins, which forms the ground for our predictive powers in terms of their subcellular localization and membrane orientation, does not necessarily apply to vesicles. By extension, the same should be considered in regards to other vesicle-borne molecules whose characteristics in the vesicle might not conform to what has previously been reported in cells.

4.3 The proteome of tumor tissue resident EVs of colorectal cancer patients (Paper IV)

The realization in the past decades that EVs play a crucial role in cell-to-cell communication and, as such, that they might play a major role in cancer biology has prompted significant growth in this field. Numerous cancers have already been studied from the perspective of EV biology (51, 179, 204-207). Colorectal cancer is one of the most prevalent cancers worldwide (wcrf.org, January 2019), and a deeper understanding of the disease mechanism is of vital importance. Thus, we set out to investigate the extracellular vesicular proteome of the tumor tissue microenvironment. To this end, tumor tissue and macroscopically appraised healthy-looking tissue (non-tumor) was collected and subjected to an EV extraction and isolation protocol followed by relative quantitative proteomics by TMT-labeling and mass spectrometry. This resulted in two separate sets (Set 1 and Set 2) containing paired tumor and non-tumor samples (Figure 8).

Taking the relative quantitation into account, we set a cut-off to distinguish relatively up or down-regulated proteins. A 2-fold change in relative abundance as well as a p-value of 0.05 or lower was chosen. This cut-off set apart 399 proteins in Set 1 as differently expressed in EVs and 592 proteins in Set 2. Amongst the overlapping proteins found in both sets, 125 met the criteria for the cut-off in both sets. The assertion of their misregulation in the tumor tissue-derived EVs of colorectal cancer patients was thus doubly confirmed. Some of these proteins were collagen alpha-1 (XII) chain (COL12A1), protein arginine deaminase, type IV (PADI4), and RNA cytidine acetyltransferase (NAT10), as an example. These have been implicated in colorectal cancer one way or another (208-211). COL12A was recently suggested to be associated to poor prognosis and has been implicated in other cancer types as well and associated to drug resistance (208, 212). PADI4 has been implicated in cancers and can both regulate transcription by histone modification as well as indirectly repress p53 activity (209). NAT10 has been suggested as both a marker and as a therapeutic target because it is able to increase motility in colorectal cancer cells and to promote EMT (210, 211). However, prolyl endopeptidase FAP (FAP) and prostaglandin G/H synthase 2 (PTGS2), which were the proteins with highest positive fold change in Set1 and Set 2, respectively, were not found to make the cut-off in both sets. PTGS2, having been previously associated with poor prognosis in cancer, could only be identified in Set 2, and FAP was present in both sets (213, 214). Even though present in both sets, FAP only made the cut-off in Set1. The reason for this was that one of the samples deviated in fold-change, thus forcing the p-value above the cut-off limit. To

not lose the proteins which showed a steady trend of being up-regulated, a second criterion was devised to include those proteins that made the first cut-off in one of the sets and that among all of the 10 patients (5 in each set) at least 8 of them showed a 2-fold fold upregulation in tumor EVs. Through this sorting, FAP was included, as were several other proteins, such as DEFA1. Both FAP and DEFA1 play a role in cancer, where FAP has been shown to promote angiogenesis and is correlated with poor prognosis while DEFA1 has been observed at elevated levels in colorectal cancer patients (215, 216). Also, strong positive correlations could be seen between FAP, COL12A1 and FN1 (fibronectin) as well as between DEFA1 and PADI1. FN1 has previously been found on breast cancer-derived EVs and has been proposed to be a potential biomarker candidate (217). Together, these proteins might present a lucrative panel with potential prognostic value. As these are just the tip of the iceberg, it will be interesting to see what other potential signatures might be hiding in the dataset.

