Diplomarbeit

Characterization of isolated human trophoblastic extracellular vesicles

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Zusammenfassung

Hintergrund:
Die menschliche Plazenta zeigt ein gewebespezifisches Expressionsprofil von extrazellulären Vesikeln vor, die während der Schwangerschaft in die mütterliche Zirkulation abgegeben werden. Kenntnisse über diese extrazellulären Vesikel, insbesondere über Exosomen aus plazentaren Trophoblasten, die bei physiologischen Zell-Zell Kommunikationen eine Funktion haben, sind spärlich und nicht ausreichend erforscht. Verschiedene Studien haben eine Korrelation zwischen Exosomen und pathophysiologischen Vorkommnissen während der Schwangerschaft nachgewiesen. Extrazelluläre Vesikel spielen wahrscheinlich bei der Entstehung von Krankheiten wie Präeklampsie oder Schwangerschaftsdiabetes eine große Rolle, deshalb ist ein besseres Verständnis des molekularen Aufbaus von extrazellulären Vesikeln fundamental.

Hypothese:
Die Proteinzusammensetzung von plazentaren Exosomen beeinflusst einerseits deren Größe und Funktion, andererseits könnte es auch Auswirkung auf die Wechselwirkung mit Zielzellen haben. Wir erwarten uns, dass über die Zusammensetzung der Proteine von Exosomen, welche aus mütterlichen Plazentaperfusaten und Plasma gewonnen werden, und der Funktion der Plazenta ein Zusammenhang besteht.

Methoden:
Mütterliches und fetales Plasma sowie Perfusionsmedium wurden für die Isolierung der extrazellulären Vesikel verwendet. Die spezifischen, mit plazentaren Exosomen angereicherten, Fraktionen wurde mittels mehrerer Differential-Zentrifugationsschritte auf Basis der Gradientendichte isoliert. Der Nachweis von Proteinen, die mit plazentaren Exosomen assoziiert sind, erfolgte mittels Western Blot und anschließender Analyse der Plasma- und Perfusatfraktionen.

Resultate:
Wir konnten nachweisen, dass keine extrazelluläre Vesikel plazentaler Herkunft im fetalen Plasma auffindbar sind. Extrazelluläre Vesikel aus der Plazenta konnten

aus mütterlichem Perfusaten isoliert und nachgewiesen werden. In allen mit Exosomen angereicherten Fraktionen wurden die spezifischen Proteine PLAP, Syntenin, TSG 101 und CD63 nachgewiesen.
Im Gegensatz dazu konnten weitere bereits publizierte Proteine nicht bestätigt werden: Die Exosom-spezifischen Proteine Integrin Alpha 6 und Annexin A2 wurden auf Mikrovesikeln von Plazentaperfusaten nachgewiesen, aber nicht auf Exosomen.

Conclusio:
Die von der humanen Plazenta sezernierten zellulären Mikrovesikel und Exosomen konnten aufgrund der unterschiedlichen Größe und ihrer Proteinzusammensetzung charakterisiert werden. Eine eindeutige Zuordnung der assoziierten Proteine ist aber aufgrund der Größenverteilung und ähnlicher Biogenese der Vesikel nicht möglich. Ob die Anreicherung von zellulären Proteinen an extrazellulären plazentaren Vesikeln mit möglichen Interaktionen an Targetzellen assoziiert ist, bleibt Gegenstand weiterer Untersuchungen.

Abstract

Background:

Trophoblastic cells of the human placenta release extracellular vesicles into the maternal blood. To date, the knowledge regarding human trophoblastic extracellular vesicles and in addition extracellular vesicles released to the fetus, especially exosomes, is sparse and insufficient. Different studies suggest an association between exosomes and pathophysiological occurrences during pregnancy. Extracellular vesicles may play an important role in intercellular communication. A better understanding of the composition of extracellular vesicles is therefore fundamental.

Hypothesis:

The protein profile of placental exosomes may not only influence their size and function. It might also effect systemic trafficking and responses of target cells. We are expecting to detect specific proteins on placental exosomes isolated from maternal plasma and feto-placental perfusates. In addition, an association between the composition of these extracellular vesicles and the function of the placenta is of interest.

Methods:

We used maternal and fetal plasma as well as maternal perfusate for our investigations. The isolation of exosomes from perfusates was performed out of collected samples from *ex-vivo* dual placental perfusion experiments. We subsequently isolated the exosomes by differential centrifugation and density gradient steps. The detection of specific exosomal and placental proteins were achieved by employing western blot analyses with antibodies targeting specific proteins of the vesicles.

Results:

We could confirm that no placental-specific extracellular vesicles are detectable within fetal plasma. Regarding the maternal perfusate, there is a correlation between the extracellular vesicles and placental origin. In all isolated exosomal fractions specific proteins namely PLAP, Syntenin, TSG101, and CD63 could be

detected. In contrast, other assessed proteins revealed a different result. Interestingly, Integrin alpha 6 and Annexin A2 proteins supposed to be expressed on all extracellular vesicles could only be detected on microvesicles but not on placental exosomes.

Conclusion:

Due to its different size and protein composition, we were able to characterize human placental microvesicles and exosomes. An exact assignment of the associated and assessed proteins is - based on the particle size distribution and similar biogenesis - not possible. It is of high importance that further investigations are carried out as there is still a lack of understanding regarding specific placenta derived exosomes and their characteristics.

It still remains unclear if proteins of placental extracellular vesicles are interacting with targeted cells.

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Glossary and abbreviations

AB	Apoptotic bodies
ESCRT	Endosomal-sorting complex required for transport
EVs	Extracellular vesicles
EX	Exosomes
FasL	Fas Ligand
GDM	Gestational Diabetes Mellitus
MV	Microvesicles
MVB	Multivesicular bodies
nm	Nanometer
NTA	Nanoparticle tracking analysis
PE	Pre-eclampsia
PLAP	Placental Alkaline Phosphatase Protein
PS	Phosphatidylserine
PSA	Prostate specific antigen
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TEM	Transmission electron microscopy
TSG 101	Tumor susceptibility gene 101
TRAIL	TNF-related apoptosis-inducing ligand
µm	Micrometer

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1. Introduction

Amongst its many functions, the placenta is responsible for the intercellular communication between mother and fetus. Trophoblasts, characterized as the first cellular barrier between the mother and the unborn, release extracellular vesicles (EVs) including apoptotic bodies (AB), microvesicles (MV) and exosomes (EX) into the maternal blood. There are many theories described that those vesicles could indeed play an important role for many physiological and pathophysiological aspects during pregnancy. Many efforts have recently been put into investigating how these different cellular fragments potentially interact with specific cells and its subsequent signaling. Nowadays, it is believed that EVs and especially EX potentially do have the capability of acting as biomarkers for both diagnostic and therapeutic successes (1).

In pregnancy, in many studies the occurrence of EVs derived of the human placenta has been shown, but it should also be mentioned that different isolation and characterization protocols were used for the isolation of placental EVs which hamper a comparison of results from functional assays (2).

The distinct composition of EVs may play an important role in intercellular communication, independently whether they act locally or at a distance from the origin cells. It is therefore essential to characterize those particles thereby getting new insights regarding their potential impact on different biological processes. For example, there is already data available suggesting that microRNA (mRNA) and proteins on EX may influence processes on target cells (1). While proteins might affect the cellular signaling, the mRNA modulates gene transcription and translation of targeted cells (3). However, a better understanding of the composition of these EX is necessary to get a deeper understanding of the particle functionality and potential clinical applications in this research area (1).

1.1. The human Placenta

1.1.1. Intercellular communication in the placenta

The placenta is responsible for the physiological exchange processes of gasses, nutrients and cellular waste between the fetal and maternal blood. The human placenta is a complex and dynamic organ. This endocrine active organ has been

neglected as source and model for different open research questions in pregnancy since it is considered as waste after birth. Due to improved molecular techniques over last decades the placenta has been put in perspective as an organ and model for physiological and cellular processes (4,5).

In fact, there is no direct communication between the fetal- and maternal blood circulations. The transport of oxygen to the fetus and backwards carbon dioxide and other metabolic wastes to the mother is based on the bidirectional transfer across the so called syncytiotrophoblastic layer which lines the chorionic villi (6). In addition, the placenta fulfills also several other functions. These include the secretion of endocrine factors, cytokines and growth factors into the maternal blood which may affect both, the mother but also the fetus (7). Furthermore, the placenta possesses regulatory effects linked to immune tolerance processes which is of high importance to ensure that the fetus is accepted by the maternal organism (8). Finally, as already mentioned, the placenta releases EVs such as EX into the maternal circulation. Specific aligned characterization and isolation protocols of EVs are still lacking (1). The focus of my thesis is to work on an extended characterization of EX of trophoblastic origin.

1.1.2. Morphology and physiology of the human placenta

The placenta has a weight of about 470g and the form of a thick disc. It is connected to the fetus via the umbilical cord that contains two umbilical arteries and one umbilical vein (5,6). After the end of fourth month placental growth, the tissue consists of a fetal part, the chorion, and a maternal part, the so called decidua (9).

The compact decidua plate is well grown together with the uterine muscle. The basal plate on the maternal side is structured in subunits called cotyledons. These cotyledons are formed by internal decidual septa that extend from the basal plate into the intervillous space (9). Between the chorion and the decidua plate there is a villous tree structure, which is formed by vessels branching out between those two plates. The intervillous space is filled with blood that originates from maternal spiral arteries. More precisely, the substances from the mother are transported via the maternal blood to the intervillous space and the syncytiotrophoblasts, the epithelial covering of the placental villi. This is exactly where the exchange takes place. The blood drains back through venous orifices to the uterine vein (6).

The fetal side of the human placenta is composed of the umbilical cord, that is located in the center of the tissue. Large vessels originating from the cord spread across the whole chorion plate in order to distribute the blood across the tissue. Also the chorion plate is covered by the thin amnion (6).

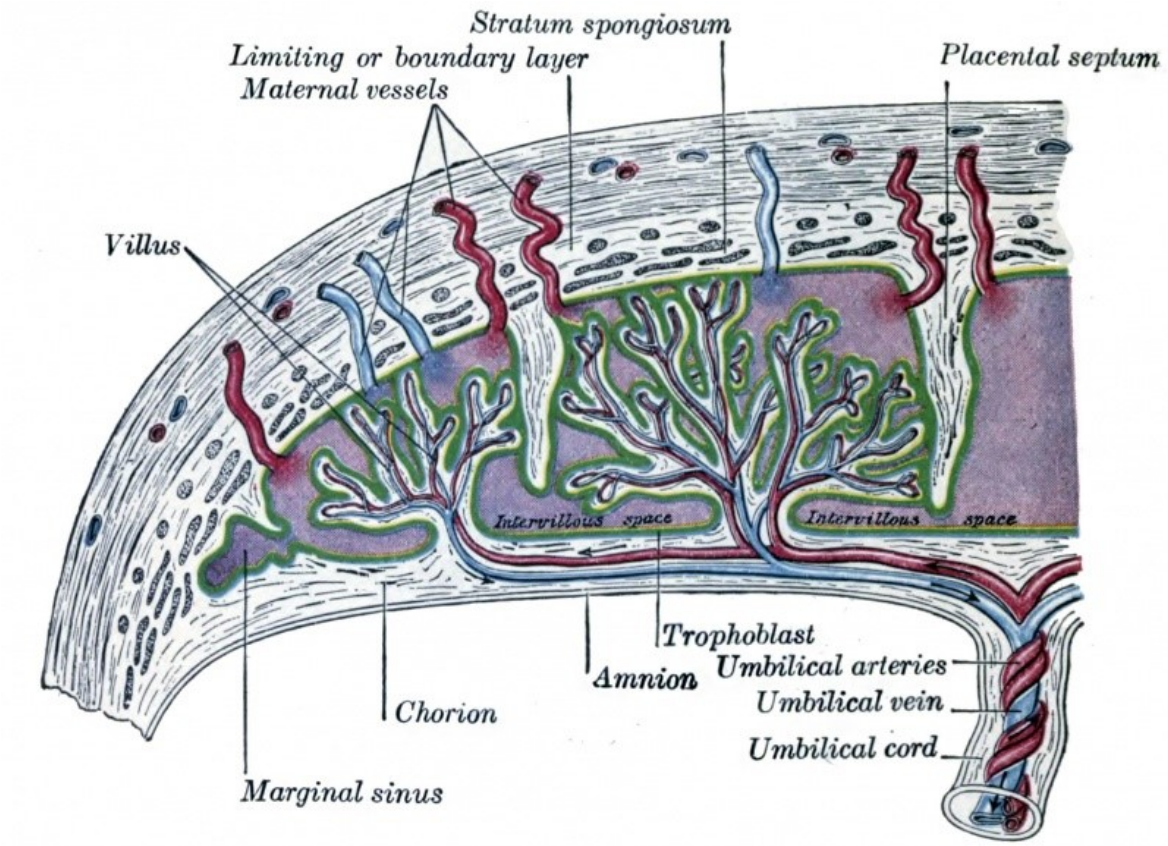


Figure 1 Illustration of the anatomy of the placenta (10)

1.1.3. Placental transfer of substances

The structure of the placental tissue allows to diffuse actively or passively substances through this biological multicellular barrier. Within the placenta, many permanent or inducible cytochrome P-450 isoenzymes are expressed which facilitates to metabolize substances and thereby decreasing the fetal exposure (4). The transport of substances from the maternal to fetal circulation depends on many factors such as molecular weight, ionization and lipid solubility (11). Substances have to pass several barriers from the maternal to the fetal circulation and *vice versa*. In fact, the substances have to pass an intricate histological barrier – first the syncytiotrophoblasts, then the villous stroma and finally the fetal capillary wall. There are many different variables that affect maternal to fetal transfer: (i) maternal plasma concentration, (ii) carrier-protein binding of the substance, (iii)

receptor expression, (iv) maternal blood flow rate within the intervillous space, and available trophoblast surface area for exchange, represent just some of them. The substances which pass the placental barrier by diffusion possess a molecular weight less than 500 Da. This is the case for oxygen, carbon oxygen or water. Other substances like insulin or steroid hormones pass the placenta very slowly. Moreover, hormones originating from the placenta enter the maternal and/or fetal circulation, but interestingly with a different concentration in both the fetal and maternal circulation (6).

