Isolation and characterization of exosomes from bovine milk

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Exosomes are extracellular vesicles of endosomal origin with a diameter of 30-150 nms that contribute to cell to cell communication and may be involved in interspecies communication on the nano-level. Exosomes contain wide array of bioactive components from micro RNAs to proteins and bioactive lipids. Milk is a unique platform of information from mother to infant in mammals with unique content of growth factors and immunogens. Some of these messages of biological programming can be transferred via exosomes.

Bovine milk contains vesicles of all sizes in very high concentrations. The abundance of vesicles in milk provides a platform for commercial scale purification of bioavailable extracellular vesicles, including exosomes. Isolation of exosomes from such complex media as milk provides challenges with high fat and protein content with similar physiochemical properties as milk exosomes. Here we demonstrate an efficient protocol for isolation of milk exosomes with the use of differential centrifugation followed by secondary purification with size exclusion chromatography or sucrose gradient centrifugation. Resulting samples were analysed for exosome enriched proteins with western blot for ALIX, TSG101 and CD81. Further analysis was done with mass spectrometry to identify the complete protein profile of isolated vesicles that included also exosomal marker proteins HSC70, CD81 and CD9.

Key words

Exosomes, western blot, bovine milk, mass spectrometry, chromatography

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1. Introduction

Exosomes are extracellular vesicles (EV) with a diameter of 30-150 nms that form in multivesicular bodies (MVB), a certain type of late endosome with multiple inner vesicles that form by inwards budding of the endosome membrane. Exosomes were first characterized by Bin-Tao et al. when they visualized the secretion of ferritin receptors in EVs in the maturation of sheep reticulocytes *in vitro* utilizing ^{125}I- labelled anti transferrin receptor antibodies. (Bin-Tao Pan, Kathy Teng et al. 1985). This was thought to be a garbage disposal system for the cells to remove no longer needed components. It was afterwards discovered that exosomes are important part of the cell to cell communication with various functions such as transporting RNA and various proteins from cell to another along with bioactive lipids in their membranes (Sjöstrand, Ekström et al. 2007, Michel Record, Kevin Carayon et al. 2013).

Exosomes are present in multicellular organisms in all bodily fluids such as, blood (Caby, Lankar et al. 2005), urine (Trairak Pisitkun, Rong-Fong Shen et al. 2004) and breast milk (Admyre, Johansson et al. 2007) and the secretion of exosomes has been characterized from several different cell types as stated in recent review articles (Simpson, Jensen et al. 2008, Simpson, Lim et al. 2009, Sedgwick, D'Souza-Schorey 2018). Exosomes are not spontaneously budded vesicles from the cell membrane but precisely secreted from MVBs when MVBs fuse with the cell membrane. However the differentiation between the two vesicle groups is often difficult, since both groups can contain similar membrane lipid profile, surface proteins and can contain similar intravesicular cargoes.

Formation of exosomes is conserved in mammals and plants and homologous proteins are found in archaea to induce membrane budding.(Brooke L. Deatherage, Brad T. Cookson 2012, Micali, Neumann et al. 2011, Regente, Pinedo et al. 2012) Bacteria have been shown to secrete membrane enclosed vesicles with yet unknown mechanisms. (Brooke L. Deatherage, Brad T. Cookson 2012) This universality of exosome mediated cell signalling could be a platform for interspecies communication with highly conserved and sophisticated mechanisms. The system includes similar mechanisms that some viruses (such as HIV) utilize in infections. (Guillaume Van Niel, Gisela D'angelo et al. 2018) Exosomes or exosome-like vesicles have been also isolated and characterized from numerous plants such as Arabidopsis, rice, carrot, and several citric

fruits. Most of plant exosome studies are considering MVBs and exosomes as a pathogen defence mechanism and as a form of unconventional protein secretion (Hansen, Nielsen 2017).

The protein composition of exosomes varies along with the cells of which the vesicles are secreted from (Smith, Lee et al. 2015) and the influence of exosomes to the recipient cells is widely unknown. This variability in the molecular composition may provide insight to possible disease related and systemic biomarkers and could lead to invention of new diagnostic tools. Variability also provides the possibly to use exosomes as bioengineered vesicles to transport molecules to specific tissues with high specificity and biocompatibility. (Suresh Mathivanan, Justin W. E. Lim et al. 2010) This novel concept of engineered biological nanovesicles is a promising possibility as a transport system for delicate and unstable components to cells.

1.1 Biogenesis of multivesicular bodies

Exosomes form inside endosomes during inwards budding of the endosome membrane thus creating a multivesicular body. This budding can happen two separate ways; independently and dependently of endosomal sorting complex required for transport (ESCRT). Dependent mechanisms require cargo sorting and clustering by ESCRT complexes 0 and I which lead to budding of specific vesicles by ESCRT II and scission of that vesicle by ESCRT III. Independent mechanism for vesicle budding is facilitated by spontaneous or syntenin guided clustering of cargo which is followed by cytoplasmic sorting and association with endosome membrane bound proteins. The resulting cluster is then spontaneously released by vesicle budding of membrane that is enriched with ceramides. (Guillaume Van Niel, Gisela D'angelo et al. 2018)

There are 5 ESCRT complexes each with specific protein composition and function. Complexes are ESCRT-0, -I, -II, -III and VPS4 complex (Vacuolar protein sorting). (Schmidt, Teis 2012) The following ESCRT proteins are described as named in yeast for simplicity, respective mammalian proteins can be seen in review by Hurley (Hurley 2015). ESCRT 0 is composed of heterodimer of proteins Vps27 and Hse1 that associate on flat clathrin-coated domains or phosphatidylinositol-3-phosphate (PI(3)P) rich membrane and recruit ubiquitinated (Ub) proteins by five Ub binding domains (Schmidt, Teis 2012, Hurley 2015, Hurley, Emr 2006, Hanson, Cashikar 2012). This localization is done by FYVE domain in the complex that has specific zinc finger

domain that recognizes PI3P and LIEF or LIEL peptide sequence that binds to clathrin β -propeller domains. (Hurley, Emr 2006) The 0-complex recruits ESCRT I complex by binding directly to amino terminus of Vps23 (TSG101, tumour susceptibility gene 101, in humans) by carboxyl terminal P(T/S)AP motif. 0-complex is not present in plants which therefore utilize a different mechanism for starting of MVB pathway. (Schmidt, Teis 2012)

ESCRT-I is a hetero tetrameric protein complex with one copy of each Vps23, Vps28, Vps37 and Mvb12. Multiple binding domains are located on the stalk like structure of Vps23 and Vps37, among these are the PTAP motifs of HIV-1 gag proteins. On the other end is the linker for the ESCRT-II complex that binds to Vps28 carboxyl terminal helical domain via GLUE (Gram-like ubiquitin) domain in Vps36. (Hurley, Hanson 2010) ESCRT-I complex along with –II is able to induce the inward budding of the membrane. (Schmidt, Teis 2012)

ESCRT-II is another tetrameric protein complex with 2 subunits of Vps25 and one of Vps22 and Vps36. The proteins form a Y-shape structure that has extending binding sites for ESCRT-I domain and for ESCRT-III subunit Vps20. The interaction with complexes I and III is characterized by specific protein motives that bind specific sequences in each counterpart. GLUE domain is responsible for binding to PI3I and ubiquitin. (Hurley, Hanson 2010, Hurley, Emr 2006)

Final ESCRT complex III consists of multiple subunits of Vps proteins. Vps2, Vps20, Vps24 and Snf7 are mandatory for complex function in yeast. (Hurley, Emr 2006) These core proteins are accompanied with accessory proteins Did2 Vps60 and Ist1 in yeast and 9 more proteins in humans. (Hurley, Hanson 2010) Vps20 protein is myristoylated and the myristylation could link the complex to the membrane, however during isolation the complex is found to be cytosolic and not membrane associated. (Hurley, Emr 2006) The ESCRT III complex is highly stable once associated to a membrane and will remain intact until Vps4 hydrolyzes ATP to disassembly the complex. (Hurley, Emr 2006) Vps4 complex is inactive in monomer or dimer form and recruitment by separate complex drives the assembly to ESCRT-III complex interaction. The interaction between complexes is most likely done by microtubule-interacting and transport-motifs. (Schmidt, Teis 2012).

The functionality and interaction of ESCRT machineries is not completely understood and analysis of different subunits by knockout mutants can lead to cell death. Yeast models are most used for studies but present similar difficulties as higher organism models. Initial budding is started by ESCRT I and II complexes in unilamellar vesicle studies *in vitro.* These complexes localize to the neck of the budding membrane. Similar effect can be achieved my ESCRT III complex proteins Snf7 and Vps20 but in much higher concentrations; 15 nM for I and II and 600 nM for Snf7. (Hurley, Hanson 2010) This curved surface provides a platform along with ESCRT II complex for ESCRT III complex formation and scission of membrane bud. In yeast Snf7 is only mandatory protein for membrane bud scission with other proteins provide the interaction platform for the attachment of possible ESCRT III fiber-structure. (Schmidt, Teis 2012, Hurley, Hanson 2010, Hanson, Cashikar 2012) ESCRT machinery is heavily involved in many other membrane associated functions such as cytokinesis (ESCRT III, Vps4 and ESCRT I along with ALIX), viral budding (same as in cytokinesis), membrane lesion repair and nerve cell proliferation among others. (Hurley, Hanson 2010)

1.2 Protein composition and sorting of cargo

Protein composition of EVs varies one vesicle to another with major differences between different sources of EVs. This great variance between vesicles provides challenges and equal possibilities in analysis of EVs. Liquid biopsies are a rising trend with more sensitive and non-invasive analytical methods to screen for disease and systemic condition biomarkers. Exosomes could provide the next level of information with the unique protein, nucleic acid and lipid profiles that could be connected to the systemic state of patient. However, much more research is needed to confirm unique biomarkers and validate the source of such biomarkers to the cells in distress or afflicted by some condition.

Proteins can be targeted to exosomes via signal sequences such as myristylation tag and phosphatidylinositol-(4,5)-bisphosphate-binding domain. These simple plasma membrane anchors can be used to target cytosolic proteins or bioengineered proteins to bind into exosome membranes. (Shen, Wu et al. 2011) Monoubiquitinylation, multiple monouniquitinylations and farnesylation of proteins targets proteins for endosomes to form multivesicular bodies as stated in recent review by Raposo et al., Llorente et al. and Piper and Katzmann (Hessvik, Llorente 2018, Guillaume Van Niel, Gisela D'angelo

et al. 2018, Piper, Katzmann 2007). These signal sequences along with higher level of oligomerisation of the membrane proteins seem to support the sequestering of proteins to the extracellular microvesicles. These common features still do not explain the intravesicular proteins (excluding ubiquitinylation), since the formerly mentioned sequences guide proteins to membranes of exosomes. Term piggyback was used to describe the co-localisation of a random protein along with vesicle cargo protein in a recent review article. (Yang, J., Gould 2013) ESCRT machinery contains proteins with catalytic activities that cleave ubiquitin from the proteins transported to the lumen of vesicles inside MVBs and therefore multiple proteins with no apparent signal sequence can be effectively localized inside exosomes with the removed ubiquitin tag. (Hurley, Emr 2006)

Protein sorting is controlled by ESCRT complexes I and III along with chaperone protein, heat shock cognate 70, to transport proteins to multivesicular bodies in the late endosomes. Process was described as microautophagy for the similarities with regular autophagy by Sahu et al. Heat shock cognate interacts with other cytosolic proteins with electrostatic interactions and propagates their transport into late endosomes. These interactions are more efficient and targeted with cytosolic proteins containing KFERQ-sequences.(Sahu, Kaushik et al. 2011) With the important role of protein sorting and transporting of heat shock cognate 70, it can be used as a enriched exosomal protein marker to use in western blot.

ESCRT machinery influences the molecular composition of exosomes in mammals. The downregulation of ESCRT machinery via short hairpin RNAs transported via lentivectors that resulted in changes in the number of secreted exosomes as well as in the surface protein profile. Downregulation of ALIX resulted in an increase of MHC II (Major histocompatibility complex II) on the exosome membrane and effected in the CD63 and HSC70 protein quantity on the membrane. TSG101 downregulation on the other hand decreased the total exosome secretion in HeLa cells. (Colombo, Moita et al. 2013) Influencing the ESCRT machinery via lentivectors could provide a route for engineering exosomes to overexpress certain proteins or using the ESCRT pathways to transport specific molecules to exosomes. For engineering to be safe and efficient fundamental understanding of the exosome formation is needed and further studies need to be done.

Release of exosomes is balanced with the option of lysosomal degradation of MVBs. Determination of which pathway MVBs take is regulated to some extent by ISGylation, a similar modification than ubiquitylation, of TSG101 (among other proteins) targets the MVB for lysosomal degradation. (Carolina Villarroya-beltri, Francesc Baixauli et al. 2016) Transport of MVBs to plasma membrane is dependant with the interaction of cytosolic structure proteins actin and microtubules, actin interacting protein cortactin and Rab GTPases. (Hessvik, Llorente 2018) Release of exosomes requires membrane fusion with MVB and plasma membrane. However the actual mechanism of membrane fusion of MVB and cell membrane to release exosomes is still unknown and separate protein machinery has been characterized for different cells and exosomes carrying different cargo. (Raposo, Stoorvogel 2013) SNARE proteins are included in the release of exosomes from the multivesicular bodies by the fusion of membranes upon vesicle release. Interaction with SNAREs and VAMP7 (vesicle associated membrane protein 7) and the R-SNARE protein YKT6 have been shown to be important on exosome release, with inhibition of SNARE-VAMP7 interaction or YKT6 expression leading to decrease in exosome release. (Hessvik, Llorente 2018)

There are no universal or entirely specific markers for exosomes to use for quantification and to use to isolate exosomes. The commonly used markers for exosomes CD9, CD81, CD63, ALIX, TSG101 and FLOT1 are also present in microvesicles and can be included in apoptotic bodies and other secreted vesicles (Guillaume Van Niel, Gisela D'angelo et al. 2018). However these proteins seem to be enriched in exosomes or in exosome like vesicles to a varying degree. It is not yet clear if the expression of these markers is completely cell, exosome or function related and could these differences be used as a mean to distinguish specific vesicle populations. With the limitations of the characterization in mind, the enrichment of these markers is still a way to show the presence of extracellular vesicles in the sample. With even better methods for exosome isolation in development the future goal is to characterize the possible subpopulations of exosomes depending on their size, density, cargo, morphology, function or some completely different yet unknown factor and analyse the vesicles on the subpopulation or even single vesicle level using high sensitivity analytics.