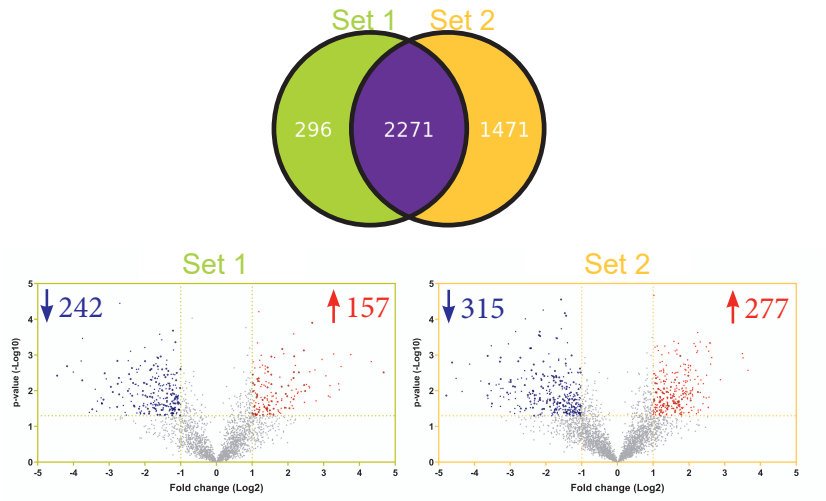

Figure 8. Quantitative difference of proteins in tumor tissue-derived EVs. Venn diagram showing the number of identified proteins in Set 1 and Set 2 and the overlap between them as well as volcano plots with the cut-off at p-value 0.05 and fold change of 2 between tumor-derived EVs and non-tumor-derived EVs. Red = Increased in tumor EVs. Blue = Decreased in tumor EVs.

A more general analysis of the data was made as well through a PPI map. By studying the interactions in a dataset, one can potentially pick out machineries or processes that are represent in the dataset. Such a PPI network was constructed with only the proteins found in both Set 1 and Set 2. Two clusters were clearly formed, one consisting of upregulated ribosomal subunits and one

consisting of downregulated components of the mitochondrial electron transport chain and these clusters were dominant characteristics of the EV proteome in this dataset. Interpreting this was made all the more difficult by the fact that EVs were the subject of studiy. If they mirror the cells, meaning that the same expression patterns are true at a cellular level as well, this would put the tumors at a state of high activity but low energy. An explanation for such a cellular state can be found in what is known as the Warburg effect which is a cellular mechanism that is observed in some cancers where tumor cells shut down mitochondrial respiration and increase glycolysis (218). Although no clear indication of elevated glycolysis was found in the EVs, this could be tied to the elevated ribosomal activity as it has been proposed that the Warburg effect is a strategy by which cells gain more raw material for the biosynthetic processes needed for growth (218). However, rather than being a reflection of the parent cell, the EV composition might just as well be a consequence of intracellular trafficking. Thus, without context, it's difficult to draw a conclusion. All in all though, these data demonstrate a fundamental difference between secreted EVs from tumor tissues and those secreted from non-tumor tissues.

4.4 The proteome of purified blood-circulating EVs (Paper III)

Blood samples are likely one of the most common biofluids collected in a clinical setting. It is relatively non-invasive and can be used for a myriad of tests and is thus used in the clinic on a routine basis (219). The fact that blood carries circulating vesicles makes this particular biofluid interesting not only from a cell-communication standpoint, but also makes it a very attractive source for potential biomarkers (192). Working with blood brings about a number of complications though, especially from a mass spectrometric perspective, where the extreme complexity and dynamic range pose a formidable obstacle (220). Thus, by isolating vesicles from blood, both the range and complexity is likely to be greatly reduced and might allow for a more relevant and deeper analysis. There are, however, further complications when it comes to the isolation of EVs from blood because EVs share many characteristics with other circulating lipid particles, which also happen to be characteristics bt which EVs are usually isolated, namely size and density. Lipid particles in the vascular circulation include lipoproteins such as high-density (HDL), low-density (LDL), and very low-density (VLDL) lipoproteins as well as chylomicrons that overlap with EVs with regards to size

(Chylomicrons and VLDL) and/or density (HDL) (221, 222). Two popular purification methods, density floatation and SEC, are thus not able to purify blood-derived EVs by themselves, but if used in succession a two-dimensional separation should theoretically be able to eliminate contaminating blood particles (Figure 9).