1.2. Extracellular vesicles

Beside metabolites, proteins, lipids and polysaccharides, the systemic circulation of multicellular organisms contains mobile membrane-limited vesicles. Those group of called EVs include AB, MV and EX (12). EVs play an important role in many intercellular processes and may contribute to pathophysiological effects. Thus, in the literature all these components are often differently described which to some extent leads to confusion (13). Among EVs, EX have received the most attention in the last years (14).

To date, cell to cell contacts as well as secreted molecules are all well described, but EVs are a third group particles with the capability of intercellular communication that may lead to many new discoveries (15).

Most human cells produce EVs that usually contain proteins, lipids and different RNA species. This might on the one hand be responsible for the spread of different diseases. However, on the other hand there is big potential for therapeutic treatment using EVs (16).

There are still many unknowns in distinguishing between MV and EX. Thus, there is already fundamental knowledge about EVs and also about the size, morphology and composition of each group (15). Table 1 shows an overview of specific differences between AB, MV and EX.

Table 1 Overview of the different characteristics of known extracellular vesicles, modified from György, Szabó et al. (12)

	Exosomes	Microvesicles	Apoptotic bodies
Size range	50-100 nm	100-1.000 nm (100-400 nm in blood plasma)	1-5 µm
Mechanism of generation	By exocytosis of MVBs	By budding of the plasma membrane	By cells from blebs of cells undergoing apoptosis
Isolation	Differential centrifugation and sucrose gradient ultracentrifugation 100.000-200.000g, vesicle density 1.13 - 1.19 g/mL	Differential centrifugation 18.00-2000g	Most studies use co-culture with apoptotic cells instead of isolating apoptotic bodies
Detection	Transmission electron microscopy, western blotting, mass spectrometry, flow cytometry (bead coupled)	Flow cytometry, capture based assays	Flow cytometry
Best characterized cellular sources	Immune cells and tumors	Platelets, red blood cells and endothelial cells	Cell lines
Markers	Annexin V binding, CD63, CD81, CD9, LAMP1, TSG101	Annexin V binding, tissue factor and cell-specific markers	Annexin V binding, DNA-content

1.2.1. Molecular composition of EVs

The protein content of EVs is based on endosomal associated proteins such as Rab GTPase, SNAREs, and annexins of different cell types. EVs are enriched in many plasma membrane proteins such as tetraspanins (e.g. CD63, CD81). Other studies show that EVs are composed of proteins that are associated with lipid rafts, including glycosylphosphatidylinositol-anchored proteins and flotillin. Especially for EX it has been shown that they are highly enriched in fatty acids such as cholesterol, sphingomyelin, and hexosylceramides. Concerning MV, there is not that extensive knowledge about their lipid or protein composition and whether there is an enrichment compared to the cell of origin (15).

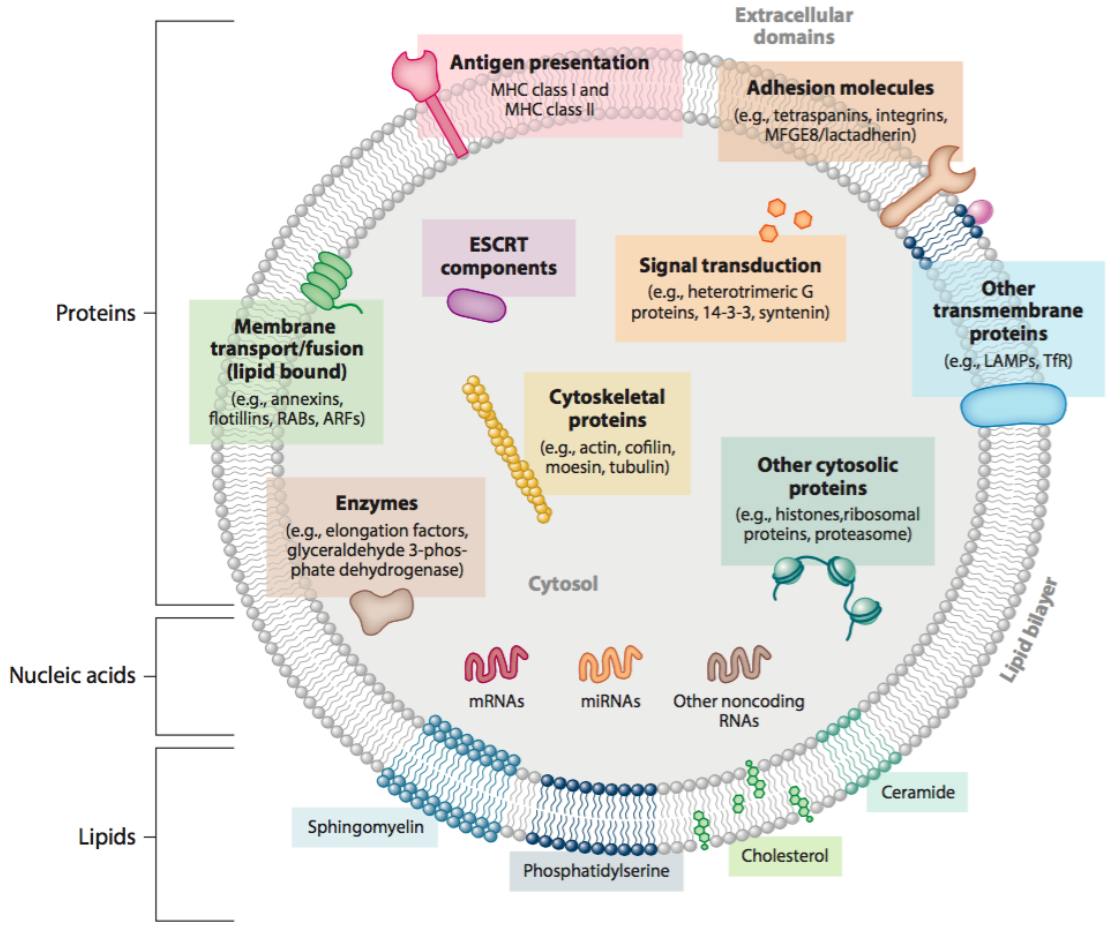


Figure 2 Schematic illustration demonstrates the main composition of EVs and membrane orientation of EVs. – reproduced with permission from Prof. Graca Raposo (17)

1.2.2. Physiological functions of extracellular vesicles

EVs play an important role in the intercellular communication both between local or distant cells. EX also possess an immunological function by acting as antigen presenting vesicles (16).

Like other cells, tumor cells also release EX and MV and it was shown that this may lead to angiogenesis and tumor cell migration in metastases. Such tumor cells, with their immune-suppressive molecules, can inactivate immune cells and suppress immune responses. Regarding EVs originating from intestinal epithelial cells, they can be involved in antigen presentation in inflammatory conditions – even at a distance (15).

1.2.3. Apoptotic bodies

The key difference between AB and other EVs is that they are only produced during programmed cell death (18).

Apoptosis is a normal process caused by the aging of cells and it is intended to maintain cell populations in tissues. In fact, it is also an important defense mechanism when cells are damaged or during immune responses. There are also different apoptotic stimuli and different conditions which induce apoptosis (19).
AB themselves are released as blebs of dying cells (12). They have a size between 1000 to 5000 nm. Their main markers are Annexin V, DNA and histone. However, there is no standardized protocol for the isolation of AB. In general, they are the final step of controlled cellular fragmentation and contain cell organelles such as mitochondria or ribosomes (13,18).
AB are usually phagocytosed by macrophages. This generally happens near the apoptotic cell. The reason for this is that phagocytosis is induced by the interaction of recognition receptors on the phagocytes and altered composition of the AB's membrane proteins. The DNA fragments of AB can eventually lead to genetic information transfer (18). Indeed the horizontal transfer of oncogenes, the presentation of T-cell epitopes upon uptake by phagocytic cells, and representation of B-cell autoantigens are further key functions of AB (12).

1.2.4. Microvesicles

It is important not to use the same terms for EVs and MV, as the latter are only a subpopulation. The size of MV is described by 100-1000 nm in literature and there are also different morphological shapes within MV (13). In general they tend to be a larger, more distinct, and more heterogeneous population (16). Like EX, MV carry proteins and RNA. They can be detected by specific markers like flotillin-2, selectins, integrins, metalloproteinases and phosphatidylserines located on their membrane. Their formation can be described as an outward budding of the plasma membrane (13). In fact, the separation of MV is done by the contractile machinery, which facilitates the approach of two opposing membranes towards each other. Then the membrane is cut on specific regions of the membrane. This process can only be found on specific places of the plasma membrane as specific molecules like lipids and proteins must be contained to ensure the survival of the released MV (20). The release is induced by activation of receptors on the cell surface combined with an increase of intercellular Ca^{2+}. MV are reported to mainly be products of platelets, red blood cells and endothelial cells. The key functions of MV, amongst others are: having a pro-coagulant activity, secretion of IL1b, contribution to the pathogenesis of rheumatoid arthritis, induction of oncogenic

cellular transformation, and feto-maternal communication (12). Similar to bacteriophages and viruses, MV could also have an important role in the horizontal gene transfer (16).

1.2.5. Exosomes

Dr. Johnstone was the first one who observed EX during the golden era of electron microscopy. The term was chosen due to similarities with “reverse endocytose” (18). EX are defined as membrane-bound vesicles in a size range of 50-100nm that contain specific endosomal protein markers. In addition to this, their density is described as being 1.12-1.19 g/mL (21).

Regardless of their origin, nearly all of them contain proteins involved in membrane transport and fusion (e.g. Rab GTPases, annexins, flotillin), multi-vesicular body biogenesis (e.g. Alix and TSG101), processes requiring heat shock proteins (hsc70 and 90), and integrins as well as tetraspanins (e.g. CD63, CD9, CD81 and CD82) (14).

They differ from other EVs regarding their biogenesis and are also specific for their endosomal origin (14). As EX contain cellular signals from microRNA, proteins, and lipids, they have the ability to potentially treat conditions such as cancer, autoimmune diseases, and pregnancy related diseases. The biogenesis and the secretion of EX is already well described. Nevertheless, knowledge of their uptake into immune and non-immune cells like cancer cells still has to be investigated (22).

On the ExoCarta database an overview of most of the molecules which are known to be present in EX can be found (23).

Biogenesis of exosomes

The formation of EX requires the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), Rab, coat complex subunit and Sec-1 proteins (22).

Basically, the process starts with an endosomal complex that contains different intraluminal proteins and RNAs and leads them to their proper destinations: either lysosomes or cell surface membranes (18).

First, multi-vesicular bodies (MVBs) are constructed by invagination of endosomal limiting membrane into the lumen. These MVBs already contain RNA and proteins delivered from the plasma, but proteins can also be added from the Golgi complex

by two different pathways: either by the endosomal-sorting complex required for transport (ESCRT) machinery or by a ceramide/tetraspanin-dependent pathway. Depending on activation signals, MVBs dock onto the plasma membrane. Rab, SNAP and SNARE proteins regulate the subsequent fusion. There are also some EX that are directly released by fission of the plasma membrane (22).

Generally, we can divide endosomes into three distinct stages: Early endosomes (A) are formed within the endosomal network and can either lead to recycling endosomes (B) or to late endosomes (C) by intake of multiple small intraluminal vesicles. For this process the amount of special membrane proteins called tetraspanins has to increase. CD9 and CD63 are two tetraspanins that play an important role in the EX pathway and they can furthermore be used for EX identification. TSG 101 and Alix are also among those markers that are enriched in EX, but more data is still needed for a secure determination (18).

The endosomal sorting complex required for transport (ESCRT) machinery also has other important roles in this pathway. ESCRT can be divided into 4 multi-protein complexes: ESCRT 0, ESCRTI, ESCRTII, ESCRTIII. PIP3, ubiquitinated cargos, and the curved membrane topology are essential for ESCRT-I and ESCRT-II related functions. They are indeed responsible for the budding of the membrane and in addition to this, ESCRT completes the budding. For ESCRT, Alix is an essential recruitment protein (18).

Subsequently, the late endosomes fuse with the plasma membrane and release EX into the extracellular space (18).

Different forms of EX biogenesis are associated with different cell types and further research is needed to fully understand the differences (14).

MVBs regulation of EX secretion: It is still not described if different MVBs emerge together or if they originate independently. In addition to this, neither the morphology nor the composition is fully defined and further research has to be done. The largest group of MVBs usually fuse with lysosomes; sometimes they can also be a temporary storage of proteins. However, the distinct MVBs also have different regulatory systems for fusion with the plasma membrane. The hypothesis exists that fusion is regulated by Ca^{2+}. On the other hand, autophagic conditions prevent EX secretion by leading MVBs to fusion with autophagic vacuoles (14).

It is also important to mention that the often confusing terminology of EX and MV can be prevented by referring to the biogenesis of those EVs (18).

