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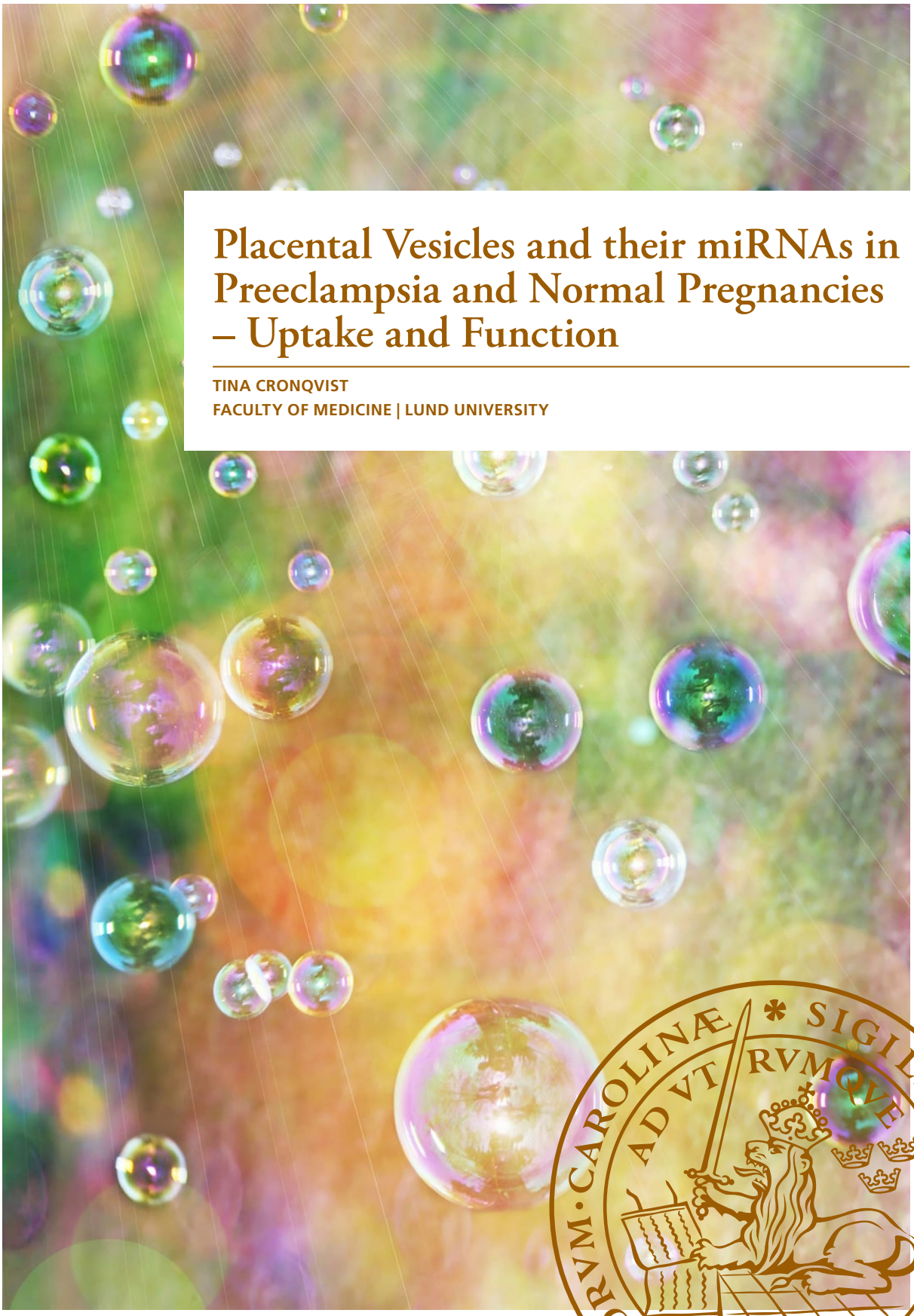

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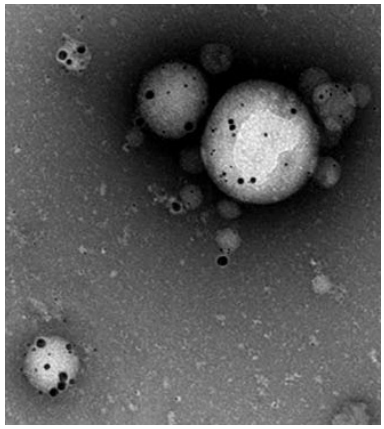

Syncytiotrophoblast extracellular vesicles from perfused normal placentas, treated with antibodies against tissue factor and CD63 as well as the primer hsa-mir-222, labelled with colloidal gold of different sizes. Cropped from paper II, fig 1.

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Tina Cronqvist

LUND
UNIVERSITY

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Abstract

The present thesis focuses on the pregnancy associated syndrome preeclampsia (PE), a potentially fatal syndrome with acute as well as long-term consequences for both mother and child. There is no cure except termination of the pregnancy, no certain way to predict the disorder and only symptomatic treatment. PE develops in two stages. In the first stage, the placenta is inadequately implanted, leading to oxidative stress and release of placental factors into the maternal circulation. In the second stage, the released factors cause endothelial dysfunction and the clinical symptoms. Furthermore, there is growing evidence of long-term cardiovascular consequences after PE

We and others have studied the possible links between the first and second stage. Central to this thesis is the placentally released syncytiotrophoblast extracellular vesicles (STBEVs), which like other extracellular vesicles carries miRNAs and can be taken up by recipient cells where they affect target cell gene expression. We focus specifically on the STBEV role in endothelial dysfunction. In paper I, we describe the interaction between specific miRNAs and trophoblast differentiation, as well as how these are affected by hypoxia. In paper II, the miRNA content of STBEVs is analysed, and how this is affected by foetal haemoglobin. In paper III, an analysis of placenta specific miRNAs in normal and PE STBEVs is performed, as well as studying the uptake of STBEVs and transfer of miRNAs to primary endothelial cells. In paper IV we further elucidate the specific uptake mechanisms of normal PE STBEVs into primary endothelial cells and study the effect on endothelial cell gene expression.

In conclusion, in this thesis we describe molecular mechanisms involved in miRNA regulation of target genes, the release and uptake of placental STBEVs as well as their transfer of functional miRNAs to target cells. By isolating STBEVs from perfused placentas we have been able to study vesicles with a strong physiological resemblance to the in vivo environment.

Key words: Preeclampsia, placenta, micro-RNA, foetal haemoglobin, syncytiotrophoblast extracellular vesicles, exosomes, microvesicles, endothelial dysfunction, cardiovascular disease

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Till Johan

"It begins to appear that almost everything one does to gain a livelihood or for pleasure is fattening, immoral, illegal, or, even worse, oncogenic."
Robbins and Cotran – Pathologic Basis of Disease

Table of Contents

Preface

The present thesis focuses on the pregnancy-associated syndrome preeclampsia (PE), a potentially fatal syndrome with acute as well as long-term consequences for both mother and child. It affects 3-8% of all pregnancies worldwide. There is no cure except termination of the pregnancy, no certain way to predict the disorder, and only symptomatic treatment is available.

Preeclampsia is a very complex disorder. It is a general understanding that PE develops in two stages. Firstly, the placenta is inadequately implanted, leading to oxidative stress and the release of placental factors into the maternal circulation. Secondly, the factors cause endothelial dysfunction and clinical symptoms. Furthermore, there is growing evidence of long-term cardiovascular consequences after PE.

We, and others, have studied the possible links between the first and second stages. Central to this thesis are the placentally released syncytiotrophoblast extracellular vesicles (STBEVs), which, like other extracellular vesicles, carry miRNAs and can be taken up by recipient cells where they affect the target cell gene expression. In the present work, we took a molecular approach to PE, trying to add a piece to the somewhat overwhelming puzzle. We describe molecular mechanisms surrounding the release and uptake of placental STBEVs, their miRNA cargo and effect on target cell gene expression. By isolating STBEVs from perfused placentas we have been able to study vesicles with a strong physiological resemblance to the *in vivo* environment.

In the background, the thesis describes the two-stage model of PE, possible links between the stages, and long-term consequences after a pregnancy complicated by PE. Specifically, the role of miRNAs, STBEVs and their role in endothelial dysfunction will be discussed in depth.

List of papers

The thesis is based on the following papers and manuscript and referred to in the text by their roman numerals indicated below:

I. The unique expression and function of miR–424 in human placental trophoblasts
Mouillet JF, Donker RB, Mishima T, **Cronqvist T**, Chu T, Sadovsky Y.
Biology of Reproduction. 2013 Aug 1;89(2):25

II. Syncytiotrophoblast Vesicles Show Altered micro–RNA and Haemoglobin Content after *Ex–vivo* Perfusion of Placentas with Haemoglobin to Mimic Preeclampsia
Cronqvist T, Saljé K, Familari M, Guller S, Schneider H, Gardiner C, Sargent IL, Redman CW, Mörgelin M, Åkerström B, Gram M, Hansson SR.
PLoS One. 2014 Feb 27;9(2):e90020

III. Syncytiotrophoblast derived extracellular vesicles transfer functional placental miRNAs to primary human endothelial cells
Cronqvist T, Tannetta D, Mörgelin M, Belting M, Sargent I, Familari M, Hansson SR.
Scientific Reports. 2017 Jul 4;7(1):4558.