To test this hypothesis, EVs were purified from plasma and serum first through SEC alone. As expected, eluted fractions that were most abundant in EVs also contained a large amount of contaminating lipoproteins as shown by western blots and EM. Mass spectrometry of these fractions identified only a few proteins, most of them common components of chylomicrons and LDL/VLDL, which speaks for a high abundance of contaminants in relation to EVs. This confirmed the inadequacy of using only a size-dependent isolation strategy. Therefore we next preceded the SEC with a density cushion centrifugation. This approach, apart from introducing another separation criteria, also allows for a larger starting volume as its not restricted to the 1 ml volume that is the limit for the commonly used EV SEC columns (77). When using the cushion, two bands formed, one at the top of the tube representing a low-density lipoprotein-enriched fraction and one at the intersection of the 10% and 30% Optiprep layers indicating a high-density vesicle-enriched fraction. These fractions were run on separate SEC columns, and analysis of the eluted particle-rich peaks in each sample showed a much higher abundance of particles in the low-density sample. Flotillin-1 and TSG-101, used as indicators of EVs, were found in both the low-density and high-density fractions, although TSG101 displayed a much lower abundance in the high-density fractions as compared to the low-density fractions. Apo-A1, used as a marker for lipoprotein particles, showed a much stronger presence in the low-density fractions compared to the high-density fractions.

These results might be indicative of both a high-density and low-density EV subpopulation. Alternatively, some EVs might be trapped by low-density lipoproteins through unspecific interactions. The presence of Apo-A1 in the low-density sample indicates that this is the less EV-pure of the two samples. EM further supported this notion because electron tomograms appeared much cleaner and void of structures indicative of lipoprotein particles in the high-density sample, while these were present in abundance in the low-density sample. Mass spectrometric analysis of two particle-rich fractions from the high-density band identified approximately 600 proteins in each band with many proteins commonly detected in vesicles. The two-dimensional separation procedure can be scaled up by concentrating plasma samples through pelleting with ultracentrifugation followed by density cushion and SEC. Mass spectrometry of EV-containing fractions identified more than 1,000 proteins

seemingly enriched for EV proteins as indicated through gene ontology and a good overlap with the top 100 identified proteins in EVpedia. To our knowledge, this represents a hitherto unprecedented identification count in blood-derived EVs.

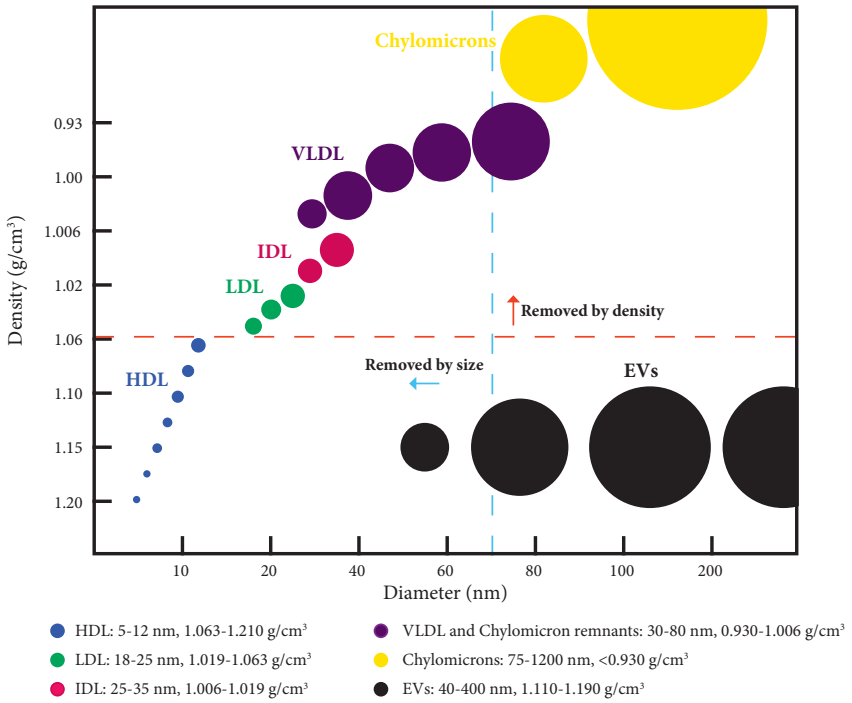

Figure 9. Theoretical overview of blood micro particles in two dimensions. The diagram demonstrates the principles on which EVs could be separated from other particles. Using a density-based method accompanied by a size-based method of separation will distinguish EVs from many other unwanted particles, and higher purity will be attained than any one method by itself could produce.