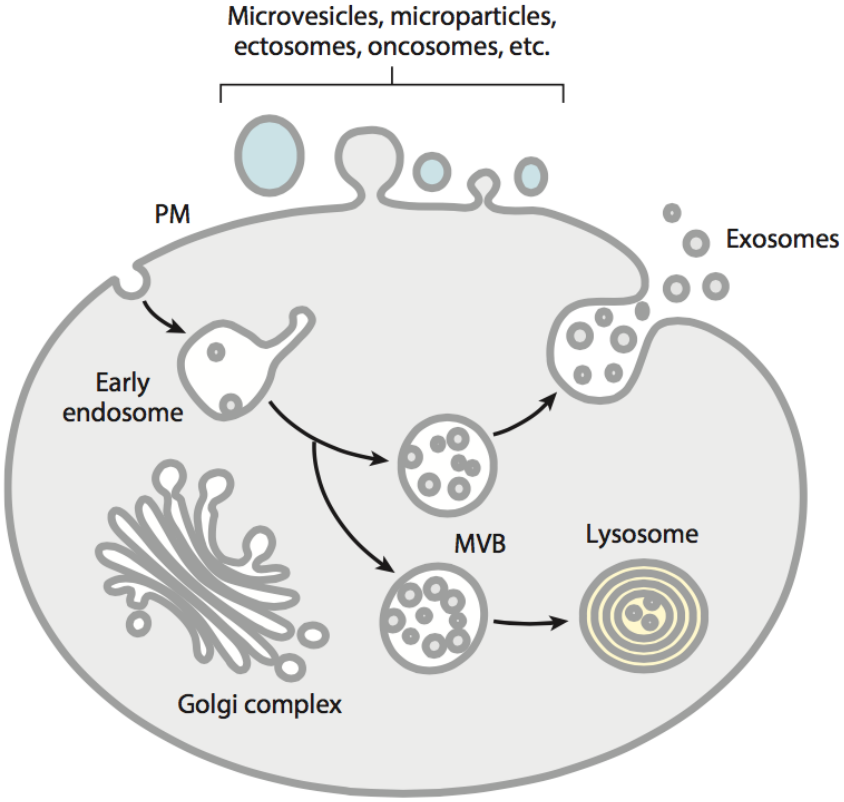


Figure 3 Different intracellular formation pathways of EVs are depicted, either by direct budding from the plasma membrane (PM) or by fusion of internal multivesicular bodies (MVB) with the PM – reproduced with permission from Prof. Graca Raposo (17)

Characteristics of exosomes

In general, EX carry proteins, RNAs, lipids, and enzymes that are all known as biofunctional molecules. The bilayer of EX is delivered by parental cells. Within this layer there are tetraspanin proteins, heat shock proteins, and some specific proteins of endosome membrane origin such as Alix and TSG101. The rigidity is higher than in MV due to their high protein content. Tetraspanin proteins within their membrane as well as Alix and flotilla are distinctive for EX (13).

The protein composition could be based on the cell type but in most cases the cell type does not influence the protein cargo. In fact, the majority of the proteins is based on the endosomes, the plasma membrane and the cytosol. Both inflammatory processes and hypoxia were observed to have a big effect on the protein composition (17).

To specify the case for placenta delivered EX, they contain fibronectin, which can lead to pro-inflammatory processes; especially in the early stages of pregnancy (24). Like MV, EX can also play an important role in genetic cross-talk (16).

When it comes to the lipid characteristics, there are few studies that are suggesting an enrichment of sphingomyelin, PS, and cholesterol, and in general of saturated fatty acids (17).

There is also a diverse repertoire of nuclide acids within EX: different small RNA like mRNA, and miRNA of various sizes, with low or undetectable levels of ribosomal 18S and 28S RNA can be found (17).

EX contain different components necessary for potential cell fusion. Syncythin-1 as a component of EX could play an important role in cell fusion. For the placenta this would mean the fusion between cytotrophoblasts. This theory is based on the fact that Syncythin-1 induces a specific fusion process as the bilayer of both cells start to fuse. This leads to the exchange of lipids (e.g. phosphatidic acid); ideally in the presence of calcium. In fact, calcium binds to phosphatidic acid, specifically to the phosphate part of the molecule and initiates the lipid interdigitation (24).

1.2.6. Exosomes in clinical diagnostic and therapeutic approaches

The better understanding of EX may be used for many clinical aspects in the near future. Both for clinical and therapeutic reasons, there are several theories regarding EX (25).

Due to their rich content of different molecules, EX are interesting for many reasons. They could potentially give us important information about their paternal cells and considering that, this could also be used to implement a potential new screening method. But patient profiling should also be taken into consideration. There are already some viral and infectious diseases that are reported to be associated with EX. As previously described, EX are very small nanoparticles and not all of them are identic, even if they have the same parental cell. However, the different compositions and biomarkers of EX could be a useful tool for identifying specific pathologies. Therefore, research is focused on finding specific biomarkers that are associated with specific diseases and also well-known markers like prostate specific antigen (PSA) are being discussed (25).

miRNA within EX is described to be associated with oncological diseases and could therefore be used as an important marker. According to Rabinowitz et al. lung cancer leads to an enrichment of miRNAs within EX. It is also important to

note that recent data shows specific protein patterns of EX for cancer diseases. A study by Liang et al. already found some tissue specific proteins for ovarian cancer that could play an important role for early diagnostics (25).

There have been many efforts lately in which EX or EX mimetic nanoparticles are used in therapeutic approaches. The idea behind this is to put specific molecules on them that should target different organs. Eventually this could be used for the delivery of therapeutic agents to specific targets. For example, there has already been success in delivering siRNAs via EX to mouse brains. These investigations were testing a therapeutic approach to Alzheimer's disease. There are also studies using EX for miRNA transport to breast cancer cells. Finally, there are studies that prove the loading of EX with conventional drugs is possible. Despite promising results, there is still a long way to go until a clinical application of EX will be possible. There are many issues that are still not clear. For example, a better understanding is needed of the organ/tissue specific tropism of different sub-populations of EX, the mechanisms of cellular uptake, the efficiency of targeting strategies, and the degree of integrity of the shuttled cargo (25).

1.2.7. Isolation of exosomes

One of the key challenges regarding EVs is the lack of standardized methods for isolation and analyzation (15). A multi-step procedure is needed to isolate EX as they need to fulfill different criteria (diameter, protein markers, and density). Therefore, differential isopycnic centrifugation, as well as the characterization of the particle size (e.g. NTA) and endosomal markers (e.g. immunoaffinity quantification) is necessary. There are different methods for extracting placenta-delivered EX that are being used by different research groups: culture of trophoblast cells (primary cell and cell lines), placenta perfusion, plasma, and urine from pregnant women. Unfortunately to date there is still a lack of standardized protocols on how to isolate placenta delivered EX and better knowledge regarding the characterization of EVs is needed (21).

1.2.8. Placental derived exosomes

It has been widely observed that EX are released during the first trimester of pregnancies, whereas MV also tend to be released during the other stages of pregnancy. The reason for this could be the different aspects of the placenta and

its functions: EX contain the immunosuppressive molecule HLA-G and other MV can lead to a pro-inflammatory state during a healthy pregnancy (24).

We know that already after 6 weeks of pregnancy the placenta releases PLAP-targeted EX into the maternal circulation (21). It has also already been described that EX from primary human trophoblast cells show resistance to viruses that affect other non-placental cells (1). Furthermore, Salomon et al. have shown that during a healthy pregnancy the amount of both placental and other EX increases significantly with the gestational age (21).

The exact function of those EX is still being investigated and there are many hypotheses regarding materno-fetal communication. In fact, the role EX play regarding immune-tolerance has been proven by available data. There is also evidence that pathological circumstances like preeclampsia during pregnancy are associated with changes in the release and concentration of EX. In this case Vargas et al. found a decrease of syncytin-2 in CD63 positive vesicles. These interesting findings lead to potential future role of EX. First, EX could predict potential pathological outcomes during early pregnancy. As already mentioned, placenta delivered EX can be detected easily by specific markers and this could be used as a prognostic tool. A screening test could identify women that are at risk in an early stage of pregnancy and could be used to reach a better outcome. Second, there is the idea of specific targeted EX that could be used to reach specific organs. As we already know EX tend to influence biological processes like immune-tolerance or cellular adhesion (21).

Placenta-derived EX also tend to indicate vasculogenesis and angiogenesis. This is essential for both the placenta, as well as for fetal circulation (26).

Table 2 Characteristics of trophoblastic EVs: overview of the most common components (1)

Characteristics of trophoblastic EVs
Distribution of phospholipids
There are about 179 detected species of phospholipids including the major ones such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SPH), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidic acid (PA), bis-monoacylglycerophosphate (BMP), cardiolipin (CL), lysophosphatidylcholine (LPC), and lysophosphatidylethanolamine (LPE). The main difference is that EX consist of phospholipids with a high level of membrane stability compared to the other EVs. In fact PC, a constitutive phospholipid of stable membrane lipids bilayer, was the highest component in EX (1).
miRNA landscape
The placenta specific C19MC miRNA is enriched within trophoblastic derived EX. In addition, all the three EVs and primary human trophoblast cells have similar miRNA contents (1).
characterization by proteomics
According to Sadovsky et al. there are about 1684 different proteins in trophoblastic EVs. Most of them are similar within different EV groups. However, there are some specific surface proteins like integrin alpha 6 or integrin beta4 as well as other tetraspanin members that are significantly enriched in EX (1).

1.2.9. Exosomes and medical disorders in pregnancies

Pre-eclampsia (PE) is a hypertensive disease which occurs in 5-7% of all pregnancies. It can eventually also lead to maternal and fetal death, especially during early onset pre-eclampsia. The main symptoms are hypertension and proteinuria. The etiology is still not completely understood but pathophysiological pathways may differ between early and late onset pre-eclampsia. This two-step disorder originating in the placenta is assumed to be the main reason for the above mentioned symptoms: Firstly, the defective trophoblastic invasion of the uterine spiral arterioles leads to decreased blood flow. Secondly, the release of proangiogenic factors leads to endothelial damage (26).

Several pathophysiological effects like maternal immune maladaptation or

increased fetal trophoblast apoptosis lead potentially to PE (27). There have been more and more studies that indicate a role of syncytiotrophoblast microparticles for the etiology of pre-eclampsia. This is where EX come into play: EX influence endothelial cell migration (21).

The number of EX continuously increases with gestational age. Pillay et al. found that early onset PE leads to an increase of PLAP+ EX while late-onset PE lead to a decrease of PLAP+ EX (26). Another study showed that syncytin-2 is reduced in CD63+ and PLAP+ EX in pregnancies with PE (21). There are many different proteins such as sFlt-1, endoglin, tissue factors and PAI that might play an important role in the pathophysiology of pre-eclampsia. It has been shown that these proteins can be found in placenta-derived EX (1). A recent study indicates 25 differently expressed proteins in EVs of patients with preeclampsia compared to healthy pregnancies. Among those proteins are annexins, integrins, histones, heat shock proteins, complement regulatory proteins, cytoskeletal proteins, and various enzymes. Most of the remaining 400 proteins were upregulated during PE while histones, integrins and CD59 glycoprotein were downregulated (27).

The differences between EX could be due to different etiologies suggesting that placenta-derived EX could influence different pathological process during pregnancy (26).

Apart from this, there are new studies that show differences in lipid composition such as enrichment of phosphatidylserine within syncytiotrophoblast EVs compared to healthy pregnancies (28). For example, the lipid marker BMP which induces the formation of intraluminal vesicles can be identified in EX but not in MV. There is indeed an imbalance during PE between EX and MV which could possibly be identified with the lipid marker BMP (24).

Gestational Diabetes Mellitus (GDM) is defined as an intolerance of carbohydrates. It appears in pregnancy and is likely to disappear after giving birth. It affects 16% of all pregnant women. Due to a high prevalence of obesity, gestational diabetes is becoming increasingly common. It can be diagnosed by an abnormal glucose tolerance test with a fasting glucose level higher than 5.6 mmol/L and glucose levels higher than 7.8 mmol/L two hours after glucose intake (29). The profile of EX during pregnancy with GDM is still not well described. However, a significant enrichment of EX has been reported compared to healthy pregnancies. Also, these bioactive EX induce the release of pro- inflammatory

factors such as TNF alpha (2).

1.3. Proteins on trophoblastic extracellular vesicles

We focused on the 10 most abundant proteins which are supposed to be enriched on trophoblastic EX, according to Sadovsky et al. (Table 3). To describe the different proteins of trophoblastic EVs, the research group performed a mass spectrometry-based proteomics assay. Interestingly, most of the data regarding AB and MV were similar, whereby trophoblastic EX were enriched with surface proteins like integrin beta-4, integrin alpha-6, CD63 or CD9. Besides, an enrichment of components of their endosomal origin like Alix or TSG101 was also described in the results. In total 61 proteins specific for trophoblastic EVs were identified (1).

Table 3 Overview of the most abundant proteins on the three different types of placental derived extracellular vesicles; modified from Sadovsky et a (1)

Apoptotic bodies (AB)	Microvesicles (MV)	Exosomes (EX)
Myosin-9/10	Myosin-9/10	Integrin beta-4
Dynein 1 heavy chain 1	Filamin A/B	Integrin alpha-6
Plectin isoform 4	Plectin isoform 4	Annexin A2/6
Filamin A/B	Dynein 1 heavy chain 1	Syntenin-1
Talin-1	Ras GTPase-activating-like protein IQGAP1	Tumor necrosis factor alpha-induced protein 3
Desmoplakin	Talin-1	Catenin alpha-1
Ras GTPase-activating-like protein IQGAP1	Alpha-actinin	Calpain-6
Fatty acid synthase	Desmoplakin	Choline transporter-like protein 2
Alpha-actinin	Annexin A2	Placental Alkaline phosphatase
Spectrin alpha chain	Fatty acid synthase	ADAM10

Detailed data about proteins associated with EX are available on Exocarta (http://www.exocarta.org) and on Vesiclepedia (http://www.microvesicles.org) (23,30).

PLAP (Placental Alkaline Phosphatase Protein)

In general, there are four known alkaline phosphatases: intestinal (ALPI), placental (PLAP), placental like (ALPPL2) and those associated with liver/bone/kidney (ALPL) (31).

PLAP is a 513 amino acid long glycoprotein of 56 kDa. PLAP belongs to the surface enzymes but it can also be found in plasma and especially cancer cells. It is therefore also known as a potential tumor marker (32).

PLAP is a protein specific marker for placenta derived EX, the exact origin are syncytiotrophoblast cells (21). As a result, this is very useful to distinguish between placental-derived and other EX (26). The exact biological function of PLAP has not been investigated yet, but it is already reported that this enzyme is capable of capturing IgG antibodies. This mechanism is linked with the fetal development by uptaking maternal IgG during the third trimester of pregnancy (33,34).

There are 3 isoforms and further 15 rare isoforms of human PLAP, that are described in literature (33).

Tetraspanins

This group of membrane proteins, also called tetraspanin superfamily, is composed of four transmembrane domains with diverse amino acids and two extracellular domains. In general, these proteins are responsible for the regulation of cell development, direct activation of target cell membrane receptors, cellular growth and motility (21).