IV. Placental syncytiotrophoblast extracellular vesicles enter primary endothelial cells through clathrin-mediated endocytosis
Cronqvist T, Erlandsson L, Tannetta D, Hansson SR
Manuscript submitted

Paper not included in the thesis

- Placenta–derived extracellular vesicles: their cargo and possible functions
  Familari M, **Cronqvist T**, Masoumi Z, Hansson SR.
  Reproduction, Fertility and Development. 2017 Mar;29(3):433-447. Review.

Abbreviations

PE	Preeclampsia
PlGF	Placental growth factor
sFlt-1	Soluble fms-like tyrosine kinase 1
ASA	Aspirin, or acetylsalicylic acid
EVT	Extravillous trophoblasts
STB	Syncytiotrophoblast
STBEV	Syncytiotrophoblast extracellular vesicles
FGR	Foetal growth restriction
miRNA	Micro-RNA
pri-miRNA	Primary miRNA
pre-miRNA	Precursor-miRNA
C14MC	Chromosome 14 miRNA cluster
C19MC	Chromosome 19 miRNA cluster
HbF	Foetal haemoglobin
sEng	Soluble endoglin
VEGF	Vascular endothelial growth factor
Hb	Haemoglobin
A1M	Alpha-1-microglobulin
EC	Endothelial cell
EV	Extracellular vesicle
MVB	Multivesicular body
STBM	Syncytiotrophoblast microparticle
TF	Tissue factor

PLAP	Placental alkaline phosphatase
CME	Clathrin-mediated endocytosis
CIE	Clathrin-independent endocytosis
NO	Nitric oxide
ROS	Reactive oxygen species
ICAM-1	Intercellular adhesion molecule 1
HUVEC	Human umbilical vein endothelial cell
CVD	Cardiovascular disease
PHT	Primary human trophoblasts
MβCD	Methyl-beta-cyclodextrin

Background

An introduction to preeclampsia

Preeclampsia (PE) is a pregnancy-associated disorder affecting approximately 3-8% of all pregnancies worldwide, causing over 70,000 maternal and 500,000 foetal and neonatal deaths annually (1, 2). The current definition for diagnosis of PE is new onset hypertension (systolic >140 mmHg and diastolic >90 mmHg) after 20 weeks of gestation, and one or more of the following; proteinuria, maternal organ dysfunction (such as kidney injury, liver involvement, neurological or haematological complications) and/or uteroplacental dysfunction (1). Eclampsia is the potentially fatal end stage of PE with grand mal seizures as the clinical presentation (3). Preeclampsia is divided into early onset (<34 weeks gestation time) and late onset (>34weeks). In early onset PE the foetus is more often growth-restricted than in late onset PE (4). Previously, PE has been classified as mild or severe, but since PE can deteriorate rapidly this classification is now advised against (1, 5).

Even though PE is a common and potentially fatal disorder, it still remains poorly understood. More and more light has been shed on the pathophysiology, and the general consensus today is that PE develops in the two-stage model first introduced by Redman in 1991 (6), and later modified in 2015 by Redman and Staff (7). Briefly, it is believed that an inadequately implanted and poorly perfused placenta releases factors into the maternal circulation, causing endothelial dysfunction and the maternal symptoms (4). A more detailed description of the placenta, the two-stage model, as well as endothelial dysfunction is presented in the following sections.

It is not possible to predict PE, but it is possible to distinguish high risk pregnancies. Risk factors include prior PE pregnancy, chronic hypertension, pregestational diabetes mellitus, maternal body mass index >30 kg/m2, antiphospholipid syndrome, and use of assisted reproduction as well as first pregnancy, family history of PE and a multiple pregnancy. Many biomarkers have been suggested for prediction, such as the angiogenic factors placental growth factor (PlGF) and soluble fms-like tyrosine kinase 1 (sFlt-1) (1, 8, 9). However, in Sweden these biomarkers have not come into clinical use yet (10). After identifying high-risk pregnant women, it has been shown that prophylactic treatment with low dose

aspirin (or acetylsalicylic acid, ASA) could reduce the risk of developing PE if given early in pregnancy (11-13). One study specifically showed that sFlt-1 inhibits trophoblast invasion, and ASA prevents the production of sFlt1, suggesting this as the mechanism by which ASA reduces the risk of PE (14).

There is no specific cure once PE has developed; only symptomatic treatment is available such as anti-hypertensive medication, as well as magnesium sulphate treatment for preventing eclampsia. Corticosteroids are used to promote foetal pulmonary maturity when there is a risk for delivery before 34 weeks of gestation (1, 8). The only "cure" remaining is induction of delivery and thereby removal of the placenta. International guidelines recommend induction of delivery from 37 gestational weeks (1, 10).

Stage 1 – the placenta

The placenta in normal pregnancy

The placenta is a temporary but vital organ, which acts as a link between mother and foetus during pregnancy, supplying the foetus with oxygen and nutrients, as well as removing waste products (15). The placenta is disc shaped and haemochorial, meaning that the foetal trophoblasts come into direct contact with maternal blood (16, 17). The placenta has two sides; the chorionic plate (foetal side) facing the foetus, and carrying the umbilical cord, as well as the basal plate (maternal side) facing the maternal endometrium of the uterus (15, 18).

After fertilisation, the embryo undergoes cell division, forming the blastomere, which consists of eight cells. After further cell division, a blastocyst is formed, consisting of two cell populations; the trophectoderm cells and the inner cell mass. The trophectoderm cells eventually give rise to the placenta and the inner cell mass to the embryo (16). Once the embryo has implanted into the uterine wall, trophoblast cells proliferate and differentiate to either villous or extravillous trophoblasts (EVTs). The villous cytotrophoblast cells do not migrate, but instead form the multinucleated syncytiotrophoblast (STB) cells (18), which function as an epithelial lining of the villous trees (Figure 1). During pregnancy, the syncytiotrophoblast cells are continuously renewed due to fusion with the underlying cytotrophoblast cell layer, with shedding of syncytiotrophoblast extracellular vesicles (STBEVs) (19). Being in the interface between the foetal and maternal sides, the STBs have several important functions, such as transport of oxygen and nutrients between the maternal and foetal sides as well as synthesis of steroids and hormones (15).

The EVTs migrate into and invade the uterine decidua and start the remodelling of the uterine arteries (18, 20). This peaks at around 9-12 weeks of gestation (16). This process also involves interaction between the placenta and the maternal uterine immune cells. Uterine natural killer cells are involved in the migration of the EVTs, and through release of cytokines and growth factors appear to facilitate the arterial conversion (21) where the smooth muscle cells and endothelium of the maternal arteries are replaced by trophoblasts. This in turn, facilitates blood flow to the placenta, turning the previously high-resistance uterine vessels into low-resistance vessels. Until the end of the first trimester (12 weeks of gestation), the maternal vessels, called spiral arteries, are plugged by EVTs, preventing blood flow and causing a hypoxic environment beneficial to trophoblast development (16, 18, 20). At the end of the first trimester, the spiral arteries are unplugged, and the maternal blood flows into the intervillous space surrounding the villous trees. (22). The villous trees consist of foetal blood vessels, which branch and form terminal villi

with a foetal capillary network. In the intervillous space, the foetal villi come into contact with, and are bathed in, the maternal blood (15, 18, 23).

Interestingly, the surface of the villous tree is protected from immunological interactions and the immunological mechanisms of pregnancy is both complex as well as not fully understood. The STBs, forming the outer layer of the villous trees with direct contact with the circulation, express no major histocompatibility complex (MHC) antigens, and the invading EVTs express an unusual combination of human leukocyte antigen (HLA) molecules on their surface. The immunological changes during pregnancy appear necessary for maternal tolerance (17, 21).

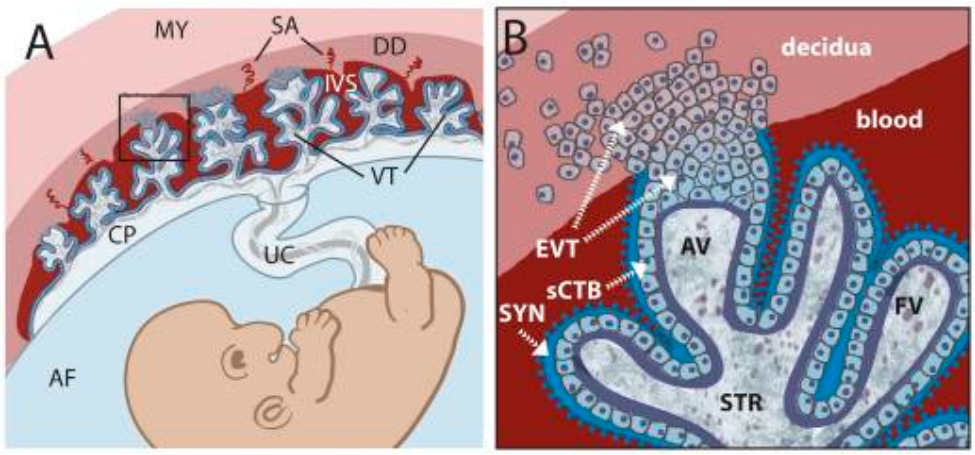

Figure 1. Overview of the placental structure.