1.3 Membrane lipid profile

Lipid profile of EVs can differ from one vesicle to another. Some differences can be seen in the lipid content of the membranes compared to plasma membrane with higher cholesterol and ceramide content in the exosome developing MVBs. (Mobius, Ohno-Iwashita et al. 2002, Trajkovic, Hsu et al. 2008) The overall lipid composition differs from the parental cells as seen in review article by Record et al. (Michel Record, Kevin Carayon et al. 2013). Inhibition of ceramide production by sphingomyelinase inhibitor leads to clear drop in the formation of exosomes when examining HeLa derived exosomes (Trajkovic, Hsu et al. 2008). This did not completely inhibit the exosome secretion which indicates ESCRT independent mechanism, which does not need ceramide for function. Lysobisphosphatidic acid (LBPA) is shown to be another important lipid for forming multivesicular bodies. Localization of LBPA among other lipids is explained in article (Kobayashi, Gu et al. 1998) with enrichment of LBPA from 1.5 % to over 15 % in the late endosomes.

Interaction with a small glycoprotein saposin (acronym for Sphingolipid Activator PrO(S)teINs) C is crucial to regulate forming of MVBs and fusion of the membranes at least in human and mice fibroblasts. Interaction with saposin C and LBPA in artificially generated unilamellar vesicles leads to increase of vesicle size from 200 nm up to 1500 nm and the growth is suspected come from spontaneous fusion of vesicles mediated by saposin C. Similar reaction was not observed with other saposins. This indicates the direct interaction with saposin C and LBPA. (Chu, Witte et al. 2005) Considering exosomes, more interesting is the effect of saposin C deficiency to the number of MVBs within a cell. The number of MVBs was over 4 times larger in saposin C deficient cells and incubation with saposin C reverted the number of MVBs back to normal levels. (Chu, Witte et al. 2005) This information could be utilized in production of exosomes to increase the number of MVBs within a cell which could lead to increased number of exosomes secreted by the cell by bioengineering saposin C deficient cells.

1.4 Isolation of exosomes

Previously the isolation of exosomes is based on differential centrifugation with various speeds to separate exosome sized particles from media. Centrifugation is done with or without gradient (sucrose or iodixanol) with exosome density ranging from 1.13-1.19 g/ml or as high as 1.21 g/ml (Michel Record, Kevin Carayon et al. 2013).

Centrifugation is time consuming and difficult to upscale due to the high g-forces ranging from 70 000 xg up to 340 000 xg (Kristine Blans, Maria S. Hansen et al. 2017) needed for exosome isolation. Many different isolation methods have been tested and compared (Shtam, Samsonov et al. 2018) such as the use of commercial kits (YAMADA, INOSHIMA et al. 2012) or the use of mild chemical treatments (Brighton E. Maburutse, Mi-ri Park et al. 2017). Different isolation methods have been more or less successful and the protocol used needs to suit the starting media to isolate the exosomes from.

The classical differential centrifugation method is also suitable for isolation of plant-based exosomes from various materials (Xiao, Feng et al. 2018). Multitude of plant based exosome studies are done with the method starting with homogenisation of the sample using a blender or from juice samples (Raimondo, Naselli et al. 2015, Ju, Mu et al. 2013, Wang, Ren et al. 2015). The disrupted cells will produce vesicles of all sizes and densities and this is made even more complex with samples containing chloroplasts that can be in the same size ranges as the exosomes. These blender-based or juice-based isolation protocols should be considered to contain exosome-like particles or nanovesicles and not be described as exosomes and the following studies using the isolated exosome-like particles need to be presented for the exosome-like particles and not for exosomes.

Differential centrifugation will not produce pure exosome isolates, since aqueous solution will always contain different size particles that will sediment along with the exosomes in the centrifugation process. The purification should be continued with a density gradient, filtration or with size exclusion chromatography to seclude protein aggregates, membrane particles of different origin and soluble proteins from the exosome isolate. The purity of the final isolate needs to suit the following analytics; as an example the exosome sample with high soluble protein content used in cell culture related studies can distort the results with the measured response of the cells being from the soluble proteins rather than from the exosomes itself. Hence the studies need to have a valid negative control to pin point the actual molecule responsible for the effect.

The commercial kits are widely based on the polymeric precipitation of exosomes with poly-ethylene glycol or similar polymer (assumption due to disclosed product specifications). Even though the kits don’t need high speed centrifugations to isolate the

exosomes, the overall yield is not comparable to the high speed centrifugation methods as seen with bovine milk (Masaharu Somiya, Yusuke Yoshioka et al. 2018) and human plasma (Stranska, Gysbrechts et al. 2018). The difference in the efficiency may also be due the wider array of pelleted vesicles by the centrifugation rather than the limitations of the precipitation chemicals. The precipitation efficiency was improved with the use of positively charged protein protamine with the exosomes charge is negative and dependent of the origin cell. Combined use of PEG and protamine yielded higher exosome concentrations in the final isolate than ultracentrifugation-based method from serum, saliva and cell culture media. (Deregibus, Figliolini et al. 2016)

Comparison between published methods is often difficult and the lack of complete protocol and used instruments may hinder the ability to replicate the results with the given protocol. Differences in centrifugation based methods are heavily influenced by rotor along with K factor, therefore centrifuge specifications must be normalized when replicating different protocols with different instruments. (Cvjetkovic, Lötvall et al. 2014) It is not enough to report just the used g-forces along with centrifugation time in the protocol, since the differences between the rotors will greatly influence the run time and the efficiency of pelleting. However, even with the equation of the different equipment used in isolation the complete yield of exosomes is essentially sample related and can be influenced by the source of exosomes. Therefore, the isolation procedure needs to suit the medium of which the exosomes are to be isolated. This is even more complicated and crucial when isolating exosomes from complex media, such as milk which is seen comparing results from bottom-up and top-down centrifugation methods by Zonneveld et al. (Zonneveld, Brisson et al. 2014) The physical properties of medium of which exosomes are going to be isolated need to be taken into consideration. More dense and viscous medium will demand longer centrifugation times or can be even unsuitable for isolation of vesicles by centrifugation. Online calculators such as http://vesicles.niifhm.ru/index.php?do=1 and https://www.beckman.com/centrifuges/rotors/calculator can be utilized to determine specific run parameters for centrifugation based isolation of exosomes.

Size exclusion chromatography has successfully been utilized to purify exosomes from bovine and human milk without the need for pelleting with high speed centrifugation. The isolated exosomes were pure from the most abundant milk-protein casein which is troublesome to prevent from contaminating the final exosome isolate. (Kristine Blans,

Maria S. Hansen et al. 2017) Size exclusion chromatography could be scaled up for bigger sample volumes, but slow flow rates and relatively delicate instruments require time and capable operating personnel for use. The combined use of size exclusion chromatography with tangential flow filtrations is shown to produce highly pure extracellular vesicle isolates from cell culture mediums (Corso, Mäger et al. 2017, Nordin, Lee et al. 2015).

Flow cytometric isolation has successfully been utilized; however, the heterogeneity of the exosome membrane proteins demands a wide array of immunolabels and the final isolate may be more homogenic than the actual exosome population in the original sample. (Wiklander, Bostancioglu et al. 2018, Suresh Mathivanan, Justin W. E. Lim et al. 2010) Isolation with the immunolabels could potentially be utilized for the differentiation of separate vesicle populations with distinctive protein expression. This could be even coupled with the engineering of exosomes to express some membrane protein that is not commonly found in these vesicles and used to isolate the new engineered vesicles from the sample. Tangential flow filtration is one of the commercially available services for isolating exosomes and is used to produce ready exosomes to use as internal standards (Lonza). Even though most exosome isolation protocols are based on ultracentrifugation the effects of high speed centrifugation on exosome structures have not been analysed. Immense g-forces could affect the elastic membranes of exosomes and possibly lead to fusion of membranes. Influence of the isolation and storage procedure can be seen with the cup-shaped morphology in the electron microscopy caused by the collapsing and distortion of membranes.

Whichever method is to be used, the results should be reported with the complete protocol to pair the results with the protocol. The isolated vesicles should be free of known contaminants such as protein aggregates and other vesicles or the results from the following analyses should take these contaminants account for. Negative controls should be carried out in all analytical methods to assess the purity of sample and therefore also define the effectiveness of the used method. Multiple separate online platforms have been established for publishing EV-related results such as evpedia.info, vesiclepedia (microvesicles.org) Exocarta (exocarta.org) and EV-Track (evtrack.org). The proteomic analyses of these databases are filled with contaminating proteins and comparison has become somewhat troublesome. The international society of extracellular vesicles (ISEV) provides free information for researchers interested in

extracellular vesicles. The massive open online course “MOOC” gives a basic understanding of everything EV-related. ISEV provides also standardized requirements for EV related publications for quality control. These standards are taken further with EV-Tracks EV-metric which is a 9 point assessment for providing quality control for the results. This checklist makes sure that the researchers remember to consider all the possible controls along with the conducted experiments.

1.5 Effects on cells

Exosomes derived from bovine milk have been analysed for use as a therapeutic agent along with exosomal loading with therapeutic compounds. Exosomes showed high chemical stability with tissue specific localization within subject rats depending on the exosome exposure method. Treated rats and cell cultures showed clear indications of anti-inflammatory responses from exosomes and higher responses from exosome loaded drugs compared to free drug molecules and PBS (phosphate buffered saline) control. (Munagala, Aqil et al. 2015) With similar affects bovine milk derived EVs reduced arthritis symptoms in mouse models, when treated with oral administration of EVs. The intake of EVs was visualized by flow cytometry and confocal microscopy. Both macrophages and ileum cells took EVs in and the intake was reduced greatly when lowering the incubation temperature to 4 ˚C. (Arntz, Pieters et al. 2015)

Exosomes isolated from bovine milk activate MAP- kinase pathway in IEC-6 cells. The cell culture was starved prior to exosome treatment, which may influence the observed change. However the kinase pathway activation was dose dependent and was greater in cells grown in exosome depleted media. (Siran Yu, Zhehao Zhao et al. 2017) This activation indicates the proliferative effects of exosomes to the recipient cells.

Exosomes from bovine milk are shown to contain proteins that are related to immune response and growth. With major differences in protein composition between mature and colostrum milk, it is clear that exosomes are precisely produced with specific cargo to transport for the recipients. In this case the calf gets a probable immune response activating surge of exosomes from the colostrum milk which then turns to growth inducing signal later post-partum. (Samuel, Chisanga et al. 2017)

Isolated exosomes have been shown to withstand simulated human digestive system and ingested by intestinal epithelial cells *in vitro*. This suggests the possibility of transport of proteins and nucleic acids from different sources via gastro intestinal cells. (Wolf,

Baier et al. 2015, Benmoussa, A., Lee et al. 2016, Mu, Zhuang et al. 2014). Exogenous RNA is already shown affect gene expression across species. MIR168a, abundant miRNA in rice, is active and binds to human and mouse LDLRAP1 mRNA and inhibits the expression of the respective protein. (Lin Zhang, Dongxia Hou et al. 2012)

The RNA in milk is shown to be stable under degrading conditions (Izumi, Kosaka et al. 2012) and are shown to be affect gene expression in mononuclear cells, HEK-293 and mouse liver cells (Baier, Nguyen et al. 2014). There are only few publications on the topic of food derived RNAs influencing gene expression directly and the subject is still highly controversial. Intake of exosomes is mediated via endocytosis (Kusuma, Manca et al. 2016, Wolf, Baier et al. 2015) and endocytosis is influenced heavily by the protein and glycoprotein content of the exosome membrane. With glycosomal competition and when the membrane proteins are treated with a protease the transport rate of exosomes is lower. (Kusuma, Manca et al. 2016) The intake of bovine milk exosomes show no cytotoxic effects as seen with mouse macrophage cells and no signs of systemic anaphylaxis was measured after 35 days of exosome treatment. (Masaharu Somiya, Yusuke Yoshioka et al. 2018)

1.6 Exosomes from bovine milk

Bovine milk exosomes are an interesting target for research as humans with western diets consume milk products and are therefore subjected to high quantities of exosomes from them. Milk exosomes are shown to be resistant to digestion and also bioavailable in different species of which derived from. (Manca, Upadhyaya et al. 2018) Recent review article combined interesting factors present in bovine milk exosomes with potential health risks in mind. The miRNA contents of exosomes were linked to multiple chronic conditions and higher risks of cancer. (Bodo C Melnik, Gerd Schmitz 2019) Indications of dangers of milk consumption could be explained with digestion resistant exosomes being the causative factor in development of chronic conditions. Therefore basic knowledge and a complete understanding of exosomes in bovine milk should be collected to determine the safety and influence of bovine milk for the consumers.

Unique protein signatures have been found with differences between mature and colostrum milk samples (Samuel, Chisanga et al. 2017, Yang, M., Song et al. 2017). Separate fractions isolated by centrifugation also are shown to have different protein

signatures as described by Benmoussa et al.(Benmoussa, Abderrahim, Gotti et al. 2019) Along with the protein contents, RNA and lipid profiles of exosomes have been characterized. Online database exocarta is designed for the characterization of exosome protein, RNA and lipid contents. This database covers 9 different species and numerous cell lines along with complex mediums of which the exosomes are isolated from (http://www.exocarta.org/).

Molecular complexity of bovine milk provides challenges for the isolation of exosomes. High fat and protein content with similar precipitation properties and density of exosomes can lead to contamination of exosome isolate with the caseins and lipid micelles (Masaharu Somiya, Yusuke Yoshioka et al. 2018). Filtration of the whey can separate larger particles from the isolate, but casein aggregates with smaller than 0,22 µm diameter will still pass the filter and be present in the final isolate. Caseins will spontaneously form micelles when the environment favours their formation with interactions between monomeric K- and B-caseins. More effective protocol could allow isolation from large quantities of abundant source of exosomes, such as bovine milk. With easier and more efficient isolation methods exosomes could be utilized for manufacturing pharmaceutical and nutraceutical products. The acetic acid isolation method was successfully used previously by Somiya et al. and effective SEC-based isolation method was utilized by Blans et al. (Masaharu Somiya, Yusuke Yoshioka et al. 2018, Kristine Blans, Maria S. Hansen et al. 2017) The bioavailability of exosomes provides a possible use of exosomes as trafficking specific cargo, such as drugs or polyphenols, to cells has been tested with promising results (Munagala, Aqil et al. 2015, Wang, Wang et al. 2014).