Tetraspanins have been detected on EX, but with the addition that the combination and proportion of specific tretraspanins on EX differ from the cells of origin. For example, CD63 protein can be observed on only 50% of EX (22). Interestingly, an enriched amount of tetraspanins was first identified in B-cell EX. This can be regarded as a genuine marker for the plasma membrane and early endosomes (15).

- **CD63**

  CD63 is a member of the tetraspanin superfamily. It has a molecular mass of 25.6 kDa. CD 63 interacts with different proteins like integrin beta 1 or CD9 (35).

CD63 has three N-linked glycosylation sites which leads to the fact, that the exact molecular weight also depends on its glycosylation pattern (36).

It has been described that, as TSG101, CD63 or CD81, all late endosomal membrane proteins are preferredly enriched in EX (21). CD63 is a unspecific marker for EX, since it has also been found on other EVs (37). There is also evidence that especially CD63 is enriched in very small vesicles with a size smaller than 50 nm (38).

- **CD9**

  CD9 plays an important role in cell adhesion, cell differentation and signal transduction (21). It is also a member of the tetraspanin superfamily (39). Studies suggest that CD9 is more likely to be a stable and an omnipresent protein within EVs compared to others (17).

  It is found on the surface of EX and has specific roles in comparision to other tetraspanins. CD9 is in fact associated with cell fusion processes linked to osteoclastogenesis, myogenesis and also fertilization (40).

TSG 101

TSG 101 is described as a very specific EX marker protein (16). The protein is located either within the cellular cytoplasm, cell nucleus or cell membrane (41). Full-length TSG 101 protein has a molecular weight of 43 kDa and holds a domain that is necessary for ubiquitin conjugates. Besides the full-length protein, a second isoform (31 kDa) has been described (62).

TSG 101 is encoded by the gene of the same name, and belongs to the part of inactive homologs of ubiquitin conjugating enzymes. This gene plays an important role in the maintenance of genomic stability and cell cycle regulation (42). Besides, TSG 101 plays an active role in cellular endosomal sorting complexes. As a component of the ESCRT-1 complex, it regulates the vesicular trafficking of ubiquitinated proteins. This tumor suppressor gene also down-regulates cell growth (21). TSG 101 is also described as a transcriptional regulator in the suppression of hormone-receptor-mediated transactivation of targeted genes (41). Mutations of this gene are associated with breast cancer; it leads to growth progression of the tumor (42).

Syntenin- 1

Syntenin-1 consists of 298 amino acids (32 kDa) (43) and is build up by repeated PDZ domains binding to the cytoplasmic domains of different transmembrane proteins (44). The protein was initially known as molecule, that is linked to syndecan-mediated signaling to the cytoskeleton. In fact, the protein is enriched within EX and is used as an EX specific marker protein (45). Syntenin is localized at membrane-related adherent junctions and focal adhesions, but it can also be found in the endoplasmatic reticulum and nucleus. It also impacts cell adhesion, the cytoskeleton, protein trafficking, and the activation of transcription factors. Due to multiple transcript variants there are different isoforms (46). Syntenin -1 supports EX production, where small GTPase ADP ribosylation factor 6 (ARF6) and its effector phospholipase D2 (PLD2) were identified as regulators of syntenin EX. Both ARF and PLD2 influence the budding of intraluminal vesicles (ILVs) into MVBs. The stimulation of EX production by syntenin-1 is based on the interaction with the intercellular Alix, an auxiliary component of the ESCRT machinery that supports viral budding (47).

Integrin beta 4

It is a common characteristic for integrin proteins that the subunit beta 4 is made up of a heterodimer and is non-covalently associated to transmembrane glycoprotein receptors. Integrins induce cell-matrix or cell-cell adhesion and are responsible for signals that regulate cell growth. More precisely, integrin beta 4 is a receptor for laminins. Together with the associated integrin alpha 6 subunit it may play a decisive role in the biology of invasive carcinomas. To date, several mutations within this gene have been described which are associated with different pathologies like the epidermolysis bullosa (48).

Integrin alpha 6 (EPR 18124)

The integrin alpha 6 protein is encoded by the *ITGA6* gene. So far, two transcript isoforms have been detected. Like integrin beta 4 the structure is built up by different polypeptides and these are responsible for cell-matrix or cell-cell adhesion. Different subunits within the integrin alpha protein family often act together; for example, integrin alpha 6 combines with beta 4 to TSP180 (49).

Annexin A2

Annexin A2 is a protein encoded by *ANXA 2* gene (50). It is a member of the annexin family, a calcium-dependent phospholipid-binding group of proteins. Annexin A2 plays an important role in cell growth regulation corresponding signal transduction pathways, and cytoskeleton remodeling. It also enhances osteoclast formation and bone resorption (51).

Annexin A2 is responsible for cell adhesion and migration by inducing dynamic remodeling processes of the cytoskeleton. Annexin A2 is located at the plasma membrane as a hetero-tetrameric complex that interacts with cytoskeleton components like filamentous actin; especially during dynamic processes like phagocytosis, pinocytosis and cell migration. In addition, many studies show an enrichment of Annexin A2 in different tumor types such as gastric, colorectal, pancreatic, breast, kidney, and vascular tumors. Moreover, preclinical studies have suggested that extracellular Annexin A2 acts as regulator, thereby predictor of adhesion, migration, homing, and invasion of cancer cells. Apart from the presence at the cell surface, the protein is described as a cytosolic monomer. However, the function of Annexin A2 as an intracellular protein is still under investigation, in particular in cancer progression (52).

2. Hypothesis

The focus of this thesis is to identify and to verify specific proteins of placental delivered EX in maternal and fetal circulations. To date, we know that primary human trophoblasts of the placenta release different EVs to the maternal circulation during pregnancy; EX are amongst these (1). However, the exact protein profile of placental EX is still not fully known.

The specific protein profile is of interest because it not only determines size and function of EX; but it also may impact systemic trafficking and more importantly the communication with maternal and/or target cells. Together, EX may have therapeutic capability regarding treatment of several pregnancy-related complications such as preeclampsia and others, by affecting physiological processes during pregnancy (1). For this reason, it is important to know more about the detailed composition of placental EX and their composition:

- Do EVs isolated out of fetal and maternal plasma, and maternal perfusates carry intracellular placental derived proteins?
- Which intracellular proteins are detectable on placental derived EX?
- Is there a compositional difference between EX isolated from maternal perfusates and maternal plasma?

3. Methods and Materials

For the isolation of placental-derived EX we used healthy maternal plasma samples at term and collected perfusates during *ex-vivo* placental perfusion experiments. Furthermore, extracellular vesicles were isolated from neonatal plasma samples. A stepwise ultracentrifugation protocol was used for the isolation of EX and obtained vesicles were characterized by using nanoparticle tracking system. Eva Grasmann, a former master student in the laboratory established the protocol. The samples were provided by the laboratory team of Prof. Wadsack and the corresponding methods of the EV-extraction are described below.

3.1. *Ex vivo* dual placental perfusion

The EVs were isolated from perfusate samples collected during an *ex-vivo* perfusion of the human placenta. This method provides an accurate model for simulating trans-placental transfer of substances (11). The placenta was dually at the maternal and fetal side perfused, directly after an uncomplicated birth. In fact, one selected placental cotyledon, visually identified as intact, was perfused by cannulating an artery-vein pair (11).

Placentae from healthy donors after given informed consent were taken and their chorionic vessels of one selected cotyledon from the marginal zones were selected for cannulation. First, one selected chorionic artery-vein vessel pair were cannulated with polyethene cannulas *(artery: fine bore polyethene tubing, Portex, 800/100/300, 1.02 mm ID; vein: fine bore polyethylene tubing, Portex, 800/100/500, 2 mm ID, Thermo Scientific Inc., Waltham, MA, USA).* Knots with surgical suture material were used in order to close side-branches of vessels within the selected cotyledon. Afterwards, the cotyledon was rinsed with 20 mL 37°C pre-warmed perfusion media using a syringe *(Injekt, Braun, Melsungen, Germany).*

In order to protect the placenta from damage, the tissue was fixed on a so-called tissue holder. The remaining non perfused placental tissue was then removed. The tissue holder was placed into the perfusion chamber keeping the maternal side up. The chamber was pre-warmed at 37°C to maintain physiological temperature during the experiments.

The tubes of the fetal circuit were connected to an infusion pump *(Argus 707v®, Codan, Salzburg, Austria).* The fetal perfusion solution was conditioned with 95 %

N_2, 5 % CO_2 by using a membrane *oxygenator (LSI-OX®, Living Systems, St. Albans, VT, USA)* to provide a physiological pH-range and approximate physiological O_2-saturation in the perfusate. The flow rate of 4 mL/min with a maximum loss of fetal perfusate of 0.2 mL/min was checked frequently during time of the experiment. In addition, during the perfusion of the tissue the back-pressure of the arterial fetal vessels was measured online by a catheter *(Micro-CathTM diagnostic pressure catheter, Millar Inc., Houston, Texas).*

Alongside the fetal perfusion, the maternal circulation was established by putting three blunt stainless-steel needles into the intervillous space and by puncturing the decidual plate, serving as maternal arteries. We used a suction tube to collect the perfusate from the intervillous space. The maternal arterial perfusion rate was 8 mL/min which was provided by an infusion pump *(Argus 707v®, Codan, Salzburg, Austria).* The maternal perfusion media was preconditioned with 75 % N_2, 20 % O_2, 5 % CO_2 by using an oxygenator *(LSI-OX®, Living Systems, St. Albans, VT, USA).*

Before the maternal and fetal circuits were closed as shown in Figure 4, both circuits were washed for about 10 minutes. 150 mL of perfusion media was provided for both the maternal and fetal circuit.

During the experimental phase levels of pH, pO_2, pCO_2, lactate and glucose were online monitored by a blood gas analyzer *(ABL 800 Basic®, Drott Medizintechnik GmbH, Wiener Neudorf, Austria).*

The perfusion lasted 120 minutes and the collected media was stored at 4°C until further processing.

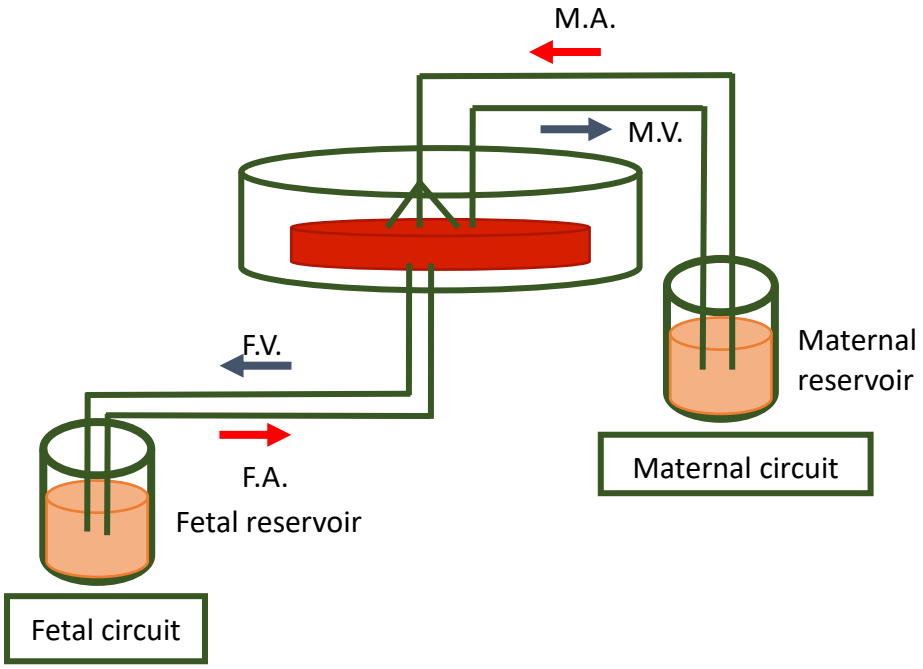


Figure 4 Schematic illustration of the *ex-vivo* dual placental perfusion setup.
Maternal, fetal venous (M.V., F.V.) and maternal, fetal arterial (M.A., F.A.) circuits were closed during perfusion experiments.

3.2. Isolation of exosomes by differential centrifugation and density gradient

For the isolation of the EX we used a protocol provided by Eva Grasmann, who worked on an isolation method for placental delivered EX in the laboratory. She used and adapted the protocol of Yoel Sadovsky and his research group (1).

Basically the gold standard protocol consists of a differential centrifugation combined with isopycnic centrifugation (53).

First, an initial volume of 150 mL collected perfusate was required for the stepwise isolation of the extracellular vesicles. All isolation steps were done at a temperature of 4°C. To pellet cells and cell debris perfusates were centrifuged (500 x g for 10 minutes) with an Allegra® X-12R benchtop centrifuge *(Beckman Coulter Inc., Brea, CA, USA)*. The supernatant was transferred and the pellet was discarded. It is very important to be careful while transferring as not to include any of the cell pellet. Then, another centrifugation step was carried out at 2 500 x g for 20 minutes in the benchtop centrifuge. Again, we discarded the pellet, which contained mainly cell fragments and AB. In addition, 132 mL of the supernatant was transferred to the ultracentrifugation tubes *(Ultra- ClearTM Centrifuge Tubes,*

38.5 mL, Beckman Coulter Inc., Brea, CA, USA) and centrifuged in the Optima XE-90 ultracentrifuge at 12,000 x g for 30 minutes in a Type 70 Ti rotor.

After this centrifugation step, the obtained pellet contained the MV population. Finally, this pellet was re-suspended in 1.5mL PBS and stored at 4°C.