Structure of the placenta (A) with an enlargement in (B), showing exravillous trophoblasts (EVTs) invading the maternal decidua (DD). The foetal vessels (blue) are bathed in the maternal blood (red). Note the epithelial lining of the villous tree consisting of syncytiotrophoblasts (in this figure abbreviated as SYN, in the thesis referred to as STB).

MY: myometrium, SA: spiral arteries, DD: decidua (uterine lining during pregnancy), IVS: intervillous space filled with maternal blood. VT: villous tree, CP: chorionic plate, UC: umbilical cord, AF: amniotic fluid. AV: anchoring villi, FV: floating villi. SYN: syncytiotrophoblast, sCTB: subsyncytial cytotrophoblasts. STR: stroma. EVT: extravillous cytotrophoblasts. Adapted from Robbins et. al. 2010 (22).

The preeclamptic placenta

The placenta is central to PE aetiology. During the first stage of PE, the placenta is poorly implanted into the uterine wall (24, 25) due to inadequate invasion of the trophoblasts and incomplete remodelling of the spiral arteries (Figure 2) (5). This leads to reduced placental perfusion (20), and also to STB stress as well as oxidative and ER stress. Furthermore, the stress leads to ischaemia and the release of different factors, such as angiogenic factors and vesicles, into the maternal circulation. The reason for this incomplete placental implantation is still unknown (20, 24, 25). Impaired placental development will result in reduced placental vascularity, reduced blood flow as well as decreased oxygen and nutrient delivery to the foetus (16). Disordered placentation can lead not only to PE but also to other pregnancy-related syndromes, such as foetal growth restriction (FGR), placental abruption, spontaneous miscarriage, pre-term rupture of the membranes and premature delivery. Early onset PE is more often associated with FGR than late onset PE (4, 5).

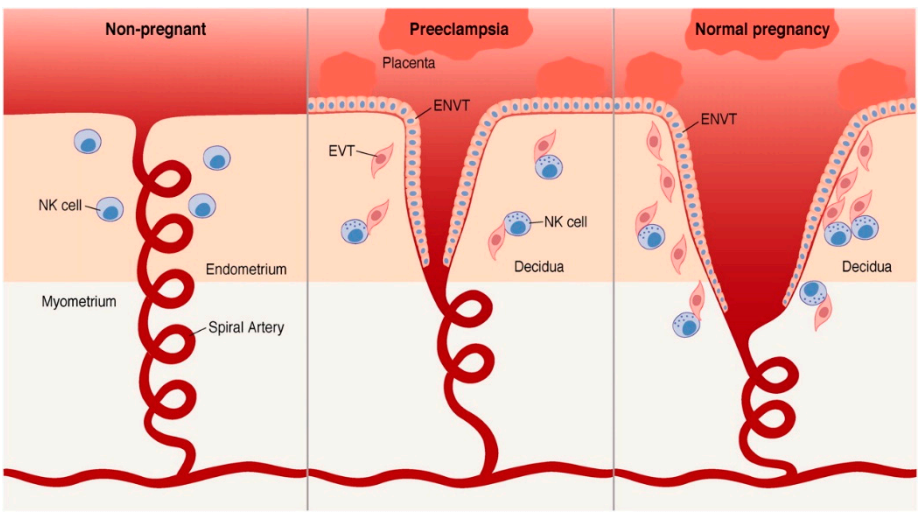

Figure 2. Spiral artery remodelling in normal and PE pregnancy.
The left panel shows a spiral artery in the non-pregnant uterus. The right panel shows spiral artery remodelling in normal pregnancy, with EVT invasion and ENVT lining of the artery, as well as interactions with uNK cells. The middle panel shows the shallow EVT invasion and decreased spiral artery remodelling associated with PE.

ENVT: endovascular trophoblast cells. EVT: extravillous trophoblast cells. Adapted from Parham 2004 (26).

Normal pregnancy is in itself an increased inflammatory state compared to non-pregnancy, and the inflammation is further increased in PE. When histologically examining PE placentas, they more often show pathological findings than seen in normal placentas. The pathology is even more pronounced in early onset PE than in

late onset (5, 20), with clear signs of abnormal implantation and stress. In some late onset PE placentas there are no obvious pathological signs of disease (20). Lesions of the PE placenta include infarcts of the villous tissue, fibrin deposition, and inflammation. Microscopically, there are signs of STB necrosis, dilation of the ER, and swelling of mitochondria, and these lesions have been shown to increase the release of trophoblastic debris. Furthermore, it has been shown that early onset PE leads to higher levels of placental stress, such as oxidative stress and activation of the unfolded protein response than seen in late onset PE (5).

It has been suggested that early onset PE is a primarily "placental" form of PE, with increased inflammatory processes in the placenta that in the end affect the maternal circulation. On the other hand, late onset PE is more of a "maternal" form of PE, where pre-existing risk factors such as obesity, autoimmune disease, and chronic arterial disease, increase the risk of getting PE by making the maternal endothelium more sensitive to even normal changes of pregnancy. However, it has also been suggested that the "maternal PE" is not entirely dependent on a sensitive maternal endothelium but is also a placental disease. The maternal pre-existing inflammatory components could impact placentation, placental size and function, and in the end may contribute to placental dysfunction and PE. In these cases, the maternal factors both affect the placenta and lead to increased sensitivity of the maternal endothelium to factors released from the placenta. In summary, there is no PE without a placenta (4).

The differences described above are most likely because PE is a syndrome and not a specific disease. The symptoms of hypertension and organ dysfunction are consistent with the diagnosis, but the aetiology might differ, with PE caused by either the placenta failing or the maternal endothelium being overly sensitive to released factors.

Placenta- and pregnancy related miRNAs

Many micro-RNAs (miRNAs) have been described throughout the human body, in different organs and cell types as well as in different diseases. The placenta and PE are no exceptions and miRNAs have been shown to be relevant both for normal placental development as well as dysfunction. Before going through placenta-related miRNAs, I will briefly describe miRNAs in general.

An introduction to miRNAs

The miRNAs are small non-coding RNA molecules considered to regulate approximately 30% of all human genes (27). The first miRNA was discovered in 1993 when Lee at al cloned the gene lin-4, which controls the larval development of *C. elegans* (28). But it was not until the year 2000 that reports were produced describing more than a hundred genes that were coding for small non-coding RNAs. These genes were all endogenously expressed and processed in a similar way (29). Today, more than 1000 human miRNAs have been discovered (30). Many miRNAs are conserved, as can be seen especially when comparing closely related animals but also when comparing between very different animal lineages, for example *C. elegans* and humans have more than a third of their miRNAs easily recognised as homolog miRNAs (29).

But how are these small RNAs formed? The miRNAs are transcribed with the help of RNA polymerase II (Figure 3) into a primary miRNA (pri-miRNA) which forms a so-called hairpin structure. The pri-miRNA is further digested by Drosha, a ds-DNA-RNA-specific ribonuclease, and released as precursor-miRNA (pre-miRNA). The pre-miRNA is then transported to the cytoplasm by Exportin-5 and thereafter cleaved by Dicer, a member of the RNase III superfamily. This results in a double stranded RNA (ds-RNA) with short 3' overhangs at each end, the miRNA:miRNA* duplex (29, 31). The true mature miRNA is formed from one of the two strands, named either the leading or the lagging strand (31), after cleavage by Helicase. The resulting mature miRNA (approximately 22nt long) binds to the RNA-induced silencing complex (RISC) which transports the miRNA to its target mRNA (29, 32).

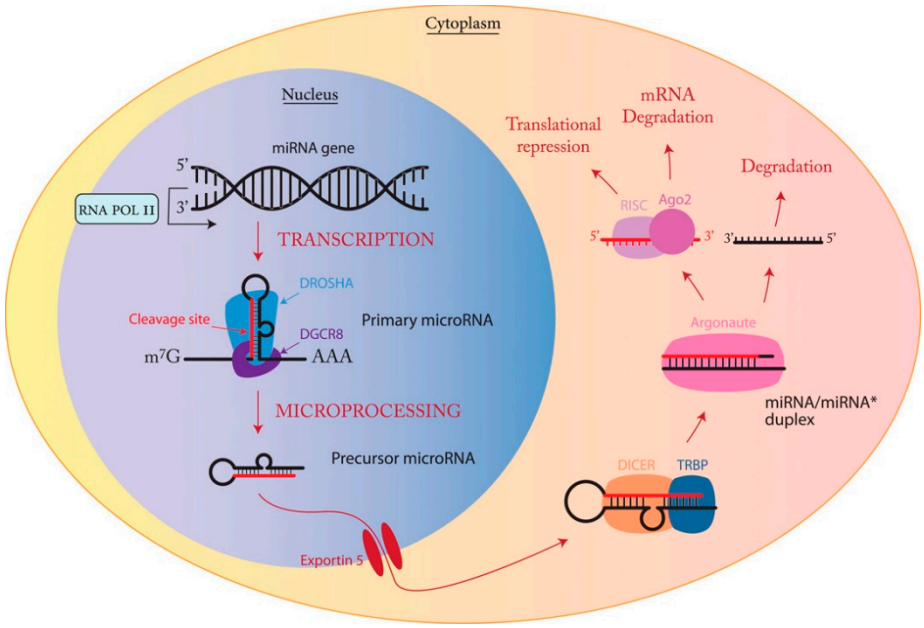

Figure 3. miRNA maturation
An overview of miRNA maturation. The miRNAs are transcribed to pri-miRNA, converted to pre-miRNA and exported out of the nucleus,where they are further modified until a mature miRNA is present. Adapted from Hemmatzadeh 2019 (33).