The objective of this study is to set up effective isolation protocol for isolation of exosomes from different bovine milk samples and characterize the proteins from the isolated exosomes. Identification of the isolated EVs as exosomes will be done by scanning electron microscopy, gel electrophoresis and western blot analysis with CanX, CD63, CD81, TSG101 and ALIX antibodies. These are the antibodies widely used for characterization of exosomes, even though there are major differences between with the proteins expressed on the exosome membrane from different exosome sources (Smith, Lee et al. 2015, Wiklander, Bostancioglu et al. 2018). This range of identification covers the minimum basis of exosome characterization described by International Society for Extracellular Vesicles in 2014 (Lötvall, Hill et al. 2014).

Isolated exosomes are quantified and characterized by nanoparticle tracking analysis and with electron microscopy with negative staining using uranyl-acetate and/or with immunolabelling using anti-CD63 with secondary antibody (donkey anti-goat) labelled with 10 nm gold particles. The NTA and EM will be done as paid service from FIMM/EV-Core at University of Helsinki.

After the isolation of exosomes proteins are digested with trypsin and analysed with LC MS/MS (quadrupole/time of flight) mass spectrometry. The differences in bovine milk sample exosomes will be analysed between different isolation procedures to examine the differences between the isolation methods.

2. Materials and methods

2.1 Sample collection

Milk from healthy Nordic Red dairy cows was obtained from Luke Minkiö research cow house (Jokioinen, Finland). Cold tank milk was collected into a glass bottle and kept cold +4 - +8 ˚C prior to analysis. Excess milk after removal of fat and intact cells was aliquoted to 40 ml fractions and frozen -80 ˚C for later use.

2.2 Isolation of exosomes

Differential centrifugation was applied to pellet the exosomes. Isolation was conducted with and without acetic acid (AA) and followed by two additional purification steps; size exclusion chromatography (SEC) and sucrose gradient centrifugation. Precipitation with ammonium sulphate was examined as a possible isolation method, but was ruled out due to difficulty to track solution density and therefore the fraction which should contain the exosomes. Flow chart of exosome purification can be seen in figure 1.

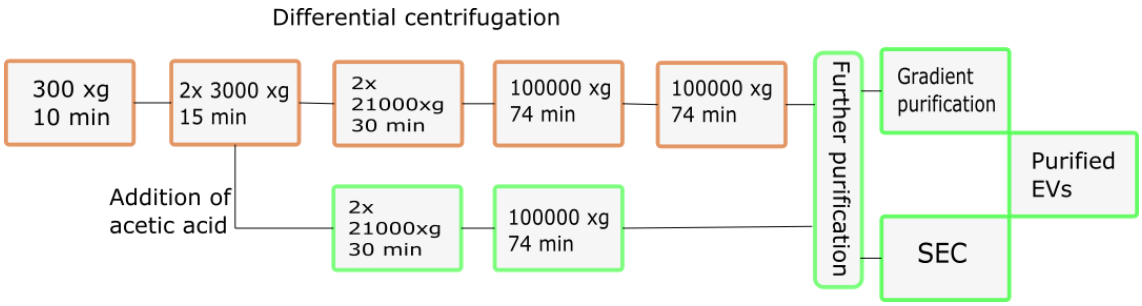

Figure 1. Flow chart of exosome isolation with differential centrifugation with or without acetic acid treatment. Consequent secondary purification with gradient

purification or size exclusion chromatography was conducted for both isolation methods to remove impurities from exosome isolates.

Fresh whole milk was centrifuged 300 xg 10 min +4 ˚C (Beckman coulter, JA-14 or JA-17, J2-21M) to pellet intact cells to prevent further cell breakage and contamination with cell debris. Separated middle layer was always used in the consecutive steps. Layer was centrifuged twice 3000 xg 15 min +4 ˚C (Beckman coulter, JA-17) to pellet cell debris, caseins and remaining fat on top. Resulting defatted milk was again centrifuged twice 21500 xg +4 ˚C 30 min (JA-17 Beckman Coulter) to further pellet caseins. Resulting clearer whey was ultracentrifuged 74 min (Beckman coulter, Type 50.2 Ti, L8-80) to pellet remaining caseins. Resulting supernatant was ultracentrifuged again as before to pellet the exosomes. Precipitated pellet was resuspended in 200 µl of PBS.

To further precipitate caseins and milk peptones the fraction after 2x 3000 xg centrifugation was adjusted to pH 3.8 with glacial acetic acid (Sigma Aldrich, MO, USA). The AA-treated sample was then centrifuged similarly as non-treated milk except the AA exosomes were pelleted already during the first ultracentrifugation. The resulting pellet was again resuspended in PBS and frozen until further analysis. Isolation is done similarly as Caby et al. and Masaharu et al. (Caby, Lankar et al. 2005, Masaharu Somiya, Yusuke Yoshioka et al. 2018)

Ammonium sulphate precipitation was tested for exosome isolation. Ammonium sulphate was added to milk to form a saturated solution. The solution was stored over night at +4 ˚C to precipitate proteins. Resulting solution was centrifuged 3000 xg 15 min +4 ˚C. The top layer was extracted and centrifuged again to separate the fat layer. The following clear solution along with the cloudy top layer was subjected to the following 21500 xg centrifugation steps as with the acetic acid treated and non-treated milk. The top cloudy layer was separated and diluted to ¼ with PBS and ultracentrifuged 40000 xg 10 min. Clear bottom layer was diluted and ultracentrifuged as the top layer. The resulting top and bottom layer were again separated and diluted ¼ with PBS and ultracentrifuged 74 min with type 50.2 Ti rotor. Resulting pellets were suspended to 200 µl of PBS. Ammonium sulphate precipitation could be utilized to isolate exosomes from milk with adjustment to the density of the solution as well as the precipitation proteins. This method needs to be optimized to accurately isolate exosome fraction. Ammonium sulphate could therefore be effective combination of similar

effects as acid precipitation Size exclusion chromatography using ÄKTA Basic protein purification system with two Superose™ 6 10/300 columns (GE Healthcare, Sweden) was applied to remove remaining soluble proteins from the final exosome isolate and to recover the exosomes from the void volume as the smaller soluble proteins are trapped in the pores of the column. PBS (137 mM NaCl (Merck, Germany), 2,7 mM KCl (Merck), 10 mM Na_2HPO_4 (Merck), 1,8 mM KH_2PO_4(Merck)) with 2 M urea (Thermo Fisher, MA, USA) was used as running buffer. Urea was used in the running buffer to dissociate the casein micelles to prevent the elution in the void volume peak.SEC was performed with and without ethylenediaminetetraacetic acid (EDTA (Sigma Aldrich, MO, USA)). 20 mM EDTA (Sigma Aldrich) was used to chelate bivalent cations from the solution in order to prevent cation related casein-casein interaction to dissociate casein micelles to prevent them eluting at the same time with the exosome void volume peak. and separates the caseins from each other. EDTA has been previously utilized by Blans et al. (Kristine Blans, Maria S. Hansen et al. 2017). Exosome pellets for SEC were first suspended in 200 µl of PBS, filtered through 0,22 µm syringe filters and adjusted to 600 µl with PBS to contain 1 M urea in the final sample. Solid urea was weighed into Eppendorf tube and the sample was filtrated on top of the solid urea. Samples were then loaded via 500 µl loop to ÄKTA. To identify proteins from the samples, bovine milk standards were used in parallel with the samples. The bovine milk standard was prepared using α-, β-, and K -casein, lactoferrin and lactalbumin in 5-10 mg/ml concentrations. All proteins for the standard mixture were manufactured by Sigma Aldrich. In first runs the milk standard proteins were run as a mixture to allow protein interaction and feasible micelle formation. In first runs samples and standard were separated with urea in the running buffer. In later run the proteins were solubilised separately in 10 mg/ml concentrations and run without the addition of urea in the running buffer to identify individiual proteins.

Gradient centrifugation was done with stepwise sucrose gradient in PBS based on a method by Luthe (Luthe 1983). The sucrose gradient was prepared step-wise by pipetting and freezing (-80 ˚C) 2 ml fractions of sucrose with decreasing densities to SW 41 Ti ultra-clear poly propylene centrifuge tubes. Fractions were as follows 45, 30, 20, 10 and 0 % w/v of sucrose in PBS. 200 µl of exosome isolate was pipetted on top of the thawed gradient tube and centrifuged 16 h, 100000 xg at + 20 ˚C. Exosomes were retrieved from the 30 % fraction with density of 1.127 g/ml and from the border of 45 %

fraction with density of 1.203 g/ml. Resulting fraction was then diluted to 22 ml of PBS and ultracentrifuged with type 50.2 Ti rotor for 90 minutes to pellet the gradient purified exosomes. Resulting pellet was resuspended in PBS and frozen -20 C until further analysis.

2.3. Validation of exosome isolation methods

2.3.1. Electrophoresis

Proteins from each isolation process were separated and visualized with 12 % w/v sodium dodecyl sulphate SDS poly acrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE with separation gel (0,192 M Tris-HCl pH 8,8 (Sigma Aldrich), 3,35 M urea (Sigma Aldrich), 12 % w/v acrylamide (19:1 Acrylamide/Bis-acrylamide-30 % solution, Bio-Rad Laboratories, CA, USA), 0,22 % w/v sodium dodecyl sulphate (SDS, Merck), 0,025 % w/v ammonium persulfate (APS, Amersham Biosciences, UK), 0,04 % v/v tetraethyl methyl diamine (TEMED, GE Healthcare)) and stacking gel (0,1834 M Tris-HCl pH 6,8, 7,5 M urea, 6 % w/v acryl amide, 0,17 % w/v SDS, 0,007 % w/v APS and 0,007 % v/v TEMED) was prepared with standard protocol. Sample buffer of 138 mM Tris-HCl (pH 6,8), 6 M Urea, 4,3 % (w/v) SDS, 22 % (v/v) glycerol, bromophenol blue (Merck) as indicator and 5 % (v/v) 2-mercapthoethanol (Sigma Aldrich) as reducing agent was mixed 1:1 with samples and incubated 30 min in 40 ˚C.

Electrophoresis was run with GPS 601 power unit (GE Healthcare) and mini-PROTEAN tetra cell electrophoresis unit (Bio-Rad) with 10-15 mA current and 100-150 V voltage with SDS running buffer (50 mM Tris, 384 mM glycine (Bio-Rad), 6,9 mM SDS). Gels were fixed with 50 % methanol (VWR) 5 % acetic acid (Sigma Aldrich) for 1 h and stained 1 h with Pro Q Emerald 300 (Invitrogen, CA, USA) glycoprotein stain and afterwards overnight with Sypro Ruby protein gel stain (BioRad Laboratories, CA, USA). Gels were destained with 10 % methanol 7 % acetic acid and washed with milli-Q water prior of imaging with ChemiDoc™ MP scan camera and ImageLab™ software (BioRad). Protein concentration was measured with DC™ protein assay (BioRad) or with Pierce BCA protein assay (Thermo Fischer Scientific) using microplate procedure and HIDEX spectrophotometer (HIDEX, Finland). Bovine serum albumin (Thermo Fischer Scientific) was used as a standard in the measurements.

2.3.2. Western blot

For detection of exosome enriched marker proteins SDS PAGE –separated proteins were transferred from gel onto a Immobilon-FL-PDVF membrane using Bio-Rad semi-dry blotting device, current of 1 mA/cm^3 and voltage maximum of 25 V, and TG as transfer buffer (Bio-Rad; 25 mM Tris, 192 mM glycine, pH 8,3 + 20 % methanol). Tris-buffered saline (TBS; 18 mM Tris, 0,5 M NaCl pH 7.5) was used as a washing and blocking buffer with the addition of 5 % (w/v) fish gelatine in the latter (Thermo Fisher Scientific). After transfer each blot was blocked with 5% blocking buffer for 1 h under shaking at room temperature and then incubated with primary antibodies anti-CanX (ST John laboratories, USA, STJ140017), anti-CD63 (ST John laboratories, STJ140029), anti-Alix (Cell Signal, Netherlands, E6P9B Rabbit mAb), anti-CD81 (Santa Cruz biotechnology, USA Texas, sc-166029 mouse monoclonal) and anti-TSG101 (Nordic Biosite, ABB-709, rabbit polyclonal) diluted 1:5000-10000 in 1% (w/v) fish gelatine in TBS-T (TBS with 1 % (v/v) Tween-20 (Sigma Aldrich) overnight at +4 ˚C with agitation. After removing the primary antibody solution blots were washed 3x5 min with TBS at room temperature with agitation and then incubated for 2 h at room temperature with corresponding secondary antibody (mouse anti-goat (Santa Cruz biotechnologies, sc-2354), anti-Rabbit HRP conjugated (Agrisera AB 10668) or anti-mouse (Santa Cruz biotechnologies, sc-516102) diluted 1:20000 in 1 % 1% (w/v) fish gelatine in TBS-T. StrepTactin™-HRP conjugate (1:50000) was added for detection of Western C protein standard (BioRad). For detection blots were incubated with SuperBright-ECL chemiluminesence reagent (Agrisera, Sweden) according manufacturer's protocol and the images were obtained using Bio-Rad ChemiDoc MP image scan camera and Imagelab software.

2.3.3. Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was applied to characterize isolated nanoparticles. NTA is based on the Brownian motion and light scattering properties of particles in solution and it produces high resolution estimates of nanoparticle size-distribution and concentration(https://www.malvernpanalytical.com/en/products/product-range/nanosight-range/nanosight-lm10) . The analysis does not separate nanovesicles form other nano-sized particles. The NTA analysis service was provided by EV Core service laboratory in Helsinki. In short the samples were analysed on Nanosight

LM14C nanoparticle tracking instrument in 6 replicates and dilutions of 1:20 – 1:20000. Resulting particle concentrations and sizes were averaged and charted with standard deviance visible on the graphs.

2.3.4. Transmission electron microscopy

Transmission electron microscopy (TEM) was applied to detect, characterize and visualize the isolated vesicles. The imaging was done at EV Core service laboratory in Helsinki according to Puhka et al 2017. (Puhka, Nordberg et al. 2017). In short, the samples were fixed with paraformaldehyde (Electron microscopy sciences, Hatfield, PA) in 0,1M NaPO4 buffer (pH 7.0), stained with 2,0 % neutral uranyl acetate, embedded in methyl cellulose uranyl acetate mixture (1,8/0,4%) and imaged with transmission electron microscopy using Jeol JEM-1400 (Jeol Ltd., Tokyo, Japan) operating in 80 kV. Images were taken with Gatan Orius SC 1000B CCD-camera (Gatan Inc., USA).