From this step on an aliquot was taken after each step for further analysis and characterization of the intermediate fractions. The remaining supernatant was filtered through a 0.2 µm filter (Nalgene® Syringe Filter, 0.2 µm, Thermo Scientific Inc., Waltham, MA, USA) in order to remove all particles larger than 200nm. Fraction enriched with EX was concentrated by reducing the volume to 4 mL using the benchtop centrifuge at 1 800 x g. For this task we used concentrator columns with a cut-off size of 100 kDa *(Vivacell® 100 Centrifugal Concentrator, PES membrane, 100 000 MWCO; Sartorius Stedim Biotech, Goettingen, Germany).*

EX are larger than 100kDa and therefore do not pass the membrane. As a result, the supernatant was diluted with 9 mL 1x PBS and pipetted to an ultracentrifuge tube (Quick-SealTM Centrifuge Tubes, 16x76 mm, Beckman Coulter Inc., Brea, CA, USA). Finally, to pellet the EX population, samples were centrifuged for 60 minutes at 100 000 x g in the ultracentrifuge using the Type 70Ti rotor. The supernatant was removed and the pellet containing the EX was re-suspended in 400 µl 1x PBS. Figure 6 summarizes all essential isolation steps for getting the EX enriched fraction out of perfusates.

To get different EX fractions we used isopycnic centrifugation, a density gradient centrifugation on a 6-40 % Optiprep *(60 % Iodixanol (w/v) in water, Sigma Aldrich, St. Louis, MO, USA)* step gradient. The gradient fractions were prepared by mixing 4.4% (w/v) mannitol *(≥ 98 %, Sigma Aldrich, St. Louis, MO, USA*), 1 mM EDTA *(Ethylenediaminetetraacetic acid, 99.0-101.0 %, Sigma Aldrich, St. Louis, MO, USA)*, and 10 mM Tris *(Tris(hydroxymethyl)nitromethane, p.a., Merck, Darmstadt, Germany),* pH 7.4.

The samples were pipetted on the bottom of ultracentrifuge tubes (Ultra-ClearTM Centrifuge Tubes, 38.5 mL), and filled up with 60 % OptiprepTM solution to a final volume of 2 mL. A 6-40 % OptiprepTM solution was utilized for overlaying the solutions; starting with the highest density coming to the lower ones at the top. 1.5 mL of each gradient was pipetted into the tube using a syringe. Samples were centrifuged for 22 hours at 100 000 x g forming a continuous density gradient in

which EX fraction is floated according to their theoretical density, which is 1.13 – 1.19 g/mL (13).

After this centrifugation in 1.5 mL steps, the fractions were removed from the top to the bottom. In order to have a sufficient protein concentration in the fraction, another concentration step was necessary. A concentrator with a cut-off of 100 kDa (VivaSpin6, PES membrane, 100000 MWCO, Sartorius Stedim Biotech, Goettingen, Germany) was used. This was done by a dilution of 1.5 mL sample with 3-fold 1x PBS, and then the samples were centrifuged at the benchtop centrifuge at 3 000xg for 8 minutes. The sample was filled up with 1 x PBS to 6 mL and was again centrifuged at 3 000 x g for about 5 minutes until 100 µL of each fraction sample were left.

The samples were stored at 4 °C until being analyzed by Western blot and nanotracking analysis.

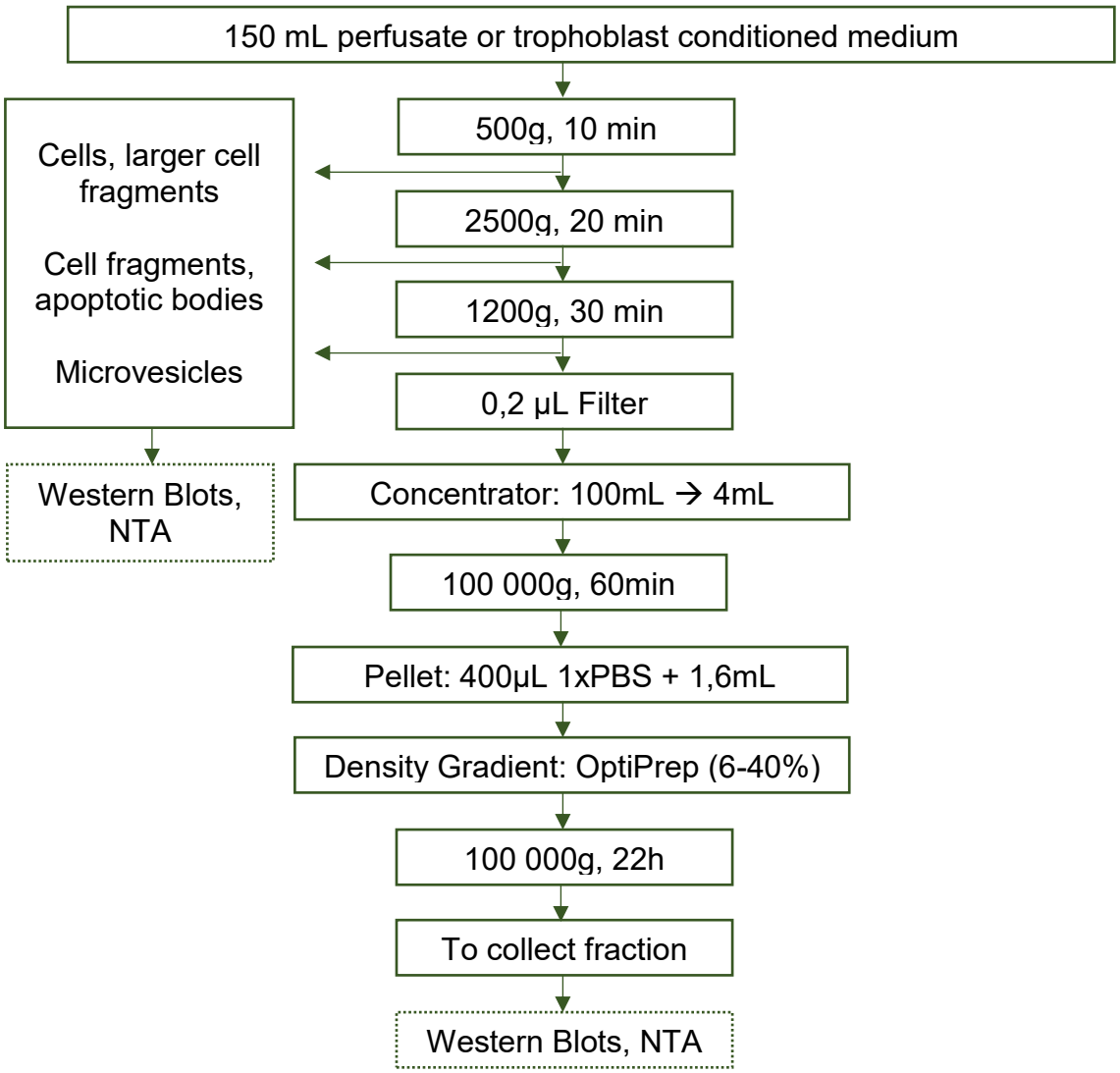


Figure 5 Detailed steps of the placental EX isolation that includes a combination of differential and isopycnic centrifugation steps.

3.3. Nano Tracking Analysis

NTA measures the size of particles in liquids such as EX by analyzing Brownian motion. In short, small particles like EX with a size between 30-100nm diluted in liquid can be measured. It is well established that the movement is influenced by the viscosity and temperature of the liquid and not by the density of the particle. For the procedure a laser beam is necessary that enters particle suspension.

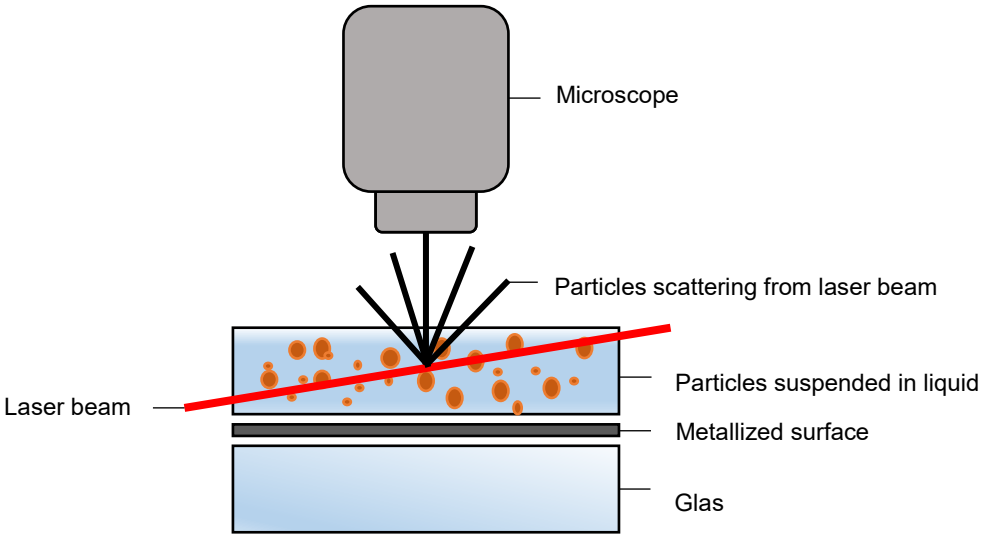


Figure 6 Scheme of the Nano Tracking Analysis (NTA)
Before the laser beam enters the liquid with the suspended particles, first it is being reflected. The low angle refraction leads to scattered light, which is captured by the microscope. Modified from Malvern Instruments® (54)

This leads to low angle refraction. The scattered light by the particles is captured by a charge–coupled device video camera that is connected to an ultra-microscope. To analyze the video, Nanoparticle Tracking Analysis (NTA) software was used. Using the Stokes-Einstein equation allows to calculate the size of the particles (55):

$$\frac{(x,y)^2}{4} = Dt;\ \ Dt = \frac{TK_b}{3\pi\eta d} = Dt$$

Whereby:

- Dt, diffusion constant, a product of diffusion coefficient D and time t
- K_b, Boltzmann's constant,
- T, absolute temperature,
- η, viscosity
- d, diameter of the spherical particle.

Nanoparticle tracking analysis of exosomes

We used the NanoSight LM10 instrument with a 488 nm laser *(Malvern UK),* camera C11440 *(Hamatsu Photonics K.K., Hamatsu, Japan),* and Nanoparticle Tracking Analysis (NTA) software Version 2.3, Build 0025 (Malvern UK) in order to detect the different sizes of isolated particles.

The different isolated fractions were diluted with 1 x PBS in order to detect up to 100 particles per image, all samples were mixed using a syringe.

We used the same measurement settings according to the laboratory protocol which was established by Eva Grasmann (56). This resulted in receiving information about the particle size distribution, the concentration of particles per frame, and a calculation of the number of particles in the sample.

3.4. Western Blot

All the collected intermediate and gradient samples during the EX-isolation were analyzed by using immuno-blotting approach.

In order to determine protein content of each fraction, the standard operation procedure of the Pierce® BCA TM Protein Assay Kit *(Thermo Scientific Inc., Waltham, MA, USA) was* carried out. This detergent-compatible formulation is based on bicinchoninic acid (BCA) to quantitate the total protein amount. Basically, it is a reduction reaction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium with a colorimetric detection of the cuprous cations. The purple-colored reaction exhibits an absorbance at 563 nm, which is nearly linear with increasing protein concentrations over a broad working range of 20-2000 µg/mL.

After determining the protein content of each fraction and diluting the samples to 2µg/mL, the same amount of Laemmli sample buffer *(Laemmli 2x concentrate, Sigma Aldrich, St. Louis, MO, USA)* was added. Proteins were denatured at a temperature of 95°C for 5 minutes.

10 µl of each of the EX enriched fractions, MV-fractions, standard samples, and human placental tissue were loaded onto wells of 10% or 20% precast Mini-Protean® TGX TM gels (BioRad laboratories Inc., CA, USA). In average, the samples on the gel were exposed to 120 V for 60 minutes.

The separated proteins were transferred to nitrocellulose membranes using the Trans-Blot® Turbo TM Transfer system (BioRad laboratories Inc., CA, USA) The

transfer efficiency of the proteins to the membrane was checked by using a Ponceau S reversible staining of the proteins.

After several washing cycles with TBS-T puffer the membrane was blocked in 5% milk in 1xTBE Buffer +0,1% Tween for one hour, subsequently the membrane was incubated with the first antibody diluted on 1% NFM/TBE overnight at 4°C.

Next, the membrane was thoroughly washed with 1xTBE Buffer + 0,1% Tween for one hour, changing the washing solution every 10 minutes, followed by incubating with the second antibody diluted in 1% NFM/TBE for 1 hour. After this, the washing process with 1x TBE Buffer + 0,1 Tween was repeated for one hour again. For identification of the specific protein bands Pierce West Pico and Femto mixed 1:1 for 5 minutes was used. The pictures were taken with the Biorad Imaging System.

With the intention to get reliable results, we tested our antibodies in different dilutions. Table 4 shows the optimized dilutions of antibodies that we used for our Western Blots.

Table 4 Summary of utilized antibodies of proteins and its corresponding second antibody for the visual detection of the bands. The dilutions of each antibody was tested and is listed in the table as well as the molecular weight of the proteins (in kDa).

Antibody	Dilution	Second antibody	Dilution	Molecular weight of the protein	Company
PLAP	1:500	Rabbit IgG	1:2000	70 kDa	Abcam ab133
CD63	1:500	Mouse IgG	1:2000	30 kDa	Abcam ab821
TSG101	1:1000	Mouse IgG	1:2000	46 kDa + 31 kDa	Abcam ab 83
Syntenin	1:750	Mouse IgG	1:2000	32 kDa	Abcam ab 199
Integrin beta 4 (M126)	1:2000	Mouse IgG monoclonal	1:2000	202 kDa	Abcam ab290
Integrin alpha 6 (EPR 18124)	1:2000	Rabbit IgG monoclonal	1:2000	127 kDa	Abcam ab181
CD 9 (EPR2949)	1:1000	Rabbit IgG monoclonal	1:000	25 kDa	Abcam ab927
Annexin A2	1:2000	Rabbit IgG polyclonal	1:2000	38 kDa	Abcam ab418

4. Results

In order to identify specific surface proteins on EX and other EVs we performed immunoblots and tested specific marker proteins. We investigated fetal and maternal plasma samples and collected maternal medium perfusates.