A miRNA generally downregulates gene expression in one of two post-transcriptional ways, either by repressing the target mRNA or by cleaving it (29, 34, 35). This is decided based on the complementarity between the miRNA and mRNA, where the miRNA binds to the 3'UTR region of the mRNA (29, 36). If there is sufficient complementarity, the miRNA will direct cleavage (29), while imprecise binding causes translational repression by blocking or altering the normal function (31). The imprecise binding between miRNA and mRNA means that miRNAs can bind to several different mRNAs and thereby have a broader regulatory potential (36). However, there is also evidence suggesting that under certain conditions and in specific cell types, miRNAs can lead to up-regulation of genes (37-39).

There are miRNAs expressed throughout the human body and several have tissue- or cell-specific expression as well as specificity to a certain stage of development. It is likely that every cell type, at different stages of development, has a specific miRNA expression profile (29). For example, heart and vascular cells have specific miRNA expression profiles which are altered during certain pathological conditions

(40). The miRNAs are involved in many physiological and pathophysiological processes in the human body. They have been shown to play an important role in the development and regulation of cellular differentiation and the expression of tissue specific genes. The miRNAs are involved in cancer, where they can act as oncogenes or tumour suppressors (30) and are also important in angiogenesis (41).

It has also been shown that miRNAs are abundant in plasma and serum. Normally, RNAs in plasma or serum are very sensitive to degradation if handled improperly but serum miRNAs have been shown to remain stable after extreme conditions such as boiling, pH changes, freezing and extended storage. This resistance might be due to the transportation in extracellular vesicles (42). Furthermore, it has been shown that miRNAs can be shuttled from one cell to another and thereby regulate gene expression in recipient cells (43, 44).

As mentioned before, some miRNAs have been shown to be tissue-specific. The placenta expresses both placenta-specific miRNAs as well as an abundancy of specific miRNAs that are also commonly expressed in other tissues (31), which will be discussed in detail below.

Placental and pregnancy-related miRNAs

In the placenta, several miRNAs are abundantly or even exclusively expressed. Studies on human placentas have shown that miRNAs are particularly abundant in this organ and exhibit a distinctive expression profile (32). The placental miRNA profile is dominated by three miRNA gene clusters; the chromosome 14 miRNA cluster (C14MC), the chromosome 19 miRNA cluster (C19MC) and the miR-371-3 cluster (45-47), with varying degrees of expression throughout pregnancy (45, 46). The C14MC is a large gene cluster, containing 52 miRNA genes, with some miRNA members predominantly expressed in the placenta (47). The C19MC is also large, containing 46 miRNAs (48). It is imprinted (49), and almost exclusively expressed in the placenta as well as trophoblast-derived cell lines (32, 45, 50, 51). Finally, the miR-371-3 cluster is much smaller, consisting of only three miRNAs (50).

The miRNAs of the placenta are essential for placental development and function (32). Different miRNAs are suggested to be involved in events such as trophoblast differentiation (52), EVT function and migration (53), spiral artery remodelling as well as in angiogenesis (54). It has even been shown that miRNAs belonging to the C19MC can have antiviral effects (55). In paper I we study the role of the abundant placenta-derived miR-424 in trophoblast differentiation.

During normal pregnancy, several studies have reported an increase in specific miRNAs in the maternal circulation, mainly those connected to the placenta-specific miRNA clusters (51, 56). The placenta-specific C19MC miRNAs as well as other

pregnancy- and placenta-related miRNAs have been found both in plasma and exosomes (50, 51, 57-59). It has been shown that placental cells can release extracellular vesicles containing miRNAs, which are taken up by recipient cells (51, 58, 60).

The levels of different miRNAs vary throughout pregnancy. In the first trimester, C14MC levels in the maternal circulation are high and then decrease towards the third trimester, whereas C19MC levels rise throughout pregnancy (50). There are also increases of other commonly expressed miRNAs that have been shown to be pregnancy-related and expressed abundantly in the placenta (46, 57). For example, miR-141 and miR-424 are significantly higher in pregnant plasma compared to non-pregnant (61). Furthermore, Hromadnikova et al. analysed umbilical cord blood and found that the expression of miRNAs previously connected to cardiovascular and cerebrovascular disease, correlated with PE severity (62).

Just as miRNAs are important in normal pregnancy, they are believed to play a role in PE as well. Many miRNAs have been described to be differently expressed in PE compared to uncomplicated pregnancies (63). In the PE placenta, several miRNA are dysregulated, and miRNAs such as miR-210 and several C19MC miRNAs have been shown to be up-regulated (64-66) as also seen for miRNAs connected to angiogenesis (67). In contrast, other studies show a down-regulation of C19MC miRNAs in PE placentas (68), as well as a down-regulation of miR-424 and several C14MC miRNAs (65, 66, 69). The dysregulation of miRNAs in PE is also reflected in the maternal circulation, where C19MC miRNAs are up-regulated (70). The C19MC miRNAs have also been found in exosomes from pregnant women with different pregnancy-related conditions, including PE, where they reflect the C19MC profile of placentas from the corresponding condition (57). Taking these findings together, it is evident that the placenta is an important contributor to the circulating miRNAs present in pregnancy and may both contribute to the development of PE as well as the long-term effects on the cardiovascular system. In studies II-III we analyse the miRNA content of STBEVs, showing how it is affected by HbF as well as differences between normal and PE placentas.

The link between stage 1 and 2 – release of factors

Central to the first stage of PE is the dysfunctional placenta, which by releasing different factors causes the second stage of PE and, once the organs are affected, also causes the maternal symptoms. No single factor that is responsible for the link between the two stages has been identified, but studies suggest a variety of factors being the responsible culprits, ranging from oxidative stress, placental extracellular vesicles and anti-angiogenic factors (24, 25). Below, I will go through three links/factors that have been suggested in the literature to date; angiogenic factors, foetal haemoglobin (HbF) and STBEVs. The angiogenic factors as well as STBEVs are widely recognised as important players in the PE pathology. Studies from our group have also suggested HbF as a potential factor linking the first and second stages of PE.

Angiogenic factors

Four angiogenic factors, produced by the placenta, have been proposed as relevant to PE; increased levels of anti-angiogenic factors sFlt-1 and soluble endoglin (sEng), as well as decreased levels of the pro-angiogenic factors vascular endothelial growth factor (VEGF) and PlGF (71). The angiogenic factors are believed to be released into the circulation and cause the endothelial dysfunction that is characteristic for PE, thereby resulting in the clinical symptoms (72). It has been proposed that either PlGF alone or the sFlt-1/PlGF ratio could be used as predictors of PE (73).

In 2003, Maynard et al. (74) for the first time suggested that the anti-angiogenic factor sFlt-1 was an important part of the PE pathology. They found that sFlt-1 mRNA was up-regulated in PE placentas (74), and they and others have shown that PE patients have increased levels of sFlt-1 in the circulation. It is known that sFlt-1 antagonises the pro-angiogenic factors VEGF and PlGF, and their levels are indeed also decreased in PE serum compared to normal, correlating with the increased sFlt-1 levels (74-76). In animal models, sFlt-1 has been shown to induce PE symptoms, such as hypertension and glomerular endotheliosis (74).

The anti-angiogenic protein sEng is also increased in PE and the levels correlates with PE severity. It is suggested to play a role in the angiogenic imbalance of PE (77, 78). The sEng levels correlates to an increase in the sFlt-1/PlGF ratio (78). Using animal models, sEng has, much like sFlt-1, been shown to cause hypertension and induces vascular permeability (77).

Foetal haemoglobin

Studies from our group have suggested HbF as a potential link between the first and second stages of PE. The studies on HbF began in 2008 when Centlow et al. (79) published a study showing increased gene expression of the haemoglobin (Hb) chains Hbα and Hbγ in PE placentas, also suggesting the Hb to be of foetal origin. This finding was quickly followed up by placental perfusion studies, where placentas from normal pregnancies were perfused with xanthine/xanthine oxidase and free Hb, leading to genetic changes similar to those seen in PE. It was suggested that oxidative stress may increase the expression of Hb genes, as seen in PE placentas (80).