2.3.5. Mass spectrometry and bioinformatics

For detection of exosome enriched proteins selected exosome isolates were subjected to in-solution trypsin digestion. 100 µg of exosome protein was loaded to 3 kDa spin columns (Nanosep, Pall Co. USA) and wash buffer (8 M urea in 10 mM Tris-HCl pH 8) was added to reach at least 6 M urea concentration. Resulting solution was centrifuged until almost dry at 10000 xg at room temperature with Eppendorf minispin plus tabletop centrifuge. Sample was washed with 100 µl of wash buffer and spun dry. Proteins were reduced with 50 µl of 10 mM DTT (Sigma Aldrich) in wash buffer and again spun dry. Reduced proteins were alkylated with 27 mM iodoacetamide (Thermo Scientific) in wash buffer and mixed gently by vortexing (1000 rpm) for 1 min and spun dry. Sample was washed twice with wash buffer and the filter unit was transferred to a clean Eppendorf tube. Trypsin (Sequencing grade modified trypsin, Promega, WI, USA) was added in 1:25 relation to the sample protein quantity and the samples were digested in 37 C overnight. Resulting peptides were spun through the filter and filter was washed with 50 µl of 0,5 M NaCl.

Formic acid (Merck) was added to the resulting peptide samples to 1 % concentration to form solution < pH 3 to assure the peptide binding to C-18 columns. Solution was loaded to activated (activated with 100 % methanol and 80 % acetonitrile 0,1 % formic acid solutions) and balanced (0,1 % formic acid) SepPak C-18 vacuum columns

(Waters). The peptides were washed twice with 1 ml of 0,1 % formic acid and eluted with 80 % acetonitrile 0,1 % formic acid solution. Samples were dried using Speed-Vac (Thermo Fisher Scientific) and resuspended to 30-40 µl of 2 % formic acid in MS grade water. Samples were transferred to plastic vials and analysed using UPLC-MS/MS (Ultra high pressure liquid chromatography tandem mass spectrometry, Waters M-Class UPLC, Xevo G2 Quadropole-Time of flight tandem mass spectrometer). UPLC was run with 1 % formic acid in MS grade water as A buffer and 1 % formic acid in acetonitrile as buffer B. Gradient was run with 97 % A buffer for 15 min, 3-45 % B buffer for 2 h and 45-97 % B buffer for 15 min with C18 column (Acquity UPLC BEH 130 C18, 1,7 µm 2,1x150 mm, Waters USA). Leucine-enkephalin with mass of 556.00 Da was used as an internal standard calibration. 10 ppm peptide mass tolerance was administered to identify peptides. Identification with 3 or more unique peptides was required for a reliable identification for each protein. Proteins that pass the criteria were mapped for biological process, PANTHER pathway and cellular compartmentalization with PANTHER and DAVID online tools (http://www.pantherdb.org/ https://david.ncifcrf.gov/).

3. Results

3.1. Sample pretreatments and storage

The proper storage of milk samples is important to minimize the contamination risk with cellular and microbial debris due to cell breakage. Milk contains 120 000-130 000 somatic cells per milliliter that can break down and create nanosized vesicles with similar characteristics as exosomes. In order to prevent spontaneous nanovesicle formation due to cell breakage milk samples should be stored in -80 °C preferably without somatic cells and by avoiding multiple freeze- thaw cycles. Samples can be stored in -80 ˚C for a prolonged time (at least 2 years) without heavily influencing the vesicle content. Storage in -20 ˚C for a prolonged time (> 2 years) led to destruction of RNA in the vesicles (data not shown). Similar observation was made when AA precipitated milk samples were frozen to –20 °C. However, exosome isolates can be stored in -20 ˚C when used for protein analysis, such as electrophoresis, western blot and mass spectrometric analysis. Samples that were frozen and thawed multiple times tended to lead to exosome aggregation and distorted morphology what was seen in the

electron microscopy (Figure 6). For nucleic acid isolation and analysis exosome isolates should be stored in -80 ˚C preferably in aliquots suitable for single extraction.

3.2. Isolation of exosomes and validation of isolation methods

3.2.1 Separation of exosomes from other milk components with differential centrifugation

Milk is a complex mixture of water, emulsified fat, casein micelles, soluble proteins, lactose and minerals. Separation of exosomes from such a complex solution is a challenge especially due to the high concentration of milk caseins and soluble whey proteins. Establishing efficient methods for exosome isolation, it is crucial to identify vesicle proteome without contamination from common milk proteins. Differential centrifugation (DC) based applications were compared and validated for exosome isolation from milk as shown in figure 1. Separation of clear whey, that is relatively free of caseins, is mandatory for the pelleting of exosomes in the ultracentrifugation steps. Caseins will form a tight gel pellet in the tube if not removed previously by centrifugation or other means. This may completely prevent the pelleting of exosomes or lead to very large casein pellet with exosome content inside of matrix of caseins. This was seen with identification of exosome enriched proteins from the solubilised casein pellet with western blot (data not shown). To prevent the formation of casein pellet in the ultracentrifugation protein precipitation with low pH was utilized successfully in AA exosome to remove majority of highly abundant caseins from the sample. The comparison of DC and AA-DC suggests that AA-treatment improves protein removal from the sample (figure 2, 3 and 4). However, SEC shows soluble proteins still present in the AA exosome samples and further purification is suggested that acetic acid combined with differential centrifugation alone is not adequate to remove contaminating milk proteins. Precipitation of caseins with acetic acid will not break the exosomes since exosomes are shown to withstand acidic conditions as in digestive system and enzymatic treatments (Wolf, Baier et al. 2015, Benmoussa, A., Lee et al. 2016, Mu, Zhuang et al. 2014, Izumi, Kosaka et al. 2012).

Major issues with the sample purity; mainly casein and other soluble protein contamination were further resolved with the additional purification with gradient centrifugation or SEC as seen in figures 2 and 4.

Density gradient centrifugation was utilized successfully to remove some of the common milk proteins. However with the dilution of sucrose fractions that happened during the preparation of gradient the tubes lacked a sufficiently dense fraction to float the exosomes on top of therefore isolation of denser than 1.2 g/ml fractions were co-isolated with the exosome fraction. Resulting densities of fractions can be seen in table 1. Exosomes were recovered from 30 -40 % fractions.

Fraction/ml	Brix	Refraction	Density g/cm3	% of sucrose
1	0.6/0.7	1.3339	1.0023	0.6
2	2.1/2.2	1.3360	1.0082	2
3	5.3/5.3	1.3407	1.0209	5
4	9.6/9.8	1.3473	1.0388	10
5	15.1/15.1	1.3558	1.0615	15
6	19.7/19.8	1.3633	1.0816	20
7	24.9/25.0	1.3723	1.1036	25
8	30.3/30.1	1.3814	1.1301	30
9	36.0/36.0	1.3903	1.1513	35
10	40.6/40.7	1.3999	1.1764	40

Table 1.Sucrose density recovered from the gradient centrifugation fractions. Densities recovered from table from United States department of agriculture (greenwoodassociates.com) using the Brix index. Resulting exosome fractions are recovered from 30-40 % sucrose fractions.

3.2.2 Removal of soluble proteins with size exclusion chromatography

To further purify isolated exosomes different conditions of size exclusion chromatography were compared. First, column selection along with running buffers with different urea concentrations were evaluated with whey exosome samples. Lower urea concentration along with Superdex™ 200 column (GE Healtcare) resulted in casein aggregates eluting along with the void volume peak fractions containing exosomes. Second, the feasibility to remove protein contaminants with EDTA was

tested with whey exosome samples. The use of EDTA successfully removed majority of caseins from the samples. Absorbance was measured with 280 nm to quantify protein concentration and to get some indications from the possible RNA content with 260 nm. Chromatograms from two separate purifications can be seen in figure 2 and 3 with separate runs with bovine milk proteins along with exosomes isolates.

In third set of SEC urea was removed from the sample buffer to examine the necessity of urea in the running buffer. Separation of soluble proteins is uniform to the runs with urea with exception of K-casein eluting almost at the same time as exosomes. This can be due to poor solubility of lyophilised protein and results may differ from fresh samples. K-casein can form micelles spontaneously without the need for Ca^{2+} ions and therefore this could be the reason with the elution profile. Same peaks can be seen in raw milk and whey exosome samples which indicate the presence of K-casein in the whey exosome sample. However, in the absence of urea, EDTA allows the removal of caseins from the sample also K-casein efficiently from the sample as seen in figure 3

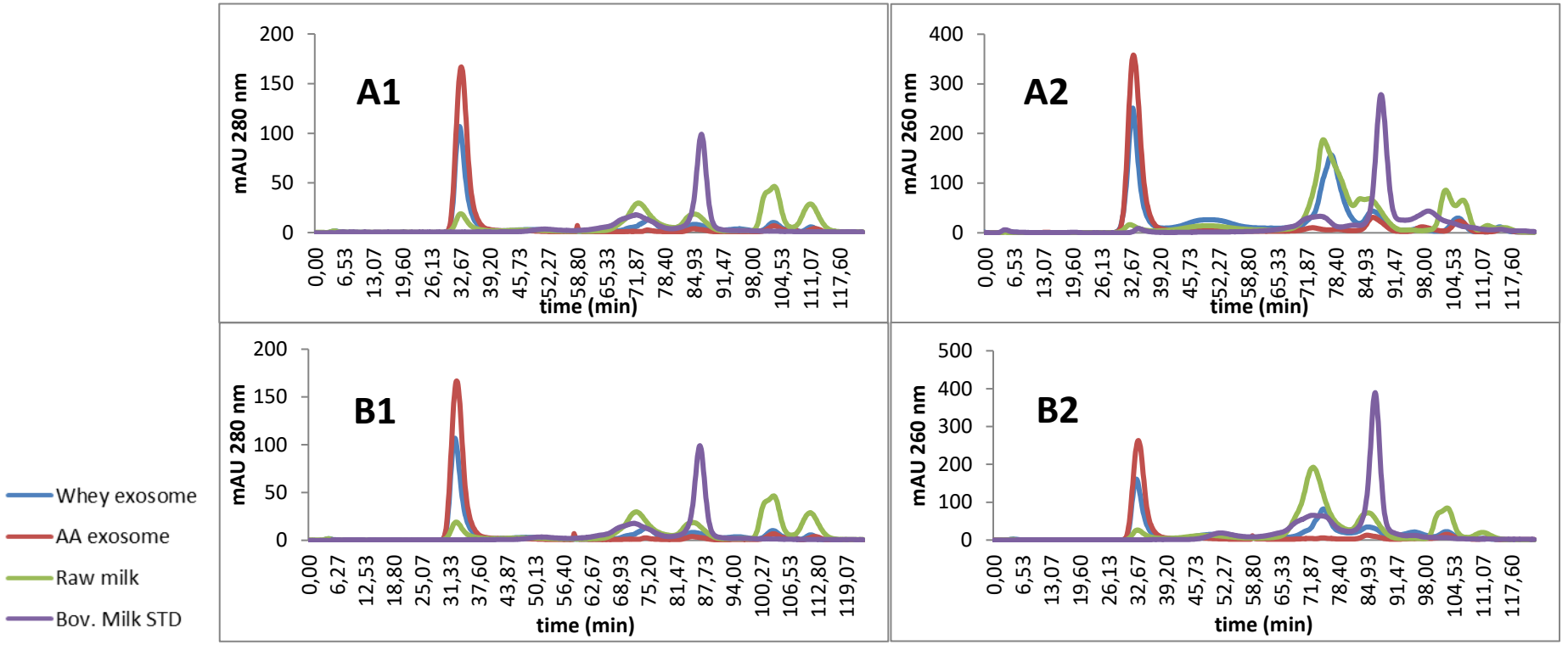

Figure 2. Chromatograms from ÄKTA basic size exclusion chromatography with 2x Superose™6. A1 and A2 are from first purification without EDTA and B1 and B2 chromatograms from second purification with 20 mM EDTA in the running buffer (2 M urea in PBS, pH 7,4 with 20 mM EDTA in the buffer in stead of 2 M urea in B run). 280 nm absorbance on the left (A1 and B1) and 260 nm (A2 and B2) absorbance on the right. Peak at 32 minutes correspondes to void volume peak; larger than 5 MDa particles including exosomes.Bov. Milk STD includes α-, β-, and κ-casein, lactoferrin and α-lactalbumin in 5-10 mg/ml concentrations. 260 nm absorbance was used to see indications of nucleic acid contents, but the absorbance was higher also with pure protein isolates in standard.

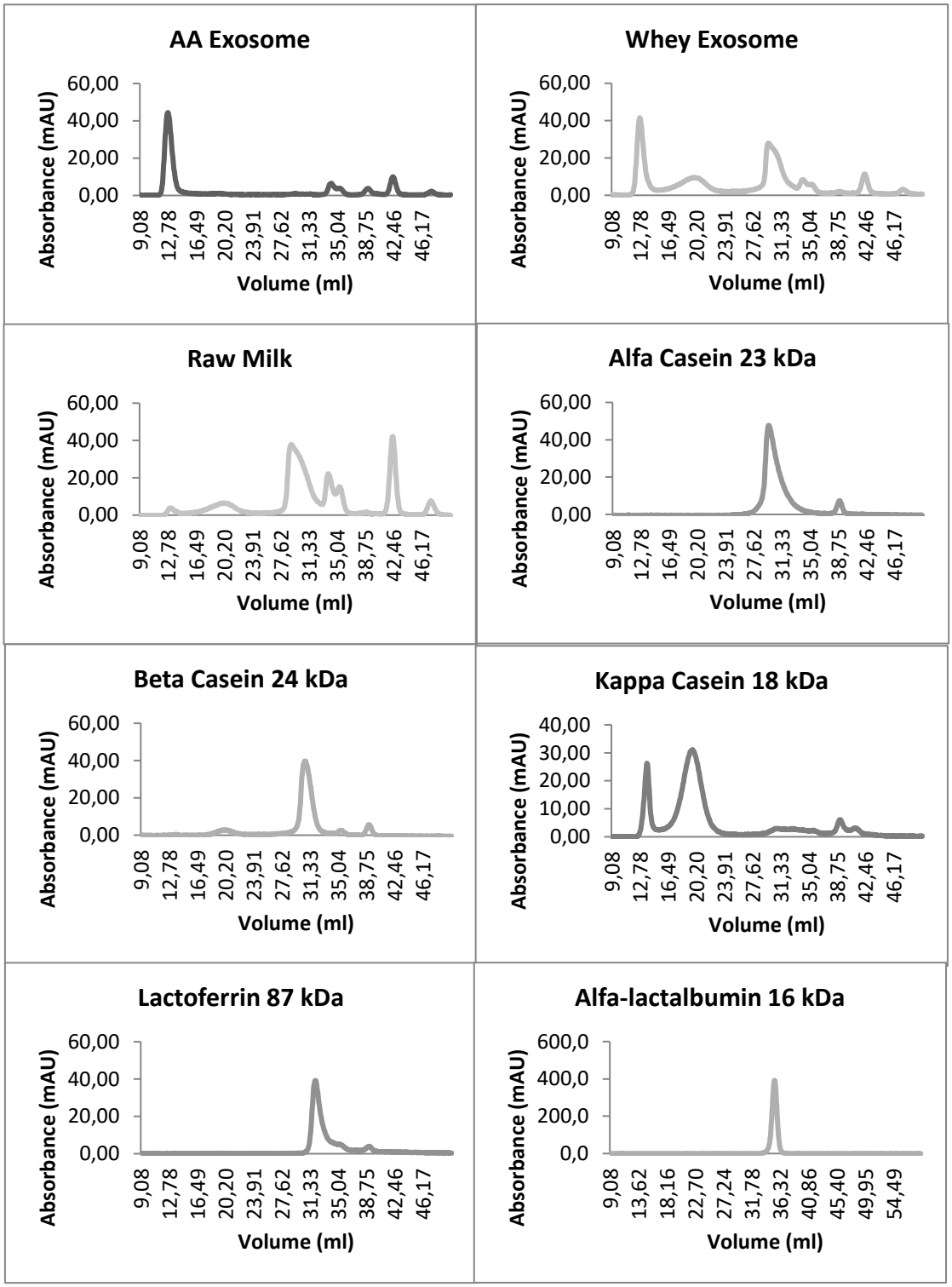

Figure 3. Separate runs of bovine milk proteins along with exosome samples of size exclusion chromatography with 2x Superose™ 6 as columns with 20 mM EDTA in PBS as running buffer. Separation was sufficient without the urea in the running buffer. AA exosome has majority of casein proteins removed by acetic acid precipitation compared to whey exosome sample. K *casein can elute in the same peak as exosomes when forming micelles without other caseins present in sample.*