4.1. Specific placental derived EV-proteins in fetal plasma samples

Using the isopycnic centrifugation method, EX were isolated based on their density gradient, making EX with low density float up and remaining in the layer that corresponds with their density.

As expected due to preliminary results, no PLAP signal as one placental specific protein was detectable in fetal plasma. This finding strongly indicates that no placental derived extracellular particles are secreted into the fetal plasma.

Interestingly, the EX specific markers TSG 101 and Syntenin could be identified in EX fractions, suggesting that these particles may be released by fetal but not placental tissue. We also tested antibodies against integrin alpha6, integrin beta4, CD9, and Annexin A2. None of them were positive.

Table 5 Master template of fetal plasma samples after centrifugation as applied for immunoblot analysis.
After isopycnic centrifugation, we analyzed the different EVs, with specific focus on EX. The different exosomal fractions are based on different density gradients.

	Sample	Protein content [µg/mL]
1	Prestained protein standard	
2	fetal plasma 12000G pellet Microvesicles	2723,00
3	fetal plasma 100000G Supernatant	11591,33
4	fetal plasma 100000G pellet	12411,33
5	Prestained protein standard	
6	Prestained Plus protein standard	
7	Exosomal fraction F13 (fetal plasma)	2386,33
8	Exosomal fraction F14 (fetal plasma)	4851,33
9	Exosomal fraction F15 (fetal plasma)	12391,33
10	Exosomal fraction F16 (fetal plasma)	11796,33
11	Exosomal fraction F17 (fetal plasma)	12439,67
12	Exosomal fraction F18 (fetal plasma)	12338,00
13	Exosomal fraction F19 (fetal plasma)	12451,33
14	Human placental tissue clontech	
15	Prestained Plus proteins standard	

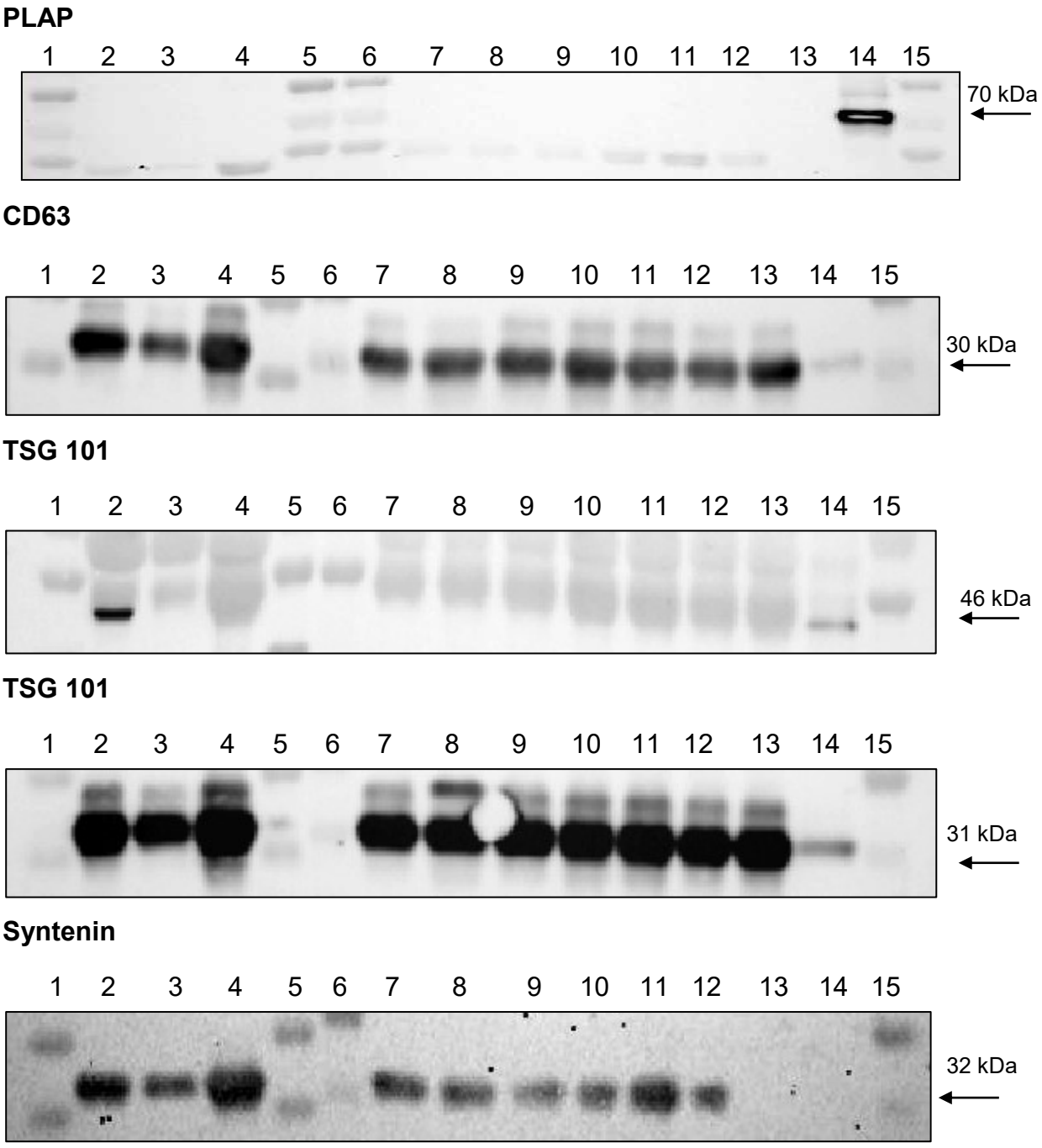


Figure 7 Western Blot analysis of exsomal marker proteins: PLAP, CD63, TSG101 and Syntenin. Anlysis of differente exsomal fractions from fetal plasma (7-13), microvesicles (2) and human placental tissue (14). Protein load: 10 µl/well

4.2. Maternal perfusate and plasma

Analogous to the fetal plasma we tested protein markers for samples of maternal plasma and perfusates.

In particular, PLAP, CD63, TSG101, and Syntenin were tested. In addition, 4 additional proteins namely, Integrin beta 4, Integrin alpha 6, CD9, and Annexin A2 were also tested by Western Blotting.

Table 6 Master template of maternal perfusates and plasma samples for immunoblot analysis.
Apart from the proteins standards, we focused on the microvesicles and the different EX fractions of maternal perfusates (F13-15) and the maternal plasma (F11-15). The fractions are based on different density gradients. Total human placental tissue served as positive control. Protein load: 10 µl/well

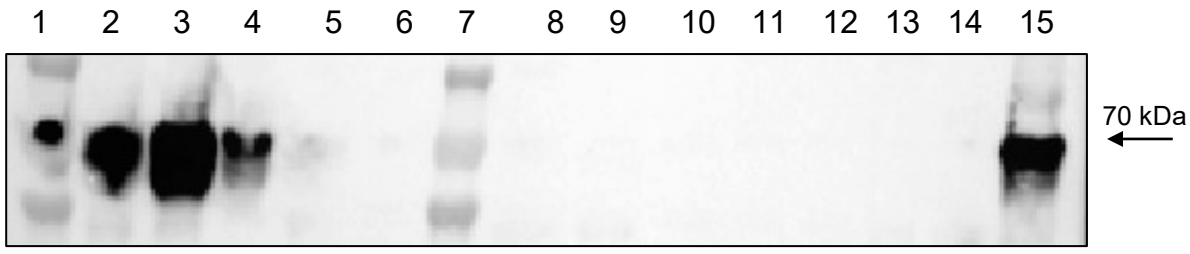
	Sample	Protein content [µg/ml]
1	Prestained protein standard	
2	100000G pellet (maternal perfusate)	15466,78
3	Microvesicles 12000G pellet (maternal perfusate)	4762,67
4	Exosomal fraction F13 (maternal perfusate)	1633,78
5	Exosomal fraction F14 (maternal perfusate)	3642,67
6	Exosomal fraction F15 (maternal perfusate)	9620,45
7	Prestained protein standard plus	
8	100000G pellet (maternal plasma)	276311,22
9	Microvesicles 12000G pellet (maternal plasma)	4428,45
10	Exosomal fraction F11 (maternal plasma)	3292,89
11	Exosomal fraction F12 (maternal plasma)	5931,12
12	Exosomal fraction F13 (maternal plasma)	7573,36
13	Exosomal fraction F14 (maternal plasma)	13684,47
14	Exosomal fraction F15 (maternal plasma)	50284,47
15	human placental tissue clontech (positive control)	

- First, we tested our EVs for placental origin with **PLAP** antibody. Interestingly, EX fractions of maternal perfusates showed only a very low PLAP signal. However, PLAP signals in our 100000 G pellet reflecting as well as in our MV of the maternal perfusate samples were particularly prominent. EX enriched fractions isolated from maternal plasma revealed no PLAP specific signals.
- Specific **CD63** signals could be detected on all EX fractions of both maternal plasma and perfusate, suggesting specificity of EVs in these isolated particles.
- **TSG 101** is one commonly used marker for EX which could be confirmed by the used placental derived EX enriched samples here. Specific signals (31 kDa) in the EX fractions from maternal plasma were slightly higher compared to maternal perfusates. We noticed a very prominent specific signal in placental MV fractions isolated from perfusates. Regarding the

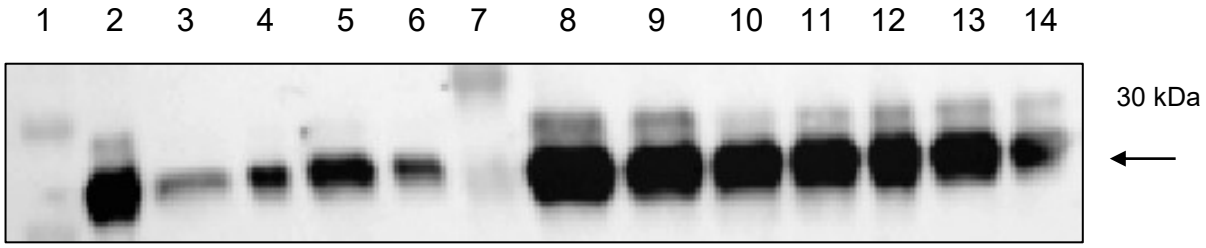
other isoform of TSG101 with a molecular weight of 46 kDa, a very low signal could be detected on the EX fractions from maternal plasma.

- **Syntenin** signals were positive in all EX fractions regardless of whether they originated from plasma or perfusate. The most abundant signal was in EX enriched fractions 13 and 14 of maternal perfusates which indicates that the placenta releases specific intracellular derived EVs to the maternal circuit.
- **Integrin beta 4** at 202 kDa was only detectable for human placental tissue. Additionally, a further signal at 25 kDa for all EX enriched fractions, for MV and human placental tissue was detectable. If this 25 kDa protein is related to a truncated integrin protein has to be proven by further investigations.
- At 127kDa a very low signal for **Integrin alpha 6** was detectable for MV apart from the signal from the placental tissue. Interestingly, there was an inexplicable low signal at 55 kDa for both maternal plasma and perfusate fractions as well as for the MV.
- **CD9** was not detectable in any probes apart from the human placental tissue. However, there were several signals at different other molecular weights, like at 55 kDa, their specificity has to be proven.
- **Annexin A2** had a strong signal at 38kDa only within the MV 12000 G of maternal perfusate, similar to integrin alpha 6. It should also be mentioned that there were band signals a bit smaller than 38kDa in the EX fractions.

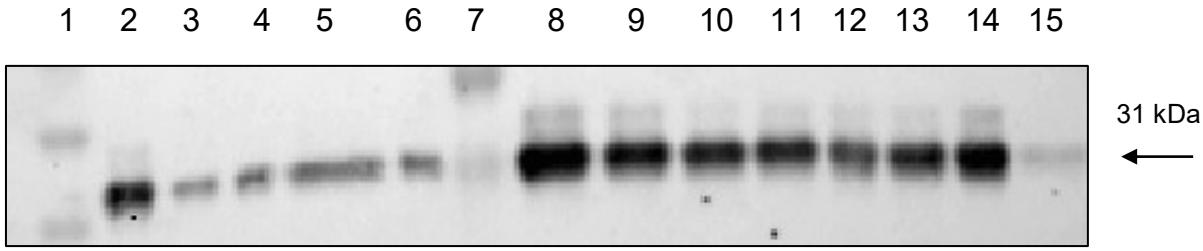
PLAP



CD63



TSG101



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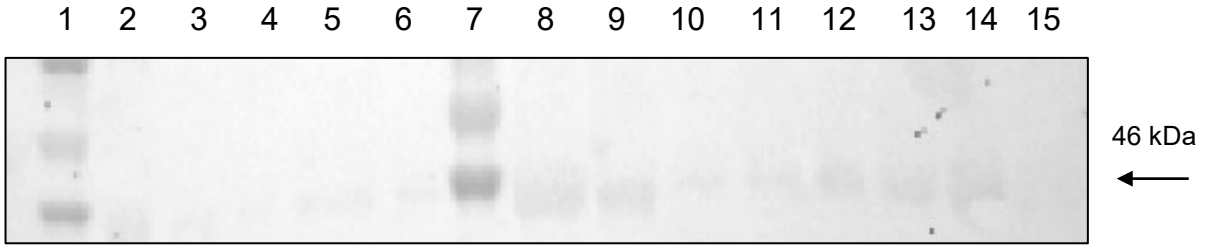
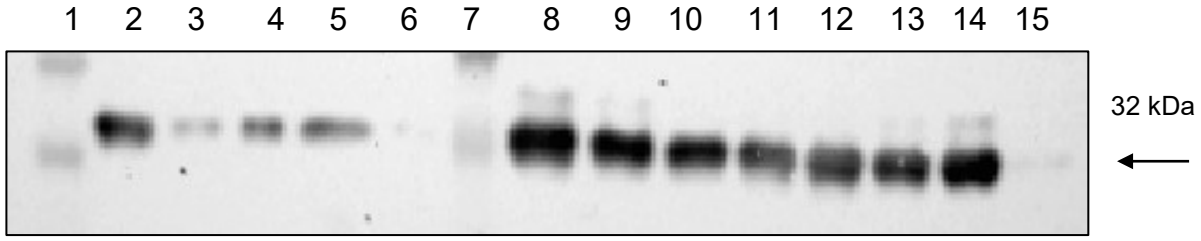
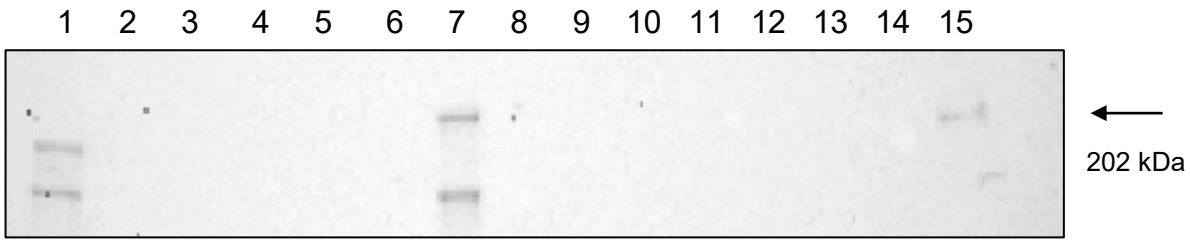