Taken together, this work demonstrates the importance of proper isolation strategies when working with blood samples. Furthermore, it shows how mass spectrometric analysis can capitalize on the reduced dynamic range of the isolated vesicles and really shows the benefit in readout when higher EV purity is achieved. As we close in on purer samples and more relevant readout, we simultaneously approach the state in which blood can be used as a biofluid for the screening of blood-borne EV biomarkers.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

Isolation and purification are at the heart of EV research. Sometimes only the former is used, and even though the point of stringent methodology has been advocated for half a decade now we still see work being published that fall short of the standard that the EV community in general expects (111). Granted, this might be due to projects that started out with outdated standards ant that have lingered too long before being published. The results of Paper I highlight the crude nature of differential ultracentrifugation with regards to the low resolution that is achieved between EVs and soluble proteins. This poor resolution also extends to EV subtypes (223). Another flaw of ultracentrifugation is the possibility that vesicles can be damaged as a result of harsh treatment though the high forces that act on the vesicle, the vesicles' interaction with the tube wall causing shearing (although evidence of this is lacking), and their pelleting, which also causes aggregations (91, 201). To remedy some of these concerns, a density cushion can be deposited at the bottom of the tube to provide the vesicles with a "soft landing" and to prevent pellet formation, arguably causing less aggregation (224). Indeed it is of a personal opinion that more researchers will probably come to adopt this method of cushioned ultracentrifugation – partly because there seems to be an overshadowing concern regarding the effect of pelleting and partly because doing so does not confer any cost increase in equipment and only a minute one in reagents. However, in the spirit of Paper I, it would be prudent to adopt the method cautiously and to evaluate the performance of the method in relation to rotor type, centrifugation duration and speed. Especially since Optiprep is a self-forming density medium, and its behavior subjected to these factors.

As Paper I demonstrates, longer centrifugations can be used to achieve higher yields. Alternatively, duration can be substituted by centrifugal force, or a compromise of the two can be applied. As shown, this can be done to a certain extent with no apparent cost of purity. What this illustrates is that the rate of EV pelleting does not taper off during such a prolonged centrifugation, meaning that shorter durations are inefficiently designed to displace all the EVs in solution to the pellet. Additionally, through a subsequent density purification, such contaminants that do pellet along with the EVs can be largely removed, but perhaps not completely, as Paper II hints at. A surprising amount of proteins succumbed to enzymatic degradation, and what is more surprising is that many of these supposedly should have been localized to the vesicular lumen (such as cytoplasmic proteins). In hindsight of Paper I and Paper II, one wonders if the vesicular proteome as we see it today is infected with incorrect

data. If that is the case, then to what extent might this be? In Paper IV, we isolated EVs from the tumor microenvironment, and the protocol included density-based purification. Mass spectrometry revealed a set of proteins specifically enriched in EVs from diseased tissue as compared to healthy tissue, which offers exciting opportunities for continued research in the project. But in the light of Paper II, one has to pose the question whether or not these enriched proteins are purely vesicular or if they are present as contaminants. It would not necessarily diminish such proteins as functional components of the disease or as potential biomarkers, but it could lead to the wrong conclusions being drawn. Also, there is a more philosophical discussion of what actually constitutes a vesicular protein and if the term "contaminant" is actually warranted in all of the cases.