3.2.3 Visualization of protein profiles of exosome isolates

To visualize the differences of the studied isolation methods 10 µg of protein was loaded to 12 % acryl amide gels for electrophoresis in sample buffer. It is clear that majority of exosome or extracellular vesicle related proteins are heavily glycosylated as can be seen from the comparison between ProQ and Sypro Ruby stains (Figure 4). The barely visible bands in the sypro ruby stained gel in the 75 kDa range are intensively stained in ProQ stain. On the other hand α and β caseins are not heavily glycosylated as seen in the ProQ gel but are in great quantities in the raw milk and whey exosome samples. The samples seem to have a uniform protein profile not dependable on the isolation method not including the casein content. The AA precipitation removes majority of caseins milk proteins but not completely. When combined with SEC or gradient centrifugation the purity of the AA- precipitated sample improves clearly. SEC is also efficient method to remove milk proteins from the sample even without AA-treatment. Interestingly caseins are clearly visible in the gradient purified exosome samples if AA precipitation is not applied. When using differential centrifugation for exosome isolation, none of the tested purification methods is efficient enough to remove milk proteins completely from the samples but the sample purity can be improved. This may be due to the gradient missing the needed denser than 45 % sucrose step. In the SEC samples caseins are purified even better from the samples with the use of EDTA in the running buffer and sample preparation. Western C was used as protein standard in the electrophoresis and the pre-stained properties of the marker seem to inhibit the full staining with sypro ruby. 3 reference bands of the standard can be seen in the Pro Q stained gel with sizes of 75 kDa, 50 kDa and 25 kDa (in picture 4) and similarly in the sypro ruby stained gel (picture 5). Samples are the same in both SDS-PAGE images and all the WB images; 1. Western C protein standard, 2. Raw Milk, 3. Whey exosome, 4. AA exosome, 5. Whey SEC exosome, 6. AA SEC exosome, 7. Whey Grad exosome, 8. AA grad Exosome.

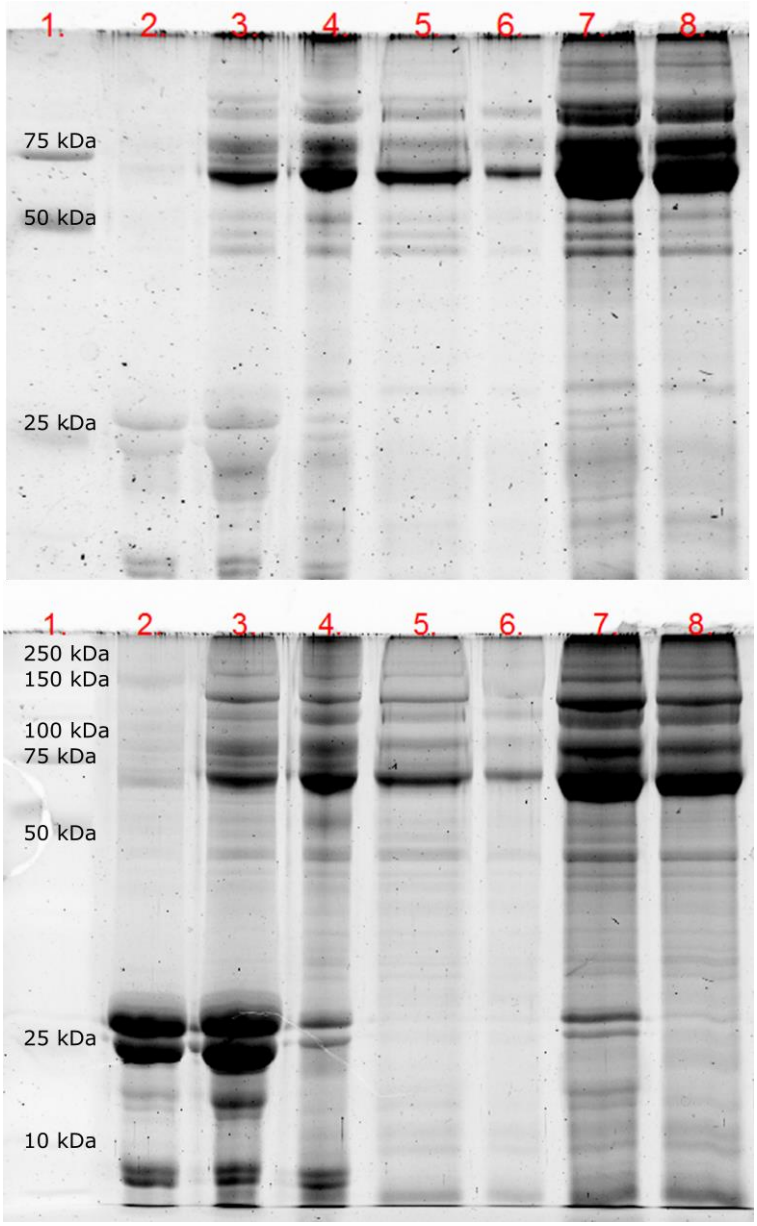

Figure 4. Milk exosome samples separated on 12 % (w/v) acryl amide gel stained by first Pro Q Emerald 300 glycoprotein stain(upper picture) and second with Sypro Ruby gel stain (lower picture). Majority of exosome fraction proteins were seen in the 75-150 kDa range with high level of glycosylation. Caseins are present in all samples in the 25 kDa range, with majority of caseins present in raw milk (2) and UC exosome (3). 1. Western C protein standard, 2. Raw Milk, 3. Whey exosome, 4. AA exosome, 5. Whey SEC exosome, 6. AA SEC exosome, 7. Whey Grad exosome, 8. AA Grad Exosome.

3.2.4 Size and morphology of isolated vesicles

To validate the success of exosome isolation the size and morphology of isolated vesicles were analysed with NTA and TEM. Exosome samples from bovine milk

contained high amounts of vesicles in the expected size range (50-200 nm in diameter, figure 5 and 6) of correct size and also vesicles of larger than 150 nms. The size range given in the literature is approximate and may vary from tens of nanometers to hundreds of nanometers depending of the cargo and origin and isolation procedure. As previously seen with other analyses the acetic acid method yielded higher particle concentrations than just ultracentrifugation alone.

The major difference between different purification methods seen in the NTA results is the absence of majority of larger than 200 nm particles in the SEC-samples. This is due to filtration alone that will clean up the larger than 220 nm particles from the exosome isolate. The SEC samples have been filtered to prevent machine damage in the ÄKTA system through 0,22 µm GLP acrodisc filters (Pall Co, USA). The resulting size distribution seen in pictures 6 and 7 along with mean particle size seen in table 2 is smaller in SEC samples compared to the unfiltered ones. This is due to filtration and not the chromatography element, since the larger particles would elute in the same fractions as the smaller particles that are not retained by the columns.

Sample	particle concentration (particle/ml)	particle mean size (nm)
AA EXO	2,17E+13	174
AA SEC EXO	1,18E+12	134,5
AA grad EXO	4,45E+12	175,3
Whey EXO	1,89E+13	188,5
Whey SEC EXO	1,02E+12	147,6
Whey grad EXO	3,25E+12	166,2

Table 2. *Exosome particle concentrations and mean particle sizes. Samples measured in 6 replicates in 1:20 – 1:20 000 dilutions and averages presented in table.*

There are still particles larger than 220 nm in the samples even after the filtration. That would indicate the permeability of larger vesicles through the filtration membrane or the aggregation of the exosomes after the SEC. There is also the possibility of spontaneous exosome membrane fusion that would easily double the particle size. Aggregation of particles can be seen in the following TEM images along with even smaller particles visible in greater amounts in the samples contradictory to NTA analysis. The quantification by NTA alone is not sufficient to profile the isolated vesicles as seen from the differences of vesicle sizes in figures 5 and 6.

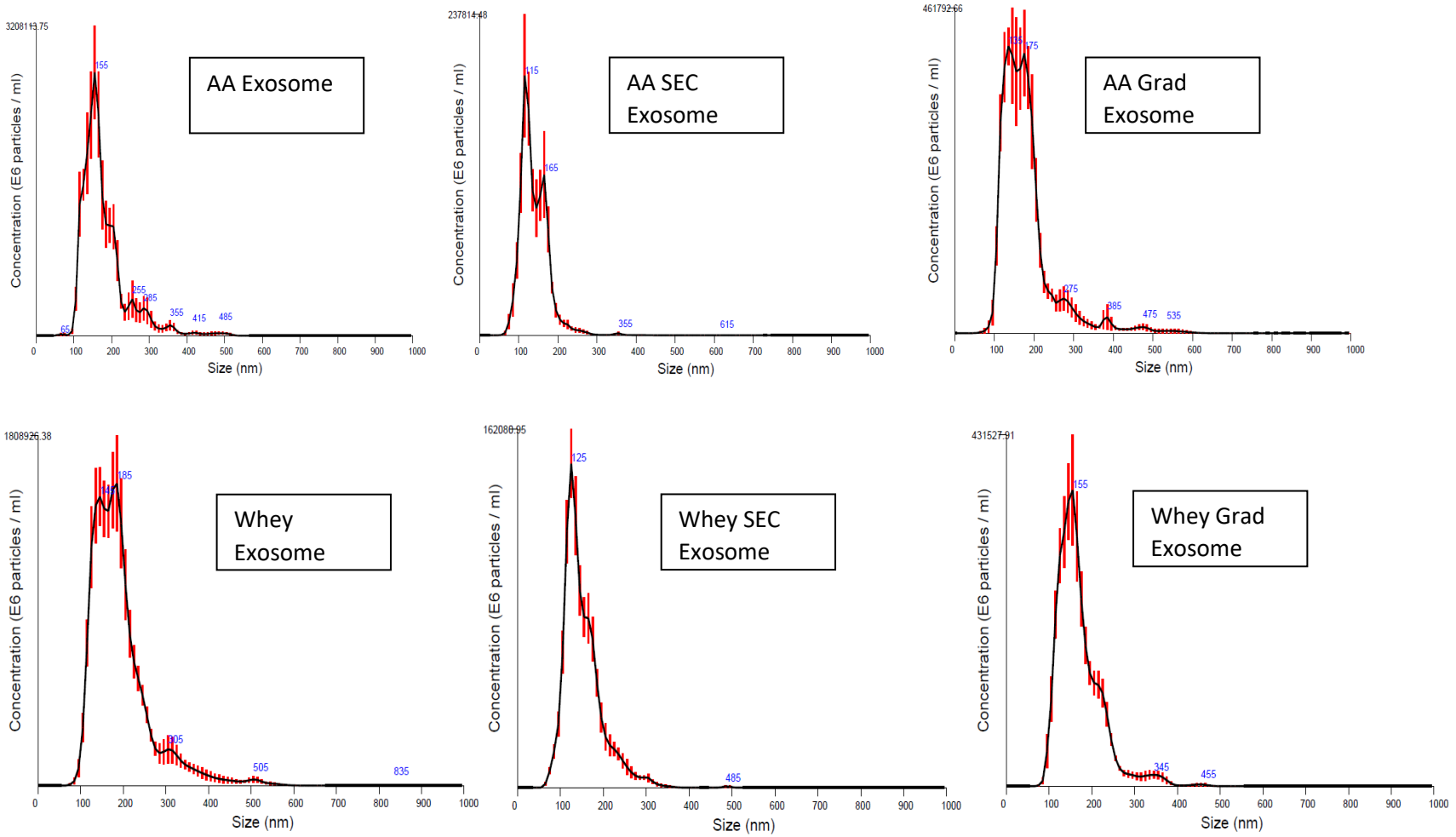

Figure 5. *Exosome particle distribution from NTA Samples from left to right are on the top 1. AA Exosome 2. AA SEC Exosome and 3. AA grad Exosome and on the bottom whey exosome particle size distribution from NTA. Samples from left are 1. Whey Exosome 2. Whey SEC Exosome and 3. Whey grad Exosome.*

Images from transmission electron microscopy show vesicles of different shapes and sizes. Smallest vesicles present in all samples range from 30 nm:s up that still present vesicular morphology with distinctive lipid bilayer characteristics. Vesicles of smaller size seem to be more prone to aggregate as seen from the clear groups and chains formed by the small vesicles (figure 6). The small sized vesicles may represent phospholipid micelles, or fat droplets commonly dispersed in milk. Largest particles present in the samples range up to 500 nm that could be plasma membrane-derived microvesicles or milk fat globules uniquely found in milk. Phospholipid trilayer surrounded milk fat globules contain fat that crystallizes in low temperatures leading to rigid bridge like structures inside vesicle as seen in figure 7B. However the differential centrifugation excludes majority of the bigger sized vesicles out from the final isolate and the seen larger vesicles can be result of membrane fusion during the isolation and storage of samples. Larger vesicle with inner smaller vesicles can be seen in the figure7B. Isolated exosomes present both cup-shape (figure 7D) and spherical morphology in all the samples with more structural distress seen in the SEC (figure 7E). This may be due to the 2 M urea concentration causing vesicle breakage due to osmotic shock. Casein can be seen in the images with higher electron density in the calcium phosphate bound in the protein. These micelles are visible in darker shades of gray as also seen by Léonil et al. (Marchin, Puteaux et al. 2007). The casein micelles can overlap the exosome structures indicating that the caseins are in interaction with the exosomes or encased to spontaneously formed membrane vesicles from disrupted cell membranes or milk fat globules.