A hypothesis was formed, according to which HbF has a role in the aetiology of PE by being overproduced in the PE placenta, inducing oxidative stress in the placenta, damaging the blood-placenta barrier, and leaking over to the maternal circulation. There, HbF causes endothelial damage and oxidative stress and consequently the symptoms of PE. Foetal and adult Hb was therefore measured in plasma, urine and placenta samples from PE and normal pregnancies. Free HbF was elevated in plasma from PE women, with higher HbF levels correlating with higher blood pressure levels (81, 82). These findings were followed up by additional clinical studies, showing that an HbF/Hb ratio combined with levels of alpha-1-microglobulin (A1M), as well as the Hb/heme scavenging system, could be used as a predictive and diagnostic biomarker for PE (83, 84).

Returning to the placental perfusions, it was shown that perfusing placentas with free Hb led to increased perfusion pressure as well as a higher foeto-maternal leakage, similar to what is seen in PE. Using electron microscopy, there was visible damage to the placental morphology after Hb perfusions, which was not seen in normal placentas. (85) In a ewe model it was shown that haemolysis, due to starvation, causes PE symptoms and structural damage to the placenta and kidneys (86). Further animal studies on rabbits, showed how HbF caused proteinuria and glomerular damage. By transmission electron microscopy, both kidneys and placentas showed intracellular as well as extracellular damage after HbF treatment (87). In paper II we show that Hb can be carried by STBEVs, possibly sheltering it from degradation. In paper III we also show that PE STBEVs carry HbF and that they deposit it in primary endothelial cells (ECs) as well as causing extensive damage to the EC membrane.

Syncytiotrophoblast extracellular vesicles

The STBEVs are a widely recognised and important factor in the PE pathology and one of the main focuses of this thesis. A more general introduction to extracellular vesicles will precede the detailed description of STBEVs.

Extracellular vesicles

Extracellular vesicles (EVs) are defined as membrane vesicles containing cytosol from the secreting cells enclosed in a lipid bilayer. Different EVs have been shown to be released by a variety of organisms and cell types into all researched body fluids, including blood, urine, breast milk and amniotic fluid (88).

The nomenclature surrounding EVs is diverse and sometimes confusing (89), since they have been named according to vesicle size as well as cell of origin (88). The general opinion today is that EVs can be divided into microvesicles and exosomes (Figure 4). The term 'microvesicles' refers to 150-1000 nm vesicles that are budded off from the cell plasma membrane. The term 'exosomes' refers to smaller (approximately <200nm) vesicles of endosomal origin (88, 90). When early endosomes mature into late endosomes, there can be an accumulation of vesicles inside, leading to the formation of multivesicular bodies (MVBs). The MVBs are often destined to fuse with lysosomes where lysosome content is degraded, but certain MVBs can also fuse with the plasma membrane of the cell and release their content (in this example, the exosomes) to the extracellular space (88). It is worth noting that the term 'exosome' is also used interchangeably with the term EV in many publications (89). It is difficult to distinguish between exosomes and microvesicles in an experimental setting, since these EV subtypes overlap in terms of size and surface markers (90, 91).

Exosomes, or rather EVs, were discovered and started to be described as early as the 1970s (88). However, it was not until a study in 1996 by Raposo et al. (92) that interest started to increase dramatically. In this study, the authors showed that exosomes derived from lymphocytes could induce a T cell response. Furthermore, around the years 2006-2008, several studies showed how exosomes and microvesicles from many cell types contained both mRNA and miRNA, which could be transferred to recipient cells and be functionally active, changing the recipient cells' protein composition and activity (43, 93-95).

It is known that EVs contain proteins that are common to many EV types, as well as proteins more specific to their origin. There is an abundance of cytoskeletal, cytosolic, heat shock and plasma membrane proteins, whereas intracellular organelle proteins are uncommon. The EV proteins are also glycosylated, with altered glycosylation patterns in different pathological conditions (96).

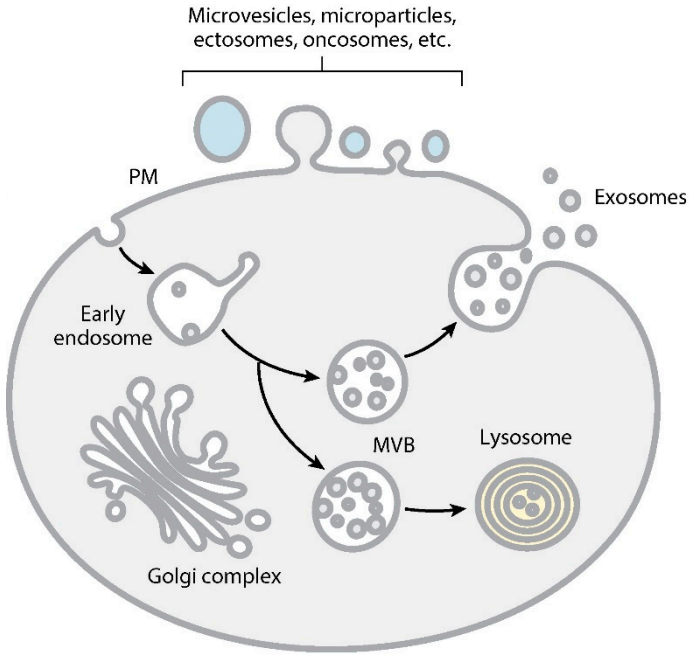

Figure 4. Extracellular vesicle subtypes
Extracellular vesicles from different cellular origins. The microvesicles are released from the cell of origin by budding off from the cell plasma membrane The exosomes are formed inside endosomal compartments and multivesicular bodies (MVB) and released through exocytosis. Adapted from Colombo et al. 2014 (88).

One of the main research areas connected to EVs is cancer, and cancer cells are shown to release excess amounts of EVs. Furthermore, the cancer cell-derived EVs are involved in both tumour development as well as metastasis (97-99). It is shown that EVs from glioblastoma can transfer mRNA, which is then translated in the recipient EC (97).

In the field of cardiovascular disease, the EVs have also attracted interest. An increase of vesicles from platelets, monocytes, ECs, red blood cells and granulocytes has been detected in plasma during atherothrombotic cardiovascular diseases (100). It has also been shown that the levels of EVs from ECs, platelets, leucocytes and others are increased in, and contribute to atherosclerosis by increased inflammation and expression of adhesion molecules. In acute coronary syndrome, the levels of pro-coagulant EVs have been shown to be increased (101).

As described above, different types of EVs are of interest in many conditions, and not least in pregnancy and PE. In pregnancy there is an increase in EVs derived from platelets and ECs (102, 103) as well as a type of EV only present during pregnancy – the placenta-derived STBEV.

STBEVs

During pregnancy, the STB cells are continuously renewed due to fusion with the underlying cytotrophoblast cell layer (Figure 1) and constantly shed vesicles (19). Besides the aforementioned microvesicles and exosomes, the placenta also releases syncytial nuclear aggregates (20-500μm) and apoptotic bodies (1-4μm) (104, 105). The STBEV levels increase throughout pregnancy and are removed from the circulation 48 hours after delivery (105).

The nomenclature for placental vesicles is, as for EVs in general, somewhat hard to grasp. The subtypes are, as already described, exosomes and microvesicles but based on the source of the vesicles, they are also named in different ways. Vesicles with a placental or syncytiotrophoblast cell origin have been named in many ways, and in the articles and manuscript connected to this thesis we have used both 'syncytiotrophoblast microparticles' (STBM, paper II) as well as 'STBEVs' (papers III-IV). The term 'placental vesicles' will also be used here as a general term for EVs released from placental tissues, explants or cells.

In 1998, Knight et al. showed how placental STBs shed what was then referred to as microvilli, into the maternal circulation (106); these were later recognised as STBEVs. Being labelled as "debris" in the beginning, STBEVs have become more and more acknowledged over the years and are now believed to be important bioactive messengers, playing a role in both normal and pathological pregnancies (107). In PE, the number of STBEVs has been shown to be increased (106, 108), although this is primarily an increase in early onset PE and not late onset (109). This could reflect the differences in placental pathology described for early and late onset PE, with a more dysfunctional placenta in the early onset PE (110).

It is also hypothesised that not only are the STBEV numbers increased in PE, there is also a change in the balance between the supposedly more beneficial exosomes and the harmful microvesicles, with an increase in microvesicles in PE (111, 112). It has been suggested that placental exosomes play a role in the fusion of syncytiotrophoblasts during normal placental development (113). Changes in oxygen tension have been shown to modulate the protein composition of placental vesicles, which could lead to changes in interaction with recipient cells (114).

It is generally accepted that PE is a disorder of endothelial dysfunction and increased inflammation (115), where STBEVs play an important contributing role (116). The STBEV exosomes are considered immunosuppressive and are probably most concentrated in the intervillous space, where they can protect the foetal-maternal interface from maternal immune attack. In contrast, the microvesicles are pro-inflammatory, immune activating and pro-coagulant. It is this effect, caused by the increased levels of microvesicles, that appears enhanced in PE (117).