Paper IV, which is in preparation, suggests numerous opportunity for improvement and further research. The results can be viewed as descriptive and preliminary at this stage and will likely be built upon in the future. Functionality could, for example, be tested on some of the highly upregulated proteins in EVs from tumor tissue. Perhaps most interesting would be the functionality of FAP as it could be involved in tumor progression (225, 226). Moreover, its suitability as a potential circulating EV-biomarker, using its enzymatic activity as a readout, could be a lucrative prospect to pursue (227, 228). As another example, the PADI4 functionality could be investigated regarding its ability to citrullinate target peptides and perhaps even regarding its ability to elicit some function in recipient cells (229). Furthermore, patient plasma could be investigated for the circulation of tumor derived vesicles (197). With the improved purity that can be attained by the combination of density and size separation as shown in Paper III, a deeper insight into the EV proteome might very well distinguish discrepancies between healthy control subjects and colorectal cancer patients.

It is through the refinement of both isolation methods and analysis methods that we can gain a deeper understanding of vesicular biology. As this refinement progresses, so will our ability to handle more demanding samples, such as those high in contaminants, low in volumes, and low in vesicles. As a consequence, it is likely that fewer model systems, such as cultured cell lines, will be used in the future and that these will be substituted with more relevant clinical samples. As far as proteomics is concerned, better separation techniques that produce a purer isolate will likely allow for smaller samples to be analyzed and more relevant results to be attained from them.

All things considered, the future of the vesicle field seems a positive one. Its growth and maturation is tended to by a highly dedicated community of

researchers who work for the betterment of the field. The coming years will no doubt see much exciting progress in the field in general, but especially so in terms of biomarker discoveries and clinical applications.

ACKNOWLEDGEMENT

"He drew his sword and, with a smooth overarm throw, completely failed to hit the troll."
-Terry Pratchett, The Color of Magic

Well, in spirit I would say that the quote reflects me as a PhD student pretty accurately. Those who have read the book, or take it upon themselves to do so, will find that the events described in this short sentence are uncharacteristic of the protagonist performing them, as he is far more likely to run from a threat than he is to face it. In this regard (and most others as pertaining to his personality and "skill") I feel that I identify with the character. The reason why I haven't run from my "troll", that is, my PhD, is undoubtedly because of the many people around me who have made the hard times that much more bearable, and the good times that much more illustrious. Thus I would like to offer my sincerest thanks and say a few words to the following:

Cecilia, my dear supervisor, it somewhat ails me to proclaim my honest opinion of you, because it can contain nothing but praise for you, something of which you usually self-medicate to the point of overdose☺. First off I have to tell you that you are nothing short of a miracle worker! Making a PhD out of me must have been the equivalent of forcing a reluctant elephant through the eye of a needle. An impossible task to say the least. As a supervisor you have excelled in every possible way! I can't thank you enough for your support throughout the years and especially for your patience (something which I despite my best efforts have failed to rob you of). I'm truly grateful for my time as your pupil and I lament that it now comes to an end. I couldn't have wished for a better supervisor nor will I likely ever find one as exceptional as you. Thank you for taking such good care of me.

Jan, my previous main supervisor and now co-supervisor. Thank you for your guidance and ability to instigate excitement in our projects. You tread through research with bold steps and you have made me realize that this is an attribute I myself need to adopt more of. I also want to thank you for giving me the opportunity to work at KRC and for bringing in so many great colleagues from all over the world whose friendships I have greatly enjoyed.

Johanna, my other co-supervisor. I am so glad to have seen your career take of at such a great pace. Your personality, with your unflinching moral integrity, serves as an example to us all and reminds us to be true to ourselves. Thank

you for the time we worked together, during which your positive energy brought me from the abyss countless times.

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Klaran, till dig har jag mer att säga än till någon annan. Men det här är ju inte rätt forum för kritik direkt ☺
Du har fått stå ut med mycket du Klaran, och då menar jag inte bara min plågsamt dåliga humor. Trots alla motgångar du mött, som har varit både tunga och frekventa, har du alltid funnit kraft att stötta mig när jag behövt det som mest. Du har en förmåga att säga precis det jag behöver höra och när jag behöver höra det. Med ditt stöd har jag haft mod nog att möta utmaningar jag annars hade flytt från. Med din kärlek har du fått pli på cynikern som så gärna vill krypa fram i mig. Tack för att du finns där för mig. Och Klaran, p. pp. p. Jah då säger vi så!

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