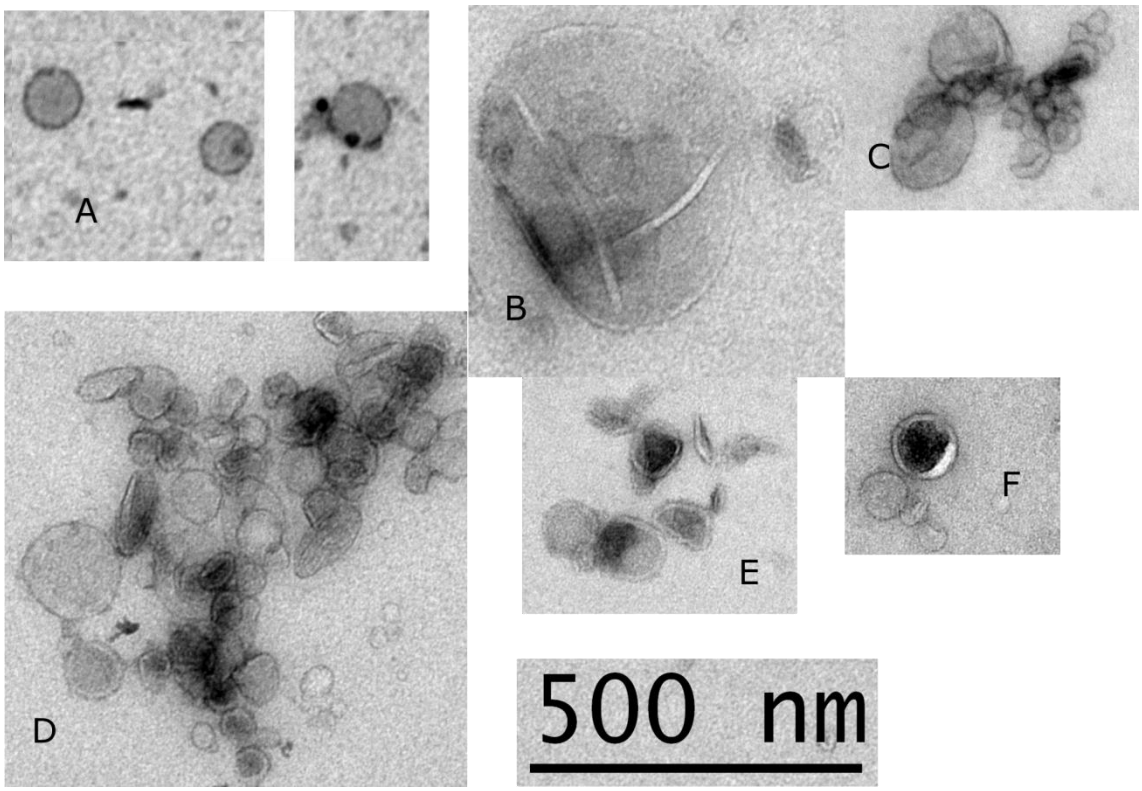

Figure 6. TEM images from six different isolation methods A) Whey exosome sample, with small vesicles present. In the right panel a vesicle labelled with two 10 nm gold particles with antibodies (Anti-CD63 and anti-goat (donkey 10 nm gold labelled)). B) Large vesicle with intraluminal vesicles present in whey SEC exosomes. This large vesicle could be a milk fat globule with rigid crystallized fat structures inside the vesicle. Milk fat globule fat will crystallize in low temperatures. C) Vesicles from whey grad exosome sample. Smaller vesicles have a tendency to aggregate as also seen in frame D. D) Isolation with differential centrifugation with acetic acid treatment (AA Exosome). Smaller micelles have a tendency of aggregating. Darker areas indicate higher electron density; casein is also present in sample. E) AA SEC exosome has vesicles with distressed morphologies present. Vesicle damage can be due to osmotic shock by urea in the first running buffer. F) Casein micelle encapsulated by a lipid bilayer in AA grad exosome sample. Scale of the bar in all of the images is 500 nm.

Smaller vesicles seem to have only single lipid layer suggesting that they are either phospholipid micelles or fat droplets from dissociated MFGs while larger vesicles show lipid bilayer. The smaller size will greatly restrict the content of the particles and the surface heterogeneity.

Antibody labelling was applied to separate exosomes from other particles present in samples. With the used CD63 it seems that CD63 is more prominent in smaller vesicles with more antibodies are bound to smaller (<100 nm) vesicles than to larger vesicles. However this may be due poor antibody specificity as seen in western blot analysis or problems with ratio of antibody and vesicles. The antibody labelling affected only a small fraction of vesicles with majority of gold labels binding to single antibodies per vesicle. Gold particles used in the secondary antibody are only present in interaction with membrane related particles and not with soluble proteins, such as high electron density containing casein for as example. However similar distribution of CD63 to smaller sized vesicles was seen in HeLa cell derived exosomes. (Colombo, Moita et al. 2013) Resulting CD63 labelled images can be seen in appendix 1 along with wide scale images of different isolation methods and in frame A of figure 6. Limitations of SEC purification can be seen in figure 6 frame B, with very large vesicle still present in sample after the SEC. This is due to the inability of the columns to separate larger than 5 MDa particles from each other.

3.3. Identification of exosome enriched markers

Milk samples were analysed with several different primary antibodies generally used in exosome studies for exosome protein marker identification. Successful identification of exosome enriched proteins was done with anti-Alix, anti-TSG101 and to some extent anti-CD81 (figure 7) and therefore identifying the isolated vesicles as exosomes. The tested CanX, CD63 and β -casein antibodies bound to almost all proteins present in the samples are could not therefore be utilised for the characterization of specific proteins. This was accompanied with absence of the correct sized bands with the CD63 and β -casein antibody (data not shown).

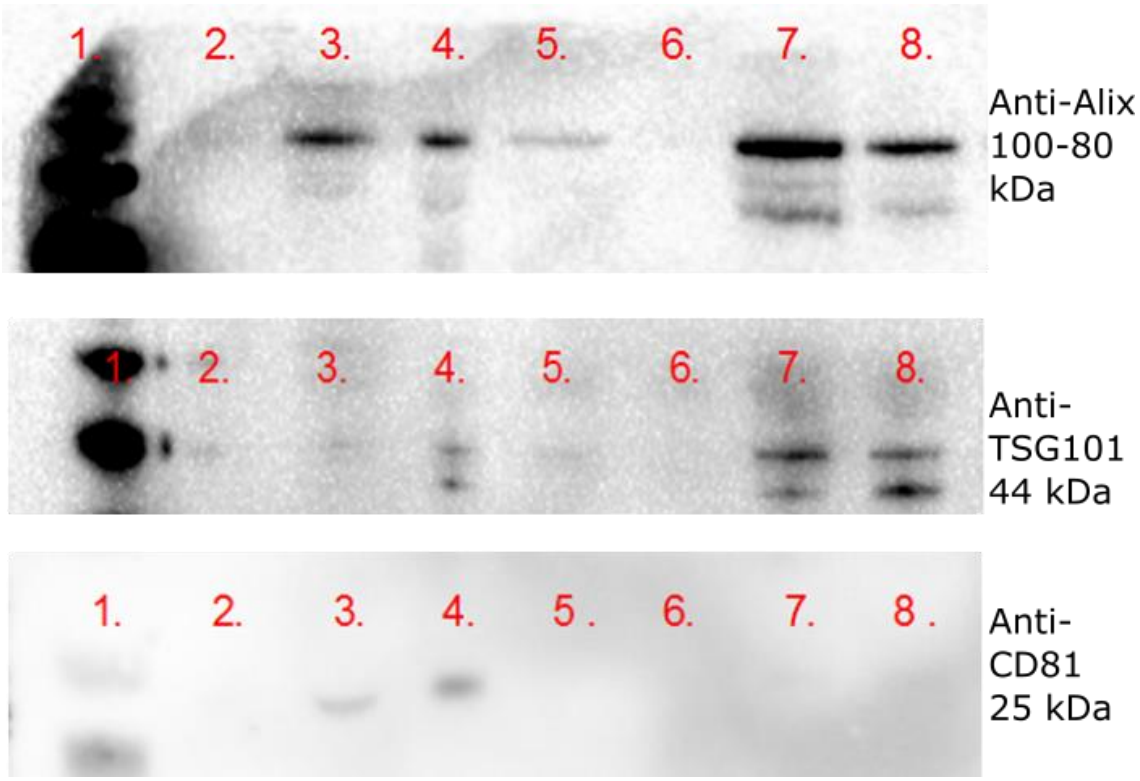

Figure 7. Western blot of milk exosomes with anti-Alix, anti-TSG101 and anti-CD81 antibodies. Top 3 bands correspond to Alix protein with size of 100-80 kDa. Anti-TSG regocnizes two bands in samples 4, 7 and 8. CD81 binds only to samples 3 and 4 with sufficient effiency for the chemiluminesence signal to be detected under the used parameters. Different location of the band is due to heavy casein concentration pushing CD81 band out of locus in sample 3. Casein concentration can be seen in figure 4. Samples are 1. Western C protein standard, 2. Raw Milk, 3. Whey exosome, 4. AA exosome, 5. Whey SEC exosome, 6. AA SEC exosome, 7. Whey Grad exosome, 8. AA Grad Exosome

Alix is 95 kDa protein by peptide sequence and the corresponding band with few others are clearly visible in all but AA-SEC exosome samples. Similar protein bands are presented on the Cell Signalling web site for anti-alix-reactivity (https://www.cellsignal.com/products/primary-antibodies/alix-e6p9b-rabbit-mab/92880?site-search-type=Products).

With TSG101 the quantity of bound antibody was much lower compared to anti-Alix resulting in lower intensity in the signal. Clear bands are only visible in AA exosome and gradient exosome samples with some faint bands in whey exosome and whey SEC

exosome samples. The intensity of the protein standard masks the more faint bands of the milk samples.

As seen with TSG101 the intensity of CD81 is lower than anti-Alix. The highest intensity of CD81 band is seen in the acetic acid exosome sample. SEC samples show no bands and really faint band is seen in gradient purified exosome samples. The different migration of CD81 bands is due to excessively high casein content still present in whey exosome sample as seen in figure 4. However the quantification of proteins via WB is only semi-quantitative and without specific protein standards to use in parallel the analysis should be done only qualitatively. Full western blot images are shown in appendix 2 with clear unspecific binding seen with all antibodies.

3.4. Proteome and functional analysis of exosomal proteins

63 proteins were identified in 5 separate mass spectrometry runs with different samples containing bovine milk exosomes. The identification was done with 3 or more unique peptides for all proteins identified. Resulting protein list was analyzed with DAVID and Panther online tools and 34 of 59 identified and gene mapped proteins were compartmentalized in extracellular exosomes as seen in table GOTERM. 50 of identified proteins were found to have molecular function (figure 8) and 20 of those proteins were described with catalytic activity. 23 of total 63 identified proteins were related to localization of proteins as seen in figure 9. On top of the resulting protein identifications there were also six uncharacterized proteins G5E5T5, G5E513, G3N0V0, G3N2D8, G5E5H5 and F1MCF8 present in the samples.

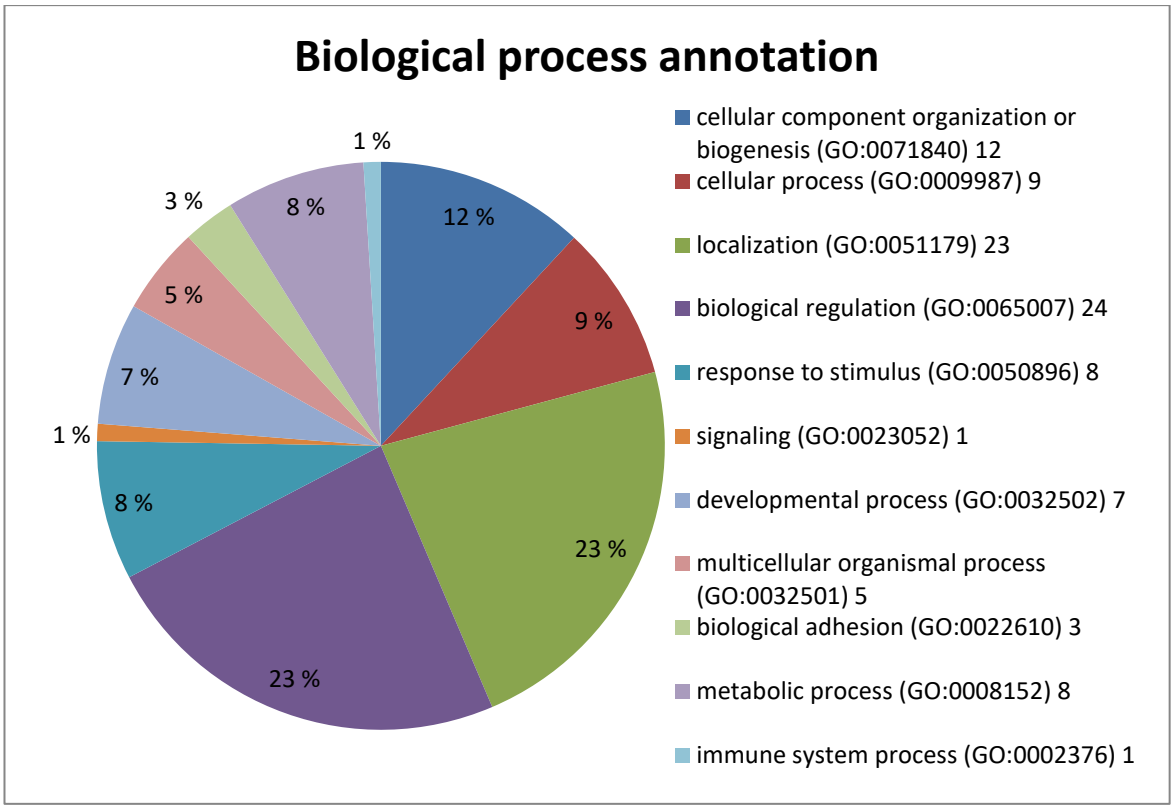

Figure 8. Proteins identified from exosomes mapped for their biological processes by PANTHER gene ontology.