In normal pregnancy, there is already an enhanced inflammatory response where mononuclear cells (primarily lymphocytes and monocytes) produce increased levels of inflammatory cytokines (108). It has been shown that STBEVs can induce such a response and increase cytokine production (116, 118). It is suggested that STBEVs activate the maternal systemic immune response, causing a controlled inflammation that might help the mother fight infection (105). In addition, vesicles from trophoblast cells have antiviral properties that are not seen in vesicles from non-pregnant individuals (119).

The STBEVs contribute to the normal systemic inflammatory response of pregnancy and appear to inhibit the T cell response (120). It is suggested that STBEVs modulate the maternal immune cell function (105), since they can be bound to and internalised by both monocytes and B cells (108, 116).

In PE, the STBEV effect on immune cells is greater than in normal pregnancy, (121), and is suggested to be a causative factor for the increased systemic inflammation seen in PE (122). Furthermore, STBEVs in general, and PE STBEVs in particular, can activate platelets, which could explain the higher risk of thromboembolism in PE. When treating platelets with ASA (mentioned previously as a PE preventive drug), the STBEV-induced aggregation is inhibited (123).

The constitution of STBEVs has been researched, showing that STBEVs are abundant in lipids such as sphingomyelin, cholesterol as well as phosphatidylcholine, phosphatidylserine and phosphatidylinositol. Sphingomyelin is increased in PE STBEVs compared to controls, which is interesting since sphingomyelin has been shown to be involved in inflammation, immune responses and oxidative stress. There is also an up-regulation of phosphatidylserine on PE STBEVs, which might reflect an activation of apoptotic pathways in placental STBs in PE (124).

The STBEVs consist of several hundred proteins, some of which are differentially expressed in PE, for example annexins and integrins (125). The STBEVs express tissue factor (TF) on their surface, shown by the ability to trigger thrombin generation. This expression is enhanced in PE which would contribute to the pro-coagulative state of the disorder (126). In a study where cancer cell line-derived vesicles contained TF on their surface, it was shown that these vesicles could transfer the TF to recipient ECs (127). If this transfer applies to PE STBEVs as well, it would contribute to a further pro-coagulant potential systemically.

A connection between angiogenic factors and STBEVs has been shown, where Flt-1 is bound to STBEVs (112), which is seen in vesicles from first trimester placentas (128). The PE STBEVs and placental first trimester vesicles carry more Flt-1 than normal (112, 128). Finally, an important marker, which is specific for STBEVs, is the placental alkaline phosphatase (PLAP), commonly used to identify or capture

STBEVs (105), and produced by STB cells. PLAP+ vesicles have only been found in plasma from pregnant women (129). However, even with a pregnancy-specific marker, the results can be hard to interpret, which became apparent when it was discovered that PE STBEVs express less PLAP (112) than normal STBEVs. This would lead to an under-estimate of PE STBEV numbers. Also, exosomes express less PLAP than microvesicles (130). The interaction between STBEVs and ECs is described in a section of its own further down.

Vesicular uptake by target cells

Extracellular vesicles in general, as well as STBEVs specifically, can be taken up by target cells. The pathways by which this occurs seem to differ depending on vesicle type as well as type of target cell.

The process where any kind of extracellular material is taken up by a cell is called endocytosis. Primarily, endocytosis is divided into clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) (Figure 5) (131). Endocytosed material can be transferred, via early and late endosomes, to either lysosomes for degradation or to different compartments such as Golgi and ER, or can be recycled back to the plasma membrane (131, 132).

The CME is dependent on cell surface receptors, where molecules or vesicles bind to specific receptors which in turn triggers the assembly of clathrin and the formation of clathrin coated pits in the plasma membrane. The pits are further formed into clathrin-coated vesicles which are pinched off using the protein dynamin. After formation of the vesicle, clathrin is uncoated and the vesicle fuses with early endosomes (131, 133). As will be described below, cholesterol is mainly a part of CIE during its involvement in lipid raft formation. But it has been shown that the absence of cholesterol also leads to flattening of clathrin coated pits (133).

The CIE is also referred to as lipid raft-dependent and is commonly divided into caveolae-dependent endocytosis, macropinocytosis and phagocytosis. Caveolae-dependent endocytosis is dependent on the protein caveolin and, just like CME, utilises dynamin. The areas where caveolae are formed are enriched in cholesterol and sphingolipids (134). In macropinocytosis, the plasma membrane forms ruffled regions dependent on cholesterol, which are used to take up the extracellular material (133). Phagocytosis occurs primarily in specialised cells, where the cell membrane protrudes around the molecule or vesicle that is to be taken up. This process is dependent on actin polymerisation (133).

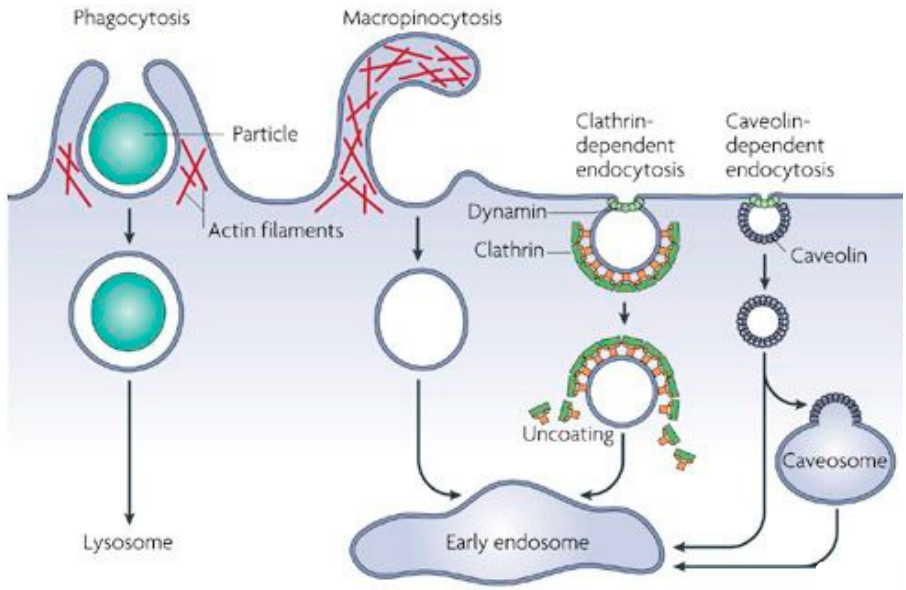

Figure 5. Endocytic uptake pathways

A simplified figure showing the endocytic pathways. Here is shown the clathrin-mediated endocytic pathway, with a clathrin coated vesicle being pinched off by dynamin. The clathrin-independent pathways phagocytosis, macropinocytosis and caveolin-dependent endocytosis are shown as well. Adapted from Mayor et al. 2007 (134).

Extracellular vesicles have been described using many different uptake pathways (Figure 6), both CME (135, 136) and CIE (135-138) as well as membrane fusion (135). There have been only a few studies investigating the specific internalisation of placental vesicles and by which uptake pathways this occurs. It has been suggested that uptake of placental vesicles occurs through internalisation of the ECs by either phagocytosis and/or endocytosis (139). Also, platelets have been shown to internalise STBEVs (123). A study by Vargas et al. (140) showed how placental vesicles from villous trophoblasts, carry the receptors syncytin-1 and syncytin-2 on their surface, and that these are involved in their uptake into BeWo cells, a human trophoblastic cell line. The syncytin proteins are otherwise present on placental cytotrophoblast cells and are involved in cell fusion into STBs (140). In paper III we show that STBEVs from placental perfusions are internalised by primary ECs, and in paper IV we suggest that this occurs mainly through CME.

Not only can EVs be taken up by recipient cells, but they can also transfer mRNA and miRNA to affect the target cell gene expression (43, 97), as is also shown in paper III. This uptake and transfer can even occur between species, for example, mouse exosomes to human cells (43). An impact on target cells can also occur by transfer of surface molecules from the EVs, as has been shown for TF on EVs, where the EV surface TF was recycled to the target EC surface (127). Furthermore, an exciting study by Delorme-Axford et al. (141) showed how primary human trophoblasts are not only resistant to several virus types, but they also secrete vesicles that transfer this resistance to recipient cells. The resistance is due to the placental C19MC miRNAs, which are packaged into the trophoblast vesicles and deposited into cells not normally expressing this miRNA cluster (141).