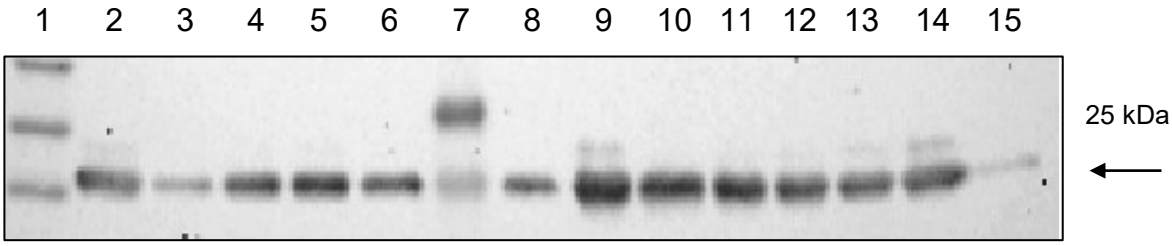
Syntenin



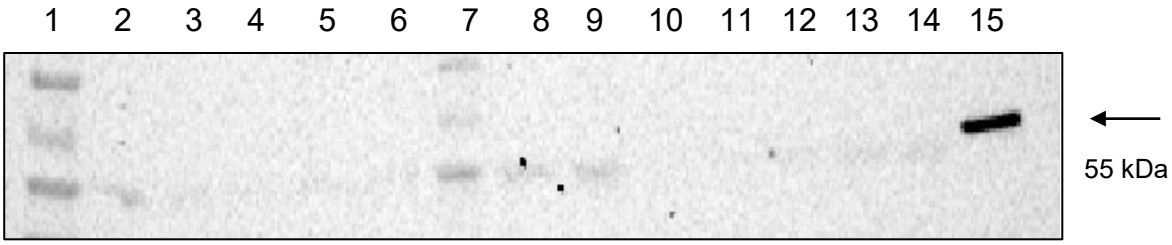
Integrin beta 4



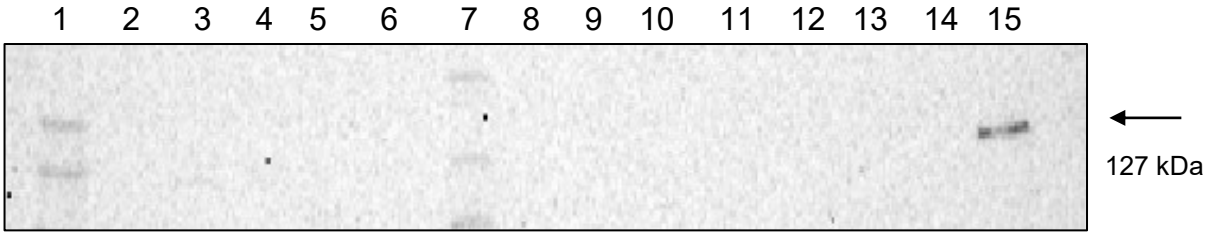
Integrin beta 4



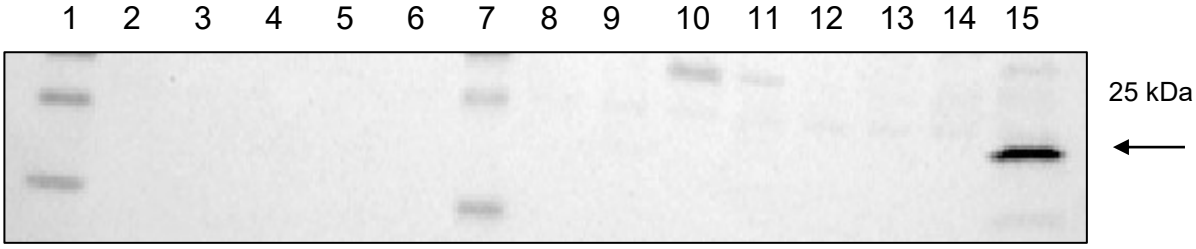
Integrin alpha 6



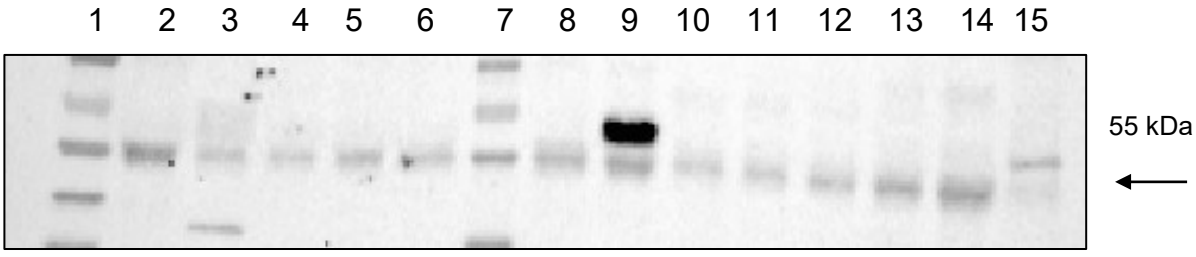
Integrin alpha6



CD9



CD9



Annexin A2

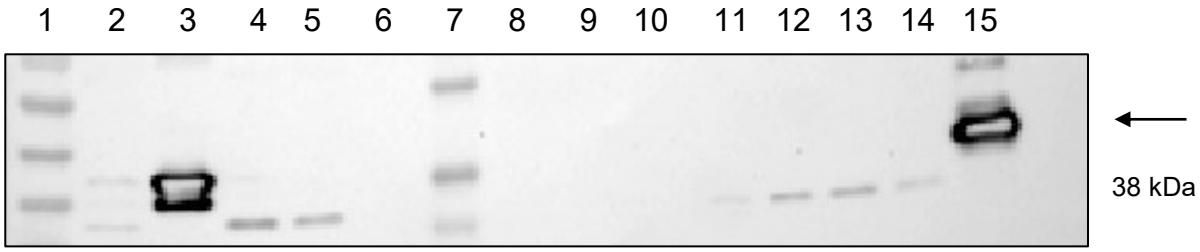


Figure 8 Western Blot analysis of exsome marker proteins: PLAP, CD63, TSG101, Syntenin, Integrin beta 4, Integrin alpha 6, CD9 and Annexin A2.
Anlysis of differente exsomal fractions from placenta perfusate (4-6), maternal plasma (10-14) and microvesicles from placenta perfusate (3) and maternal plasma (9). The pellets and supernatants after centrifugation were analyzed by specific protein markers. Protein load: 10 µl/well

4.2.1. Nano Tracking Analysis

Figure 9 shows as an example the size distribution of one EX enriched fraction (F13) isolated out of maternal perfusate, measured by NTA. The NTA analysis shows different bands with two main peaks at 88 nm and 125 nm size, followed by additional smaller peaks at 220 nm, 309 nm and 366 nm. The total mean value of five measurements of EX fraction F13 from maternal perfusate was 155.8 ± 3.8 nm. The mean size was slightly bigger than the normal size of EX, which is around 100 nm according to literature (12).

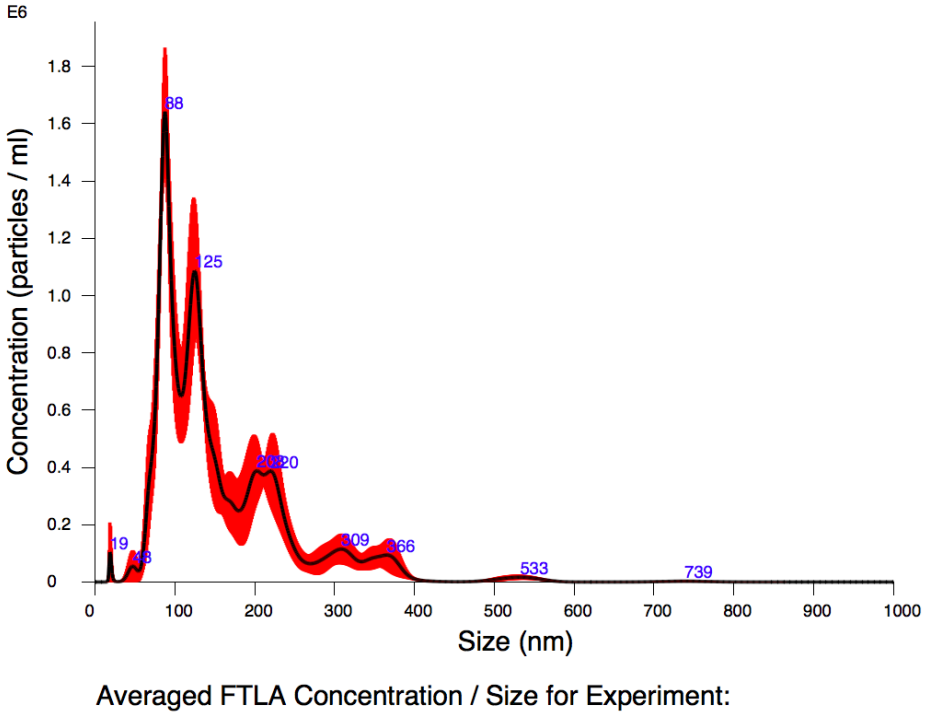


Figure 9 Size distribution of isolated exosomal enriched fractions F13 from maternal perfusate measured by NTA.
The analysis shows two main peaks at 88 nm and 125nm. The total mean value of five measurements of all peaks was 155.8 ± 3.8 nm.

4.3. Maternal plasma

Table 7 Master template of maternal plasma samples for Immunoblots
Apart from the proteins standards, we focused on the microvesicles and the different EX fractions (F11-15). The human placenta tissue was used as the positive control.

	Sample	Protein content [µg/mL]
1	Prestained protein standard	
2	12000G SN (maternal plasma)	78528,91
3	12000G filtration SN (maternal plasma)	89635,60
4	100000G pellet (maternal plasma)	276311,22
5	Microvesicles pellet (maternal plasma)	4428,45
6	Prestained protein standard	
7	Exosomal fraction F11 (maternal plasma)	3292,89
8	Exosomal fraction F12 (maternal plasma)	5931,12
9	Exosomal fraction F13 (maternal plasma)	7573,36
10	Exosomal fraction F14 (maternal plasma)	13684,47
11	Exosomal fraction F15 (maternal plasma)	50284,47
12	human placental tissue clontech (positive control)	
13	Prestained plus protein standard	

Table 7 shows the solutions including MV, EX fractions isolated from maternal plasma that we tested with specific protein antibodies.

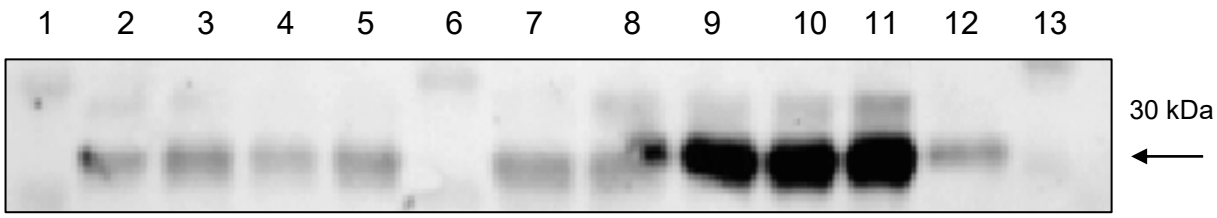
Following the assessment of the maternal perfusate, we also investigated EX fractions from maternal plasma to see if we have any differences in our results:

- MV and EX enriched fractions isolated from maternal plasma revealed no **PLAP** specific signals.
- Specific **CD63** signals could be detected in all MV and EX fractions of our maternal plasma samples, suggesting specificity of EVs in these isolated particles.
- There was a strong signal at all our EX fractions isolated from maternal plasma for the isoform of **TSG 101** with a molecular weight of 31 kDa. Regarding the other isoform with a molecular weight of 46 kDa, a very low signal could be detected on the EX fractions from maternal plasma.
- Also **Syntenin** with a molecular weight of 32 kDa could be detected in all EX fractions and MV isolated from maternal plasma.
- For **Integrin beta 4** no signal was detected in our MV and EX fractions isolated from maternal plasma for the molecular weight of 202kDa, but interestingly at the 25 kDa band.
- For **Integrin alpha 6** no signal was detected for the 127 kDa band.

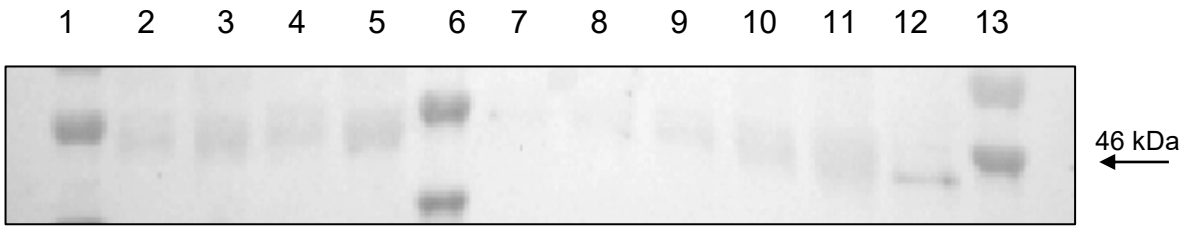
- There were no signals of **Annexin A2 or CD9** proteins in any of the EX fractions isolated form the maternal plasma or the MV of maternal plasma. It should also be mentioned that for Annexin A2 there were band signals a bit smaller than 38kDa in the EX fractions F9 – F11 isolated from maternal plasma.