The cellular compartmentalization of identified proteins localizes the proteins mainly to extracellular exosomes, plasma membrane, extracellular space and to focal adhesion. List of 7 most abundant hits of GOTERMS can be seen in table 3. This could indicate the pathway for exosome trafficking from cell to another and a possible messaging system for the target cell. With the amount of proteins identified the probability of random localization of analyzed proteins is relatively high in large categories such as plasma membrane, cytosol and extracellular space. This can be seen with the Benjamini corrected P-value of 2,1e-2 that is still statistically significant (p-value < 0,05) but the value is much higher than with the other GOTERMs. However the localization for term focal adhesion and extracellular exosomes are certainly specific for these proteins. This still does not rule out the possibility of falsely categorized proteins to the extracellular exosomes GOTERM. With mass spectrometer with higher sensitivity the list of identified proteins could be vastly improved and larger dataset would lead to better localization of proteins for their GOTERMS and biological processes.

Category	Term	Gene count	Benjamini corrected p value
GOTERM_CC_DIRECT	extracellular exosome	34	3.0E-14
GOTERM_CC_DIRECT	plasma membrane	17	2.1E-2
GOTERM_CC_DIRECT	extracellular space	16	1.4E-5
GOTERM_CC_DIRECT	focal adhesion	15	1.1E-10
GOTERM_CC_DIRECT	cytosol	15	1.1E-4
GOTERM_CC_DIRECT	membrane	13	5.8E-4
GOTERM_CC_DIRECT	extracellular region	11	1.1E-4

Table 3. Enriched cellular compartments for proteins identified from exosomes with their respective gene count and corrected p-value.

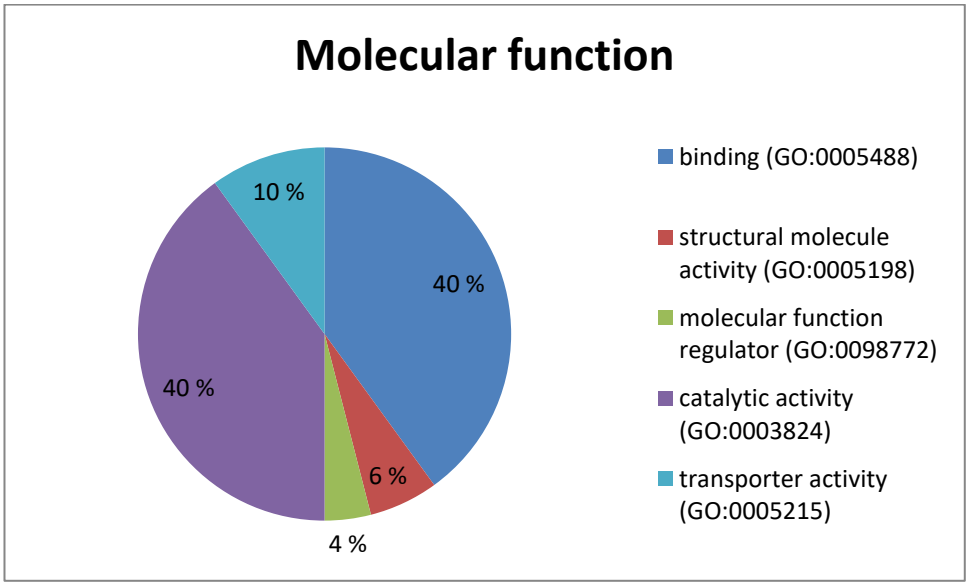

Figure 9. 50 of identified proteins found to have molecular function, mapped with PANTHER.

Entry name	Proteins	Gene names
A0A140T897_BOVIN	Serum albumin	ALB
A0A140T8A9_BOVIN	K -casein	CSN3
LALBA_BOVIN	Alpha-lactalbumin (Lactose synthase B protein) (allergen Bos d 4)	LALBA ALACTA
CASA1_BOVIN	Alpha-S1-casein (allergen Bos d 8) [Cleaved into: Antioxidant peptide]	CSN1S1

CASA2_BOVIN	Alpha-S2-casein [Cleaved into: Casocidin-1 (Casocidin-I)]	CSN1S2
CASB_BOVIN	β -casein [Cleaved into: Casoparan; Antioxidant peptide; Casohypotensin]	CSN2
CASK_BOVIN	K -casein [Cleaved into: Casoxin-C; Casoxin-6; Casoxin-A; Casoxin-B; Casoplatelin]	CSN3 CSN10 CSNK
LACB_BOVIN	β -lactoglobulin (β -LG) (allergen Bos d 5)	LGB
APOA1_BOVIN	Apolipoprotein A-I (Apo-AI) (ApoA-I) (Apolipoprotein A1) [Cleaved into: Proapolipoprotein A-I (ProapoA-I); apolipoprotein A-I]	APOA1
TRFL_BOVIN	Lactotransferrin (Lactoferrin) (EC 3.4.21.-) [Cleaved into: Lactoferricin-B (Lfcin-B)]	LTF
B2D1N9_BOVIN	ATP-binding cassette sub-family G member 2 (Breast cancer resistance protein)	ABCG2
F1MCF8_BOVIN	Uncharacterized protein	LOC112441499
F1MHI1_BOVIN	Perilipin	PLIN2
F1MUT3_BOVIN	Xanthine dehydrogenase/oxidase	XDH
F1N726_BOVIN	Glycoprotein 2	GP2
G3MYH4_BOVIN	Tetraspanin	CD81
G3N0V0_BOVIN	Uncharacterized protein	
G3N2D8_BOVIN	Uncharacterized protein	GGT1
G5E513_BOVIN	Uncharacterized protein	
G5E5H5_BOVIN	Uncharacterized protein	
G5E5H7_BOVIN	Uncharacterized protein	PAEP
G5E5T5_BOVIN	Uncharacterized protein	
NB5R3_BOVIN	NADH-cytochrome b5 reductase 3 (B5R) (Cytochrome b5 reductase) (EC 1.6.2.2) (Diaphorase-1) [Cleaved into: NADH-cytochrome b5 reductase 3 membrane-bound form; NADH-cytochrome b5 reductase 3 soluble form]	CYB5R3 DIA1
UBB_BOVIN	Polyubiquitin-B [Cleaved into: Ubiquitin]	UBB
FABPH_BOVIN	Fatty acid-binding protein, heart (Fatty acid-binding protein 3) (Heart-type fatty acid-binding protein) (H-FABP) (Mammary-derived growth inhibitor) (MDGI)	FABP3
GBB2_BOVIN	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β -2 (G protein subunit β -2) (Transducin β chain 2)	GNB2

ENPP3_BOVIN	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3 (E-NPP 3) (Phosphodiesterase I β) (PD-I β) (Phosphodiesterase I/nucleotide pyrophosphatase 3) (CD antigen CD203c) [Includes: Alkaline phosphodiesterase I (EC 3.1.4.1); Nucleotide pyrophosphatase (NPPase) (EC 3.6.1.9) (Nucleotide diphosphatase)]	ENPP3
BT1A1_BOVIN	Butyrophilin subfamily 1 member A1 (BT)	BTN1A1 BTN
HSP7C_BOVIN	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	HSPA8 HSC70
CD36_BOVIN	Platelet glycoprotein 4 (Glycoprotein IIIb) (GPIIIB) (PAS IV) (PAS-4) (Platelet glycoprotein IV) (GPIV) (CD antigen CD36)	CD36 PAS4
CD9_BOVIN	CD9 antigen (CD antigen CD9)	CD9
EZRI_BOVIN	Ezrin (Cytovillin) (Villin-2) (p81)	EZR VIL2
GDIB_BOVIN	Rab GDP dissociation inhibitor β (Rab GDI β) (Guanosine diphosphate dissociation inhibitor 2) (GDI-2)	GDI2 RABGDIB
ACTB_BOVIN	Actin, cytoplasmic 1 (β -actin) [Cleaved into: Actin, cytoplasmic 1, N-terminally processed]	ACTB
RAP1B_BOVIN	Ras-related protein Rap-1b (GTP-binding protein smg p21B)	RAP1B
RHOA_BOVIN	Transforming protein RhoA (Gb) (p21)	RHOA ARHA RHO12
GBB1_BOVIN	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit β -1 (Transducin β chain 1)	GNB1
PPIA_BOVIN	Peptidyl-prolyl cis-trans isomerase A (PPIase A) (EC 5.2.1.8) (Cyclophilin A) (Cyclosporin A-binding protein) (Rotamase A) [Cleaved into: Peptidyl-prolyl cis-trans isomerase A, N-terminally processed]	PPIA
RAC1_BOVIN	Ras-related C3 botulinum toxin substrate 1 (p21-Rac1)	RAC1
EF1A1_BOVIN	Elongation factor 1-alpha 1 (EF-1-alpha-1) (Elongation factor Tu) (EF-Tu) (Eukaryotic elongation factor 1 A-1) (eEF1A-1)	EEF1A1 EEF1A EF1A
GLCM1_BOVIN	Glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) (28 kDa milk glycoprotein PP3) (Lactophorin) (Proteose-peptone component 3) (PP3)	GLYCAM1
XDH_BOVIN	Xanthine dehydrogenase/oxidase [Includes: Xanthine dehydrogenase (XD) (EC 1.17.1.4); Xanthine oxidase (XO) (EC 1.17.3.2) (Xanthine oxidoreductase) (XOR)]	XDH

PIGR_BOVIN	Polymeric immunoglobulin receptor (PIgR) (Poly-Ig receptor) [Cleaved into: Secretory component]	PIGR
ANXA5_BOVIN	Annexin A5 (Anchorin CII) (Annexin V) (Annexin-5) (Calphobindin I) (CBP-I) (Endonexin II) (Lipocortin V) (Placental anticoagulant protein 4) (Placental anticoagulant protein I) (PAP-I) (Thromboplastin inhibitor) (Vascular anticoagulant-alpha) (VAC-alpha)	ANXA5 ANX5
ARF1_BOVIN	ADP-ribosylation factor 1	ARF1
5NTD_BOVIN	5'-nucleotidase (5'-NT) (EC 3.1.3.5) (Ecto-5'-nucleotidase) (CD antigen CD73)	NT5E NT5 NTE
RAB18_BOVIN	Ras-related protein Rab-18	RAB18
CIB1_BOVIN	Calcium and integrin-binding protein 1 (Calmyrin)	CIB1
RHOC_BOVIN	Rho-related GTP-binding protein RhoC	RHOC ARHC
NPT2B_BOVIN	Sodium-dependent phosphate transport protein 2B (Sodium-phosphate transport protein 2B) (Na(+)-dependent phosphate cotransporter 2B) (Sodium/phosphate cotransporter 2B) (Na(+)/Pi cotransporter 2B) (NaPi-2b) (Solute carrier family 34 member 2)	SLC34A2
RAB1B_BOVIN	Ras-related protein Rab-1B	RAB1B
CDC42_BOVIN	Cell division control protein 42 homolog	CDC42
RB11B_BOVIN	Ras-related protein Rab-11B	RAB11B
Q3SYR8_BOVIN	Immunoglobulin J chain (Joining chain of multimeric IgA and IgM)	JCHAIN IGJ
RAB7A_BOVIN	Ras-related protein Rab-7a	RAB7A RAB7
CD81_BOVIN	CD81 antigen (CD antigen CD81)	CD81
ABCG2_BOVIN	ATP-binding cassette sub-family G member 2 (Urate exporter) (CD antigen CD338)	ABCG2
MCP_BOVIN	Membrane cofactor protein (CD antigen CD46)	CD46 MCP
MUC15_BOVIN	Mucin-15 (MUC-15) (Component II) (Glycoprotein 4) (Glycoprotein C) (PAS3) (PASIII)	MUC15
MUC1_BOVIN	Mucin-1 (MUC-1) (CD antigen CD227) [Cleaved into: Mucin-1 subunit alpha (MUC1-NT) (MUC1-alpha); Mucin-1 subunit β (MUC1-β) (MUC1-CT)]	MUC1
MFGM_BOVIN	Lactadherin (BP47) (Components 15/16) (MFGM) (MGP57/53) (Milk fat globule-EGF factor 8) (MFG-E8) (PAS-6/PAS-7 glycoprotein) (SED1) (Sperm surface protein SP47)	MFGE8
PLIN2_BOVIN	Perilipin-2 (Adipophilin) (Adipose differentiation-related protein) (ADRP)	PLIN2 ADFP ADRP

IDHC_BOVIN	Isocitrate dehydrogenase [NADP] cytoplasmic (IDH) (EC 1.1.1.42) (Cytosolic NADP-isocitrate dehydrogenase) (IDP) (NADP(+)-specific ICDH) (Oxalosuccinate decarboxylase)	IDH1 ICDH

Table 4. Proteins identified with mass spectrometry. Proteins stained red are common milk proteins that are present in samples and not specific for exosomes.

4. Discussion

4.1 Isolation and morphology of vesicles

The molecular complexity of milk provides certain challenges in nanovesicle related work. Milk fat droplets can form with same density as exosomes and therefore will be located in the same fractions as exosomes in gradient centrifugation. These particles are accompanied with casein micelles or aggregates that spontaneously form in milk even after casein precipitation. These micelles can range from the size of single casein protein from 25 kDa to 60 - 200 nms of which normal casein micelles form (Fox, McSweeney 2003) or up to gel or cheese like complexes as done in the isolation steps. The casein micelles are highly stable and not affected by addition of urea. The used 20 mM EDTA concentration is relative to the amount of colloidal Ca^{2+} ions in casein micelles in bovine milk. (Fox, McSweeney 2003) Despite the efforts to remove majority of the soluble proteins there are still common soluble milk proteins present in the samples even after additional purification with SEC or gradient purification.

In SEC the urea concentration was assumed to be safe to not cause osmotic shock to exosomes, but from the TEM images can be seen the flaky and disrupted morphology in the SEC-samples. This is most likely from the osmotic shock caused by addition of urea accompanied with some shear stress from the chromatography column. EDTA was utilized in 20 mM concentration to dissipate formed casein aggregates to allow the separation with size as previously done by Blans et al. (Kristine Blans, Maria S. Hansen et al. 2017)

The separation of the protein fractions could be increased with stronger running buffers that could include SDS, EDTA, mercaptoethanol or DTT. However the SDS in the running buffer would lead to exosome membrane dissipation and loss of sample. The better resolution between the proteins is not mandatory since exosomes are recovered in

the void volume peak. However the difference between exosome peak and second peak needs to be clear for efficient purification of exosomes. This could be problematic with the pure K-casein close to the void volume peak but this seems to be different with isolated protein and a raw milk sample.