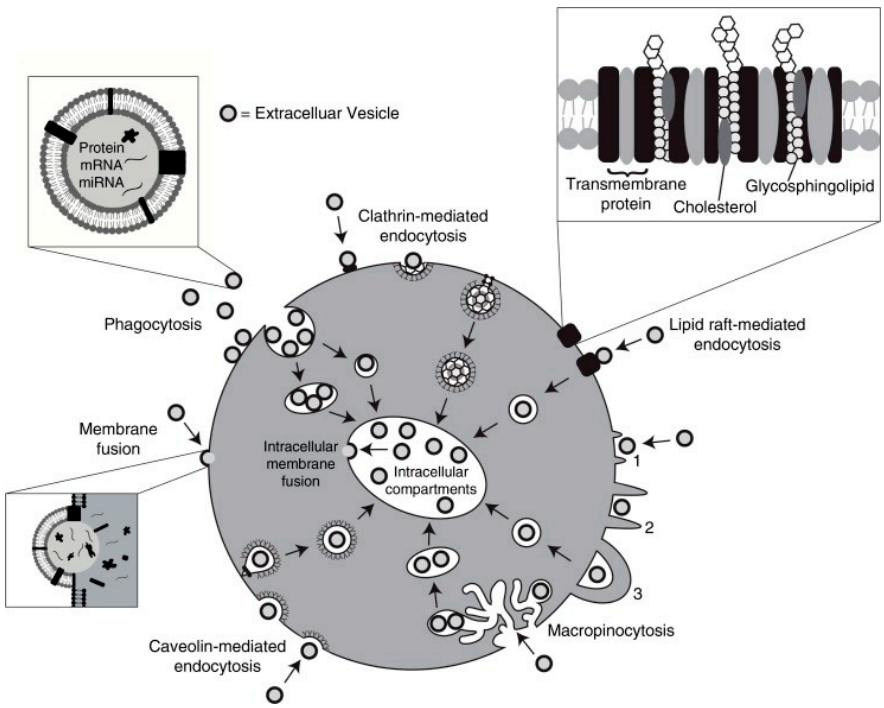

Figure 6. Uptake pathways of extracellular vesicles
Extracellular vesicles can be taken up by the recipient cells by many different pathways. Described in a review by Mulcahy et al. are; clathrin-mediated endocytosis, as well as clathrin-independent but lipid raft-mediated, endocytosis, which includes caveolin-mediated, macropinocytosis as well as phagocytosis. It has also been indicated that vesicles can fuse with target cell membrane. Adapted from Mulcahy et al. 2014 (135).

Endothelial dysfunction

What is endothelial dysfunction?

Very simply described, endothelial dysfunction refers to the endothelium losing its normal functions and vasodilation capacity, and entering a pro-inflammatory and prothrombotic state (142). Under normal circumstances, the endothelium helps to maintain vascular tone and blood flow through the vascular system and there is very little, or even no expression of inflammatory markers (143).

When the endothelium is subjected to any form of damage or alterations to its physiological function, the result is an increase in inflammatory processes as well as a loss of antithrombotic and vasodilation mediators, such as nitric oxide (NO) (142, 144). The endothelium releases NO, which besides vasodilation also has an anti-inflammatory effect and an anti-aggregation effect on platelets (142). The reduced levels of NO in endothelial dysfunction can be ascribed both to reduced endothelial nitric oxide synthase (eNOS) activity (142), as well as lower bioavailability of NO due to increased levels of reactive oxygen species (ROS) reacting with NO (144). Endothelial damage and dysfunction also includes an increase in prothrombotic and vasoconstriction mediators (144).

The term 'endothelial activation' refers to increased levels of pro-inflammatory, proliferative and pro-coagulative factors (144). Oxidative stress and ROS cause endothelial dysfunction by increased endothelial permeability and up-regulation of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), leading to increased leukocyte adhesion to the ECs (145).

Endothelial dysfunction is connected to many cardiovascular diseases and events (144), as well as to PE (146). The endothelium is central to PE pathogenesis, with factors released from the dysfunctional placenta in stage 1 of PE affecting the endothelium and causing maternal symptoms in stage 2. Many studies suggest that the endothelial dysfunction of PE is rather an activation than an actual injury occurring in the endothelium (146). It is also hypothesised that it is the angiogenic factors that cause the endothelial dysfunction (72).

Whether the endothelial dysfunction is caused by PE or a pre-existing risk factor is still unclear. When measuring flow mediated dilation – the golden standard for evaluating endothelial function (72), it has been shown that flow mediated dilation is lower in early pregnancy before the development of clinical PE features as well as during and after PE. This could in fact suggest a pre-existing condition rather than an effect by PE itself (147).

STBEVs and endothelial cells

To elucidate the role of STBEVs in the endothelial dysfunction of PE, several groups have studied how STBEVs, or placental vesicles, interact with ECs. The reports have been somewhat conflicting, probably due to differences in experimental settings (111). In 2005 Gupta el al (148, 149) published two studies comparing STBEVs derived from mechanical disruption, placental perfusions as well as placental explants, showing that they all had different properties. The conclusions were that mechanically prepared STBEVs inhibited human umbilical vein endothelial cell (HUVEC) proliferation, disrupted the EC monolayer, and also induced apoptosis to a much higher degree than STBEVs from perfusions and explants. They concluded that STBEVs from perfusions were probably more physiological due to normal STB turnover and minimal structural damage in the perfused placenta, in contrast to explants and especially the mechanical preparation, which was suspected to induce the release of necrotic material.

The most common sources of placental vesicles include (111);

- Mechanical dissection of human term placentas; villous tissue is dissected and placed in saline solution, from which the supernatant is collected and the STBEVs are isolated. In this section they will be referred to as mSTBEVs.
- Placental explants: villous tissue is dissected and cultured in medium, from which the STBEVs are later isolated. Referred to as eSTBEVs.
- Placental perfusions: method described in detail under "Methods". The vesicles are derived from placental perfusate. Referred to as pSTBEVs.
- Cell cultures: either trophoblast cell lines, or primary placental cells such as primary human trophoblasts (PHT). The placental vesicles are released by the cultured cells into the culture medium. Referred to as cSTBEVs.
- Circulating vesicles: STBEVs or a general EV population isolated from maternal peripheral blood. Some studies have also looked at plasma only, which merely brings an indication of EV effect but might also account for other circulating factors in plasma.

One of the first studies indicating an effect by mSTBEVs on ECs was performed in 1993. In that study, Smárason et al. treated ECs with normal or PE mSTBEVs, which both interfered with EC growth (150). It was soon reported thereafter that mSTBEVs from normal placentas altered the functional response of arteries with a reduction in relaxation, and electron microscopy showed that the ECs were disrupted (151). The EC disruption caused by mSTBEVs can be reversed by angiogenic factors such as VEGF and PlGF (112). The mSTBEVs did not have any effect on EC gene expression (152)

When comparing normal and PE eSTBEVs on the other hand, PE eSTBEVs have been shown to activate ECs by up-regulation of ICAM-1 to a greater extent than normal eSTBEVs. The PE eSTBEVs carry Flt-1 to a greater degree than normal vesicles, and when inhibiting Flt-1 the effect on ECs diminished. However, part of the activation was considered to occur independently of Flt-1 (128). This was supported by Xiao et al., who treated normal placental explants with sera from normal or PE pregnancies. After treatment, eSTBEVs were isolated and the eSTBEVs from the PE-treated explants had the ability to increase EC activation (153). The PE eSTBEVs also have the ability to inhibit endothelial tube formation, which could be reversed by the addition of VEGF (154)

Although many STBEV studies have been performed using the placental perfusion system, few have focused on how the pSTBEVs affect ECs. However, it has been shown that HUVECs can internalise pSTBEVs over time (122). In papers III and IV we show pSTBEV internalisation by primary ECs. Furthermore, pSTBEVs can carry eNOS, although with a decreased expression on PE pSTBEVs, indicating their role in vasodilation (155).

As an indication of cSTBEVs' effect on ECs, it has been shown that necrotic debris from trophoblast cell lines can activate ECs by increased ICAM-1 expression (156). However, when instead treating the trophoblasts with apoptotic debris, they appeared to be protected from "necrotic activation" (157). Trophoblastic debris has also been shown to affect EC gene expression (158).

A study in 1996 by Smárason et al., supported the idea of placentally released vesicles as inhibitors of EC growth. In this study, they treated ECs with normal and PE plasma, where PE plasma displayed greater inhibitory potential (159). Plasma from PE pregnancies has not been shown to activate HUVECs in the form of altered ICAM-1 expression (160).

When analysing the effect of isolated EVs from plasma, studies have not separated placenta-derived EVs from other EVs in the circulation. However, by comparing with non-pregnant plasma, the results give an indication of the effect of placenta-specific STBEVs. Plasma-derived EVs from pregnant women are bioactive and increase HUVEC migration, with the greatest effect seen in the first trimester (161). Vesicles from PE plasma cause endothelial dysfunction in HUVECs by delivery of sFlt-1 and sEng (162). One study suggested that plasma-derived EVs from pregnant women needed monocytes present in order to activate ECs (163). Treating HUVECs with PE plasma did not alter the cell gene expression (160).

In conclusion, it has been shown that EC growth is affected negatively by mSTBEVs and by plasma from pregnant women. The migration of ECs is, on the other hand, promoted by vesicles from the circulation of pregnant women. Furthermore, endothelial tube formation is inhibited by eSTBEVs, ECs are disrupted by

mSTBEVs, and an EC dysfunction is induced by circulating vesicles from pregnant women.

When it comes to EC activation, the results are also somewhat conflicting. The ECs are activated by PE eSTBEVs as well as by necrotic debris from trophoblast cells, while apoptotic trophoblast debris appears to protect from activation. Plasma from PE pregnancies does not induce EC activation, while circulating vesicles can do so in the presence of monocytes.

Finally, only a few reports have been made on STBEVs' effect on EC gene expression. Gene expression can be affected by trophoblastic debris, but no effect has been shown by mSTBEVs or by circulating vesicles. In paper III we show that specific miRNA target genes are affected by pSTBEV treatment, but in paper IV we demonstrate that EC biology genes are not affected. In fact, except for one study where pSTBEVs were internalised by HUVECs (122), our papers III and IV are the first studies focusing on pSTBEV's direct interactions with, and uptake by ECs.

Stage 2 – maternal and foetal manifestations

There is a connection between the maternal risk factors and how the maternal endothelium reacts to the placenta-derived factors. A mother with pre-existing elevated levels of vascular inflammation is probably more susceptible to even lower degrees of stress, with resulting systemic endothelial dysfunction and PE symptoms (4). Below, we will go through the second stage of PE, i.e. the maternal symptoms, as well as the long-term consequences of PE.

The PE symptoms

Preeclampsia is, as previously described, diagnosed by new onset hypertension (systolic >140 mmHg and diastolic >90 mmHg) after 20 weeks of gestation, with one or more of the following; proteinuria, maternal organ dysfunction (such as kidney injury, liver involvement, neurological or haematological complications) and/or uteroplacental dysfunction (1).

The mechanisms behind hypertension in PE are thought to be the endothelial activation and dysfunction, caused by the aforementioned released factors from the dysfunctional placenta. When the maternal endothelium is activated, there is enhanced formation of endothelin and superoxide as well as increased vascular sensitivity to angiotensin II. Furthermore, there is less production of vasodilators such as nitric oxide. All these changes in the endothelium lead to an impairment in renal-pressure natriuresis and increased total peripheral resistance, i.e. hypertension (164).

Another hallmark of PE is proteinuria and kidney damage, and the endothelium of the kidney is of course also affected by the released factors. In the PE kidney, there is evidence of glomerular endotheliosis, where glomeruli are enlarged and "bloodless" due to swollen ECs. The endotheliosis leads to decreased glomerular filtration rate (GFR) but exactly how the endotheliosis leads to the clinical sign of proteinuria has not been fully established (165, 166).

In PE, there is a 10% risk of developing the severe liver-related HELLP syndrome – characterised by Haemolysis, Elevated Liver enzymes and Low Platelets (1, 167, 168). It is important to distinguish the low platelets (thrombocytopenia) of PE and HELLP from the low platelets that can also appear in otherwise uncomplicated pregnancies. The exact mechanism and cause of thrombocytopenia is not known (167).

Neurological conditions are severe complication of PE and involve eclampsia, intracranial haemorrhage and cerebral oedema. Eclampsia is a potentially fatal clinical manifestation of grand mal seizures, and worldwide is one of the leading causes of maternal deaths (3). Women who are at high risk of developing eclampsia, having severe hypertension and proteinuria or showing neurological symptoms, are given prophylactic therapy for convulsions in the form of magnesium sulphate (1). The reason for developing neurological manifestations and eclampsia is still being debated. Endothelial dysfunction is once again considered a key player. One theory is that the severe hypertension of PE causes vasospasm and diminished cerebral blood flow, giving rise to oedema, ischaemia and tissue infarction. Another theory is that the oedema is caused by changes in blood pressure which in turn increase hydrostatic pressure, hyper perfusion and extravasation of plasma (169).

Finally, a pregnancy involves (at least) two individuals who can be affected by PE – the mother and the foetus. The most common foetal complications involve pre-term delivery and FGR. If delivery occurs before 34 weeks of gestation, the foetal lungs have not developed properly yet, and corticosteroids are given to promote foetal pulmonary maturity (1, 8). The FGR can occur both with and without PE and both syndromes are considered as results of placental dysfunction (170). In early onset PE, FGR is more common than in late onset PE (4) and is correlated to many different foetal complications such as asthma, neonatal respiratory distress, increased vulnerability to infections in early life, jaundice as well as neurocognitive disorders later in life (171).

Long-term cardiovascular consequences

Endothelial dysfunction is considered central to PE (72) and it has been shown that women with PE have increased arterial stiffness (172, 173). Whether the arterial stiffness is pre-existing and a feature of women susceptible to developing PE, or caused by the disorder itself, has not been established. However, developing PE and showing signs of endothelial dysfunction and arterial stiffness, is considered a risk for future vascular disease. In fact, compared to women with uncomplicated pregnancies, women with previous PE have a two-fold increased risk of developing cardiovascular disease (CVD) in the future (1, 174, 175), such as hypertension, ischaemic heart disease, stroke and venous thromboembolism (174). The increased risk of developing hypertension, requiring pharmacological treatment (176) is higher immediately after delivery, decreases over time, but persists as at least a two-fold increased risk 20 years post-partum (177, 178). It has also been suggested that offspring from PE pregnancies are at risk for CVD, such as increased risk of stroke as well as hypertension (179-181).

As implied earlier, the question of whether the increased risk of CVD is due to common risk factors for PE and CVD, or PE being an actual risk factor for CVD, is still unanswered. However, it has been found that women with previous PE still have increased levels of the anti-angiogenic factor sFlt-1 one year post-partum, with effects on arterial ageing (182).

Due to the fact that women with previous PE have this increased risk of CVD later in life, it is recommended to continue lifelong monitoring of cardiovascular risk factors (183). In Sweden, all women with early onset, severe or repeated PE are referred to yearly follow-ups due to the increased risk of hypertension and CVD. All women who have had any type of hypertensive disorder during pregnancy are informed about the risk factors and recommended to have yearly follow-ups (10).

Both women with previous PE (184) as well as children who have been exposed to PE in utero, have an altered miRNA expression profile in blood compared to those who underwent uncomplicated pregnancies. These miRNAs are connected to cardiovascular and cerebrovascular complications (185) and have also been found in the cord blood in PE pregnancies (62).

The present investigation

Aims of the thesis

The overall aims of this thesis were to study and describe the mechanisms by which STBEVs and placental miRNAs can affect target cells, contribute to endothelial dysfunction, and thereby play a role in the pathogenesis of PE and its long-term consequences. Specifically, the role of HbF in these processes was also investigated.

Specific aims:

Paper I. To investigate the molecular mechanisms involved in trophoblast differentiation and hypoxia, more specifically the placenta-abundant miR-424 and its effect on the expression of FGFR1.

Paper II. To analyse the effect of free Hb on STBEV release and their miRNA content.

Paper III. To compare the uptake of normal and PE STBEVs, their potential transfer of miRNA content and effect on miRNA target gene expression, as well as the influence of HbF on these processes.

Paper IV. To investigate the differences in uptake mechanisms between normal and PE STBEVs, their ability to activate ECs, and further elucidate the potential effects on EC gene expression.

Methods

The following section provides a detailed description and discussion of the human *ex vivo* placental perfusion model used in papers II-IV. For a detailed description of other methods included in this work, please see the "Materials and methods" section of the respective papers.

The human placental perfusion model

The human placental perfusion method, also referred to as dual perfusion, is an excellent way to study the human placenta *ex vivo*. Due to practical and ethical considerations, there are immense difficulties in studying the placenta *in vivo*. Since the placental physiology is species-specific, animal studies also have their limitations (17, 186, 187). The placental perfusion method has been used by many groups to study the transfer of substances, drugs as well as parasites, between the maternal and foetal sides of the placenta (186, 188, 189). It was first described in 1967 by Panigel et al. (190), and later modified in 1972 by Schneider et al. (191). The placental perfusion model is used as the source of STBEVs for papers II-IV.

Brief description of the method

For placental perfusions, term placentas are used, which are collected at the maternal ward as soon as possible after delivery. Practically, this requires close contact with the maternal ward and midwives, as well as being able to initiate perfusions at different hours of the day. For paper II, the placentas were collected at the maternal ward "Kvinnokliniken" at Lund University Hospital. The placentas were collected within 20 minutes after delivery and quickly brought to our laboratory in a saline buffer. At the laboratory, the placentas were examined, and a suitable cotyledon chosen for perfusion. In paper II, the perfusions were performed by the guest researcher and co-author Dr Karen Saljé (née May) during my very first year in the laboratory, and I assisted. A detailed description of the phases and procedures for the perfusions used in paper II was published by May et al. in 2011 (192) and a depiction of the perfusion system is seen in Figure 7.

In paper III-IV the placentas were perfused by Dr Dionne Tannetta at the University of Oxford, UK. A detailed description of these perfusions was published by Southcombe et al. in 2011 (116). A detailed methodological guide on performing placental perfusions was published in 2017 by Conings et al. (193), although one

should be observant of differences in experimental settings between different laboratories.

At the end of the perfusions, the maternal perfusate was collected, centrifuged briefly to remove cellular debris, and thereafter stored at -80°C until further use and STBEV isolation using ultra-centrifugation.