PLAP

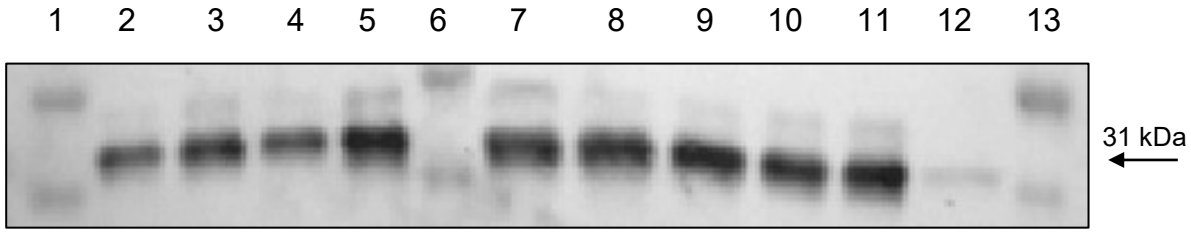
CD63



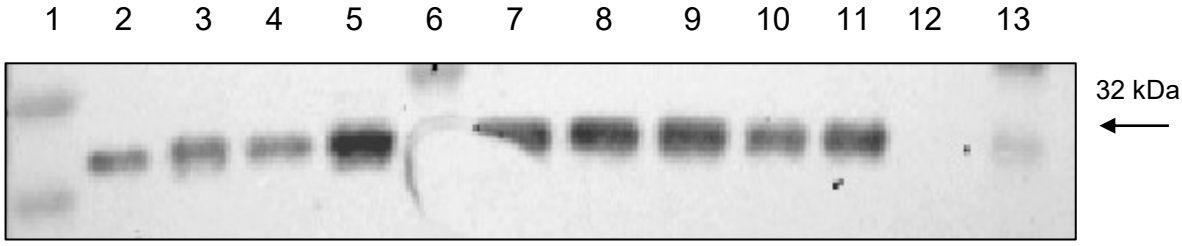
TSG 101



TSG 101

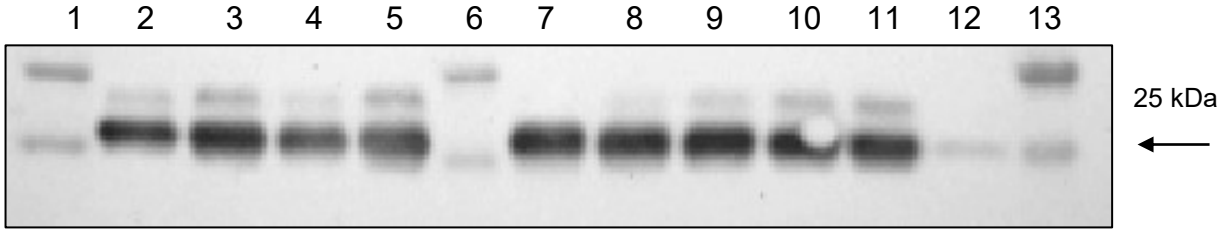


Syntenin

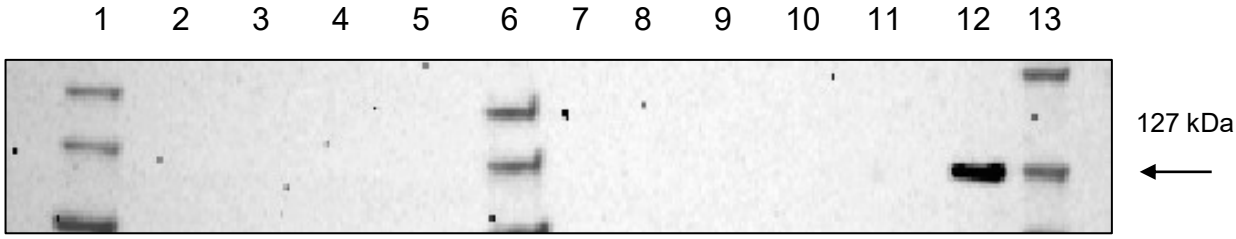


Integrin beta 4

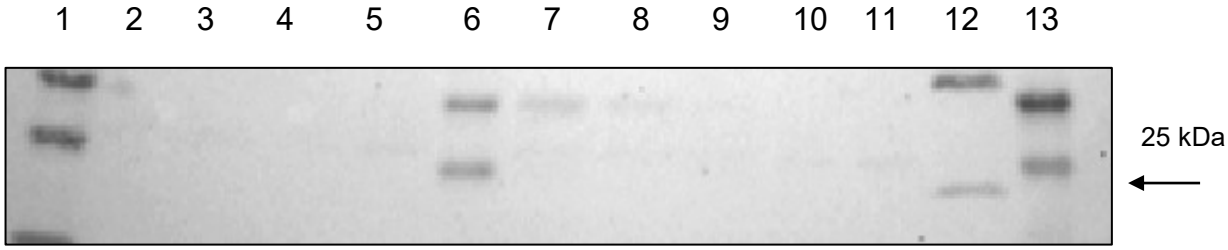
Integrin beta 4



Intgerin alpha 6



CD9



Annexin A2

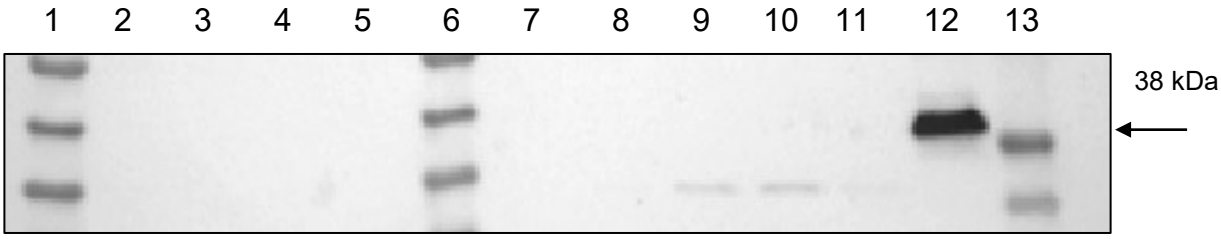


Figure 10 Western Blot analysis of exsome marker protein PLAP, CD63, TSG101, Syntenin, Integrin beta 4, Integrin alpha 6, CD9 and Annexin A2.
Anlysis of differente exsome fractions from pmaternal plasma (band 7-11) and microvesicles from placenta maternal plasma (band 5). The pellets and supernatants after centrifugation were analyzed by specific protein markers. Protein load: 10 µl/well

5. Discussion

The importance to characterize EVs exported from the human placenta, particularly EX as one subtype, is based on fundamental partly unanswered questions. First, do placental EX, either released from the maternal or fetal side of the placenta, influence systemic trafficking and are they capable to induce a response on target cells as a result of their different composition and endocytic origin? Second, do EX possess therapeutic and diagnostic potential with regards to treatment of numerous pregnancy-related complications such as pre-eclampsia and other pregnancy associated diseases (1,17)?

It is described that released EX possess features to affect intercellular communication processes. The bioactivity of EX released by trophoblastic cells is determined by the contained RNA, lipids and proteins. Nonetheless, its role within the intercellular communication is not clearly understood and it may potentially effect normal as well as pathological pregnancies (24).

As placental derived EX may impact the functionality of target cells and execute immunological effects, the core question would also be how these processes are regulated (57)? The release of placental derived EX to the maternal circulation is well described; whereby the knowledge regarding the composition of EVs is still lacking (1). For this reason, a deeper characterization of placental EVs, in particular EX, was the main objective of this thesis.

To date we know that EX differ from other EVs not only but also by their protein composition. In fact, Sadovsky et al. found that there are in total 63 proteins, which are solely expressed on trophoblastic EX, but not on other EV particles. We also already know that these EX possess an antiviral activity. The diverse repertoire of proteins suggests that different EX are linked to different functions. For instance, integrin-like proteins located at the surface of EX may have an effect by targeting local cells or cells at a distance. There is also a theory that different pathological conditions, such as the previously discussed pre-eclampsia, may influence the protein cargo of EVs (1).

It has been noted that EVs are not homogeneously composed and further investigations into this matter are needed. This might also lead to a better understanding of EVs' role in normal and pathological pregnancies (1).

In this thesis, we tried to gain deeper insight about the protein composition of placental derived EX. As there was already an established protocol for the

isolation of placental derived EVs in the laboratory, I used this for my investigations as well (56).

The results revealed that EVs, in particular EX gained from maternal perfusates, were confirmed to be of placental origin. This was assessed by checking for PLAP positive immuno-staining; a marker which has previously been shown to be a strong indicator for placental EVs (21). Additionally, CD63, a tetraspanin protein, was tested as a surrogate marker for general EV-specificity. Interestingly, not all EX fractions isolated from maternal plasma showed PLAP positive signals. We therefore assume that placental specific EX isolated from maternal plasma make only a minor proportion within the total EX fraction which is very likely composed of different EX from other maternal tissues as well, e.g. liver.

In contrast, TSG 101, a protein regulating vesicular trafficking processes, showed a strong signal in all maternal MV and EX fractions suggesting an intercellular origin of these particles (41). For the EX fractions of maternal perfusates a very strong expression of the 31 kDa TSG 101 isoform was detectable, whereas the signal for the 46 kDa isoform was weak. In contrast to previous results of our laboratory, TSG 101 signal in maternal MV fractions was also observed, indicating that TSG 101 serves as a non-specific marker for exosomes in general (56). However, further experiments are needed in order to test whether contaminations during the isolation procedure might explain this discrepancy. The higher abundancy of the 31 kDa protein could be explained by the fact that this isoform is more involved in the cellular Endosomal Sorting Complexes Required for Transport (ESCRT) - complex system (58).

Interestingly, the Western blot results on all fetal EX fractions revealed that no placenta specific proteins were detectable. Based on previous similar results we can therefore conclude that no placental derived vesicles are released from the human placenta to the fetal circulation. However, in contrast to previous studies (56), we found positive EV-specific signals in fetal plasma samples, in particular for Syntenin and TSG 101. These varying results need further detailed characterization of the origin of isolated vesicles in fetal plasma.

EX that contain integrins can also influence tumor progression by different pathways (59). Sadovsky et al. recently published that integrins may serve as an additional protein marker on EX derived from trophoblasts (1). Integrin beta 4 and Integrin alpha 6 were ranked among others within the top 10 enriched proteins for

trophoblastic EX. However, our results suggest a differentiated conclusion. CD9 did not give any signals in our perfusate and plasma EX samples. In addition, for Integrin alpha 6 only a low signal in the MV fractions of maternal perfusates was detected. Our results suggest that further investigations with more biological replicates are needed as there are still questions left open. Particularly, why some of the proteins such as Integrin alpha 6 can only be found in MV fractions, while others like CD9 could not be detected at all is unclear. It would be very interesting to extend our results by complementary experiments for Integrin alpha 6 since this protein may potentially be used as a specific marker for MVs. Similar to Integrin alpha 6, only positive signals for Annexin A2 proteins in the MV samples of maternal perfusate were identified. But for Annexin A2 a specific band smaller than 38 kDa was detectable in all our EX fractions. This is of big interest and it would be relevant to investigate whether these are undetected isoforms or modified proteins. As already mentioned the diversity of obtained results could also be an outcome result of a heterogeneous quantitative ratio between MVs and EX after isolation.

Nanoparticle tracking analysis (NTA) as our used method for analyzing particles sizes showed intriguing results. The mean size of EX fractions was, even after fractions were purified by isopycnic centrifugation, higher than 100 nm (12). For samples of fraction F13 isolated of maternal perfusates, two main peaks were detected, representing two different distinct EX subfractions, indicating either a different origin of the particles or compositional differences. Another reason for these occurrences could be aggregation of exosomes due to the long-lasting steps during the isolation. However, latest publications describe a size between 30-120 nm for EX (60). The inhomogeneity in the size of EX enriched fractions may additionally originate from swelling processes of these particles during the isolation process at 4°C (61).

Future perspectives:

Our interest in the exact composition of placental EX is based on the diagnostic and therapeutic potential of these particles. Many research groups are currently working on the understanding of the placental EX functionality and on the capability to use EX as biomarkers for pathophysiological pregnancies. Due to numerous findings in this field we are already far closer to better understand and

harness EX as a potential therapeutic and diagnostic aid. It has been shown that a distinct plasma EX profile is strongly related to women who developed GDM during pregnancy as one example (62). Furthermore, placental derived EX, which express FasL and TRAIL proteins on their surface, are capable to induce apoptosis in targeted cells and thus play an important role in the placenta's immune-protective functions and the fetus' immune privilege (57). Cell culture derived EX have already been used in a therapeutic setting, but the proteins that they contain are diverse and many of them remain unclassified (22).

Maternal to fetal communication processes at the placental barrier are still not fully understood, therefore a better understanding of EVs' functionality and their interaction with different tissue layers would enlarge this knowledge. Observations on EX crossing the blood brain barrier have already been made. One may speculate that fatty acids, which are also part of the EX cargo may impact the micro-environment of targeted cells, thereby potentially even accounting for nutritional aspects during pregnancy (24).

Clinical studies using EVs have already been lined out (25). Thus, in order to better distinguish between the different subpopulations more accurate isolation and characterization methods will be needed including technical improvements of the isolation procedure itself. Besides, developing new cutting-edge techniques that allow a more precise EX isolation and a better understanding of their composition, are further areas of interest. To manipulate the biogenesis, composition, secretion, and interaction of EX represents a challenging task in the future; thus leading to the development of new therapeutic approaches (17).

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