SEC provided a novel in-house identification of particles from the samples. With no NTA or other similar device available to identify vesicle size distribution the ÄKTA chromatography system with SEC columns provided a novel way of identifying particles from the samples in the void volume peak of the elution. This was due to the separation capacity of the columns and therefore provided a UV-based identification for larger than 5 MDa particles from samples.

The clear differences between the glycosylation of milk exosome proteins can be seen in the comparison between pro Q and sypro ruby stained protein gels. Glycosylation is crucial for function of exosomes and the loss of glycosylation will inhibit the functionality of exosomes. Use of acetic acid does not seem to have an effect on the glycosylation of the exosome proteins, since the intensities of proteins are comparable to the intensities of exosomes isolated by centrifugation alone. In this study we did not have the means to study the functional effects of the acetic acid treatments and therefore suggesting milder isolation protocol with differential centrifugation along with SEC to remove soluble proteins. This method could be utilised to isolate purer and possibly more native-like vesicles from milk. Glycosylated proteins are clearly enriched in all isolated samples compared to raw milk sample, with all samples containing 10 µg of protein loaded to the gel. The pro Q glycoprotein stain will also stain proteins with very high protein concentration and the staining of casein bands may be due to that nonspecific staining to some extent. K-casein with size of 18 kDa will be visible on the pro Q stained gels as K-casein can be glycosylated up to nine amino acid residues and can be described as a hairy protein due to the high level of glycosylation.

Nanoparticle tracking analysis and transmission electron microscopy are two recommended analytical methods for characterization of extracellular vesicles (evtrack.org). The limits of NTA accuracy can be seen comparing the results to the TEM images, since there are clearly smaller than 100 nm particles visible in all samples despite NTA showing basically no particles of this size. TEM images however confirm the aggregation of vesicles in groups and chain like formations especially in the smaller

sized exosomes. NTA is based on Brownian motion and light scattering of the particles and the resulting particle aggregates will have the characteristics of larger particles while still being built of smaller vesicles. This interaction is understandable since some of the identified proteins in isolated samples are related to cell adhesion and cellular component organization which could mediate the interaction of vesicles. The size differences between our set of samples compared to vesicles described in literature can be also due to vesicle swelling or shrinking in different solutions and different steps of storage conditions. As previously described casein is present in almost all samples as seen in TEM images. The interaction with the casein along with formation of chain like structures could explain the median size of vesicles being larger than 150 nm in all but SEC samples. The quantification of vesicles from the TEM images is not quantitative since the micrograms produced are just certain levels of the sample, not the complete 3D image.

4.2 Identification of exosome enriched markers

Antibodies used in western blot showed some levels of nonspecific binding but some migration differences could be due to the different glycosylation of identified proteins. Pro Q stained gel will provide challenges for identifying the correct bands on the membrane. This is accompanied by the lack of available protein standards to use in parallel to identify and quantify the correct protein bands in WB. Such protein standards could include CD81, CD63 or other exosome marker proteins to identify specific bands from the samples. Sample protein and standard marker concentrations need to be optimized, since the really intense bands of standard can mask the signal from the faint bands in the samples. Glycosylation also greatly influences the mobility of proteins in the electrophoresis and proteins can be located in different location than based on the protein size alone.

Still even without entirely specific antibodies, successful identification of exosomal marker proteins was done for some of the samples. Anti-ALIX antibody worked with the highest sensitivity and identification was successful from all but AA-SEC sample. Samples also contained exosomal marker proteins that were identified in mass spectrometry such as HSC70, CD81 and CD9 that could be utilized also in western blot to identify the vesicles as exosomes or to be coupled to some immuno-based isolation methods with only limiting factor being the proteins need to be membrane proteins to be

utilized in isolation. Mass spectrometry results also contain interesting uncharacterized proteins among with the enriched exosomal marker proteins. From the six uncharacterized proteins G5E5H5 has no described functionality and closest identified protein with only 78,5 % protein identity is UPF0472 protein C16orf72 (chromosome 16 open reading frame 72) from sperm whale, that has no described function. Homologous protein expressed in Zebrafish is related to cellular response to xenobiotic stimulus (uniprot entry A2BE76). The protein could be therefore related to antibodies as with the 4 other uncharacterized proteins.

The uncharacterized protein G3N2D8 is encoded by GG1 gene which is related to glutathione recycling and amino acid metabolism by similarity and gene ontology. Remaining 4 uncharacterized proteins are related to IgM antibodies according to BLAST results of amino acid sequences. It comes as no surprise that from the large quantity of immunogens present in milk fraction is also present in exosome fraction. Our data suggests that the immunogens should be localized in the vesicles and not soluble free proteins, since these would be discarded in the isolation method, first in the differential centrifugation step and second in the SEC to remove the remaining soluble proteins. This could be also one of the passageways for the mother to deliver specific immunogens to the infant as a bioavailable immuno-programming.

The amount of identified proteins is low due to the lack of systems sensitivity as previously Reinhardt et al identified over 2000 proteins related to bovine milk exosomes. (Reinhardt, Lippolis et al. 2012) The proteins identified in our experiments were also present in the most abundant proteins identified in analysis by Reinhardt et al. Contradictory to paper by Benmoussa et al we also identified xanthine hydrogenase in 100 000 xg isolated pellet compared to enrichment to fractions that they isolated by lower speed centrifugations. Some of our protein identifications could be thefore derived from co-isolated milk fat globules or fragmented milk fat globules with majority of xanthine hydrogenase is localized in milk fat globules. (Benmoussa, Abderrahim, Gotti et al. 2019) Due to the limitations of the used mass spectrometry system results are pooled from all the different isolation methods and not represented as separate lists. Major differences were found in the abundance of common milk protein related peptides between the isolation methods.

4.3 Further analysis and use of isolation protocols

As stated before the isolation procedure needs to suit the following experiments. The differences seen in the protein profile, vesicle concentration and morphology can influence greatly the suitability of the samples for different analyses. Retaining the glycosylation of exosome membrane proteins is crucial for working with cell or tissue models as an example (Kusuma, Manca et al. 2016). Keeping the limitations of different isolation procedures in mind it is mandatory to work with as pure exosome samples as possible to rule out false positive results. Discovering the complete proteome could enable the separation to separate subpopulations by their protein content as described previously by Kowal et al. (Joanna Kowal, Guillaume Arras et al. 2016) This set of proteome date could also be utilized to discard unwanted impurities in the isolation procedure. For further experiments with mass spectrometry combinatory purification with both SEC and gradient centrifugation could be utilized to exclude majority of unwanted impurities. Even further purified or sub-populations could be isolated with anti-body based methods to isolate specific vesicle populations for further analysis. This may result in loss of sample and therefore can be difficult to utilize for use in cell culture studies or feeding experiments. For these studies it would be more suitable to have a soluble protein mixture as a reference for the negative control utilized in the studies. However depending on the downstream analysis; metabolome analysis, RNA-sequencing or quantitative polymerase chain reaction analysis the experiments have to be highly controlled to identify the actual influence of the exosomes and their contents rather than the effect of cells being exposed to a stimulus.

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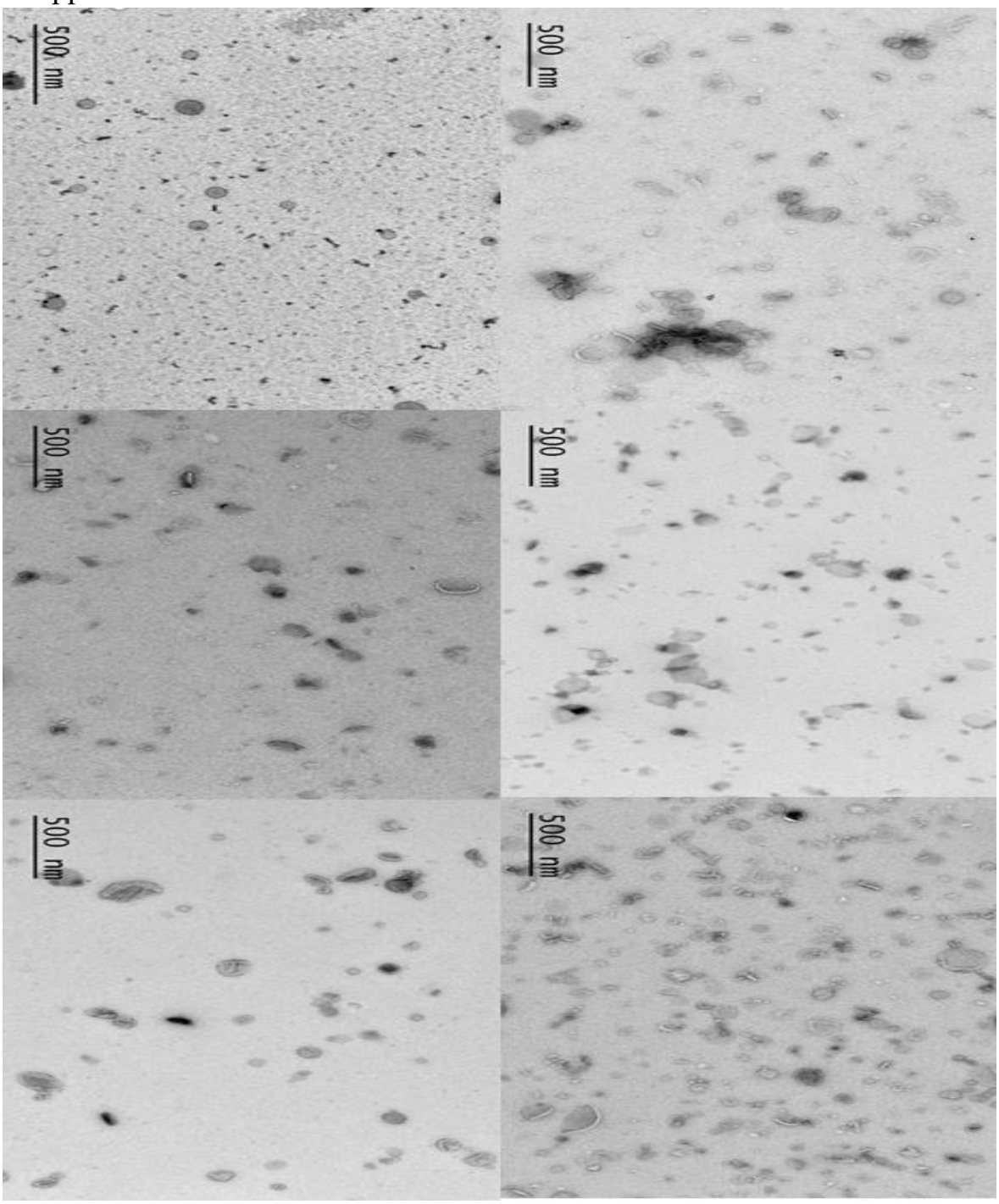
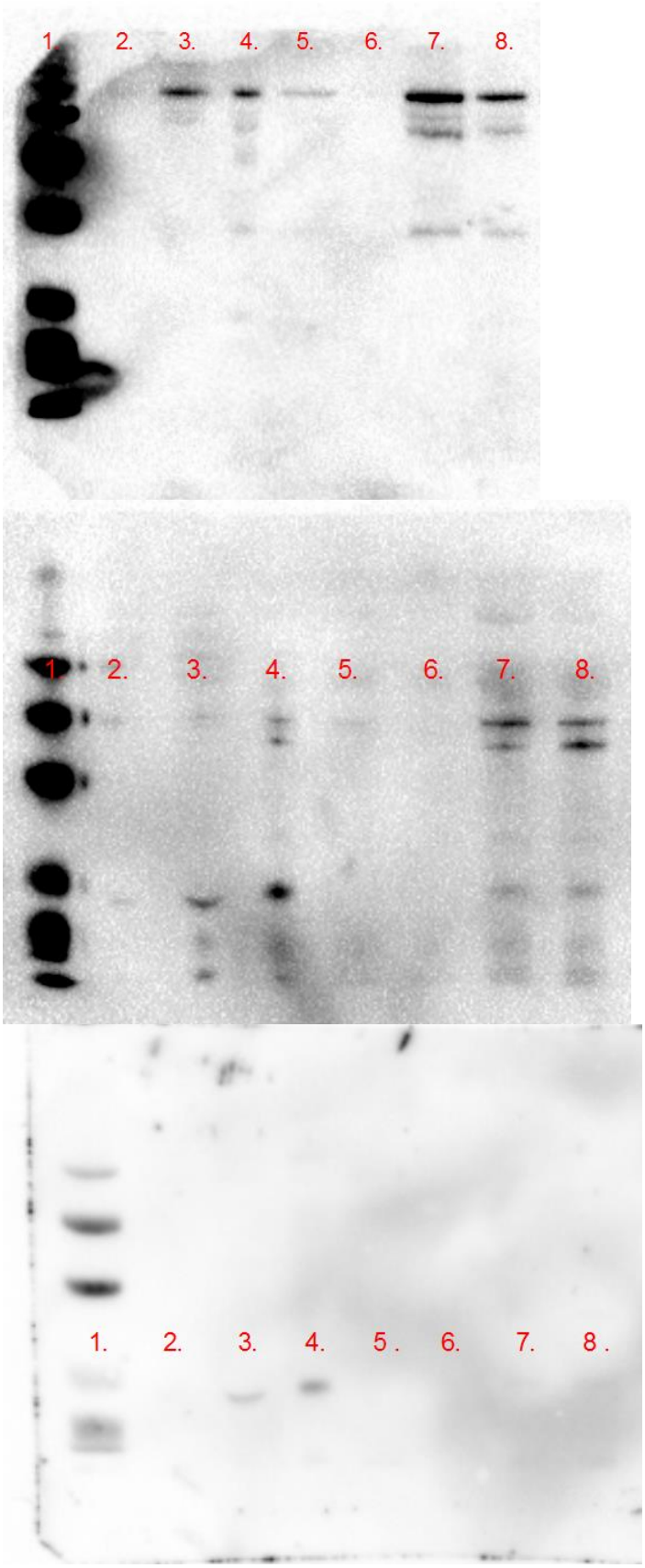
6. Appendix



Appendix 1. Wide screen images from TEM. From top left UC Exo, AA Exo, UC Sec Exo, AA Sec Exo, UC Grad Exo and AA Grad Exo.



Appendix 2, Wide screen images from western blot; top anti-Alix, middle anti-TSG101, bottom anti-CD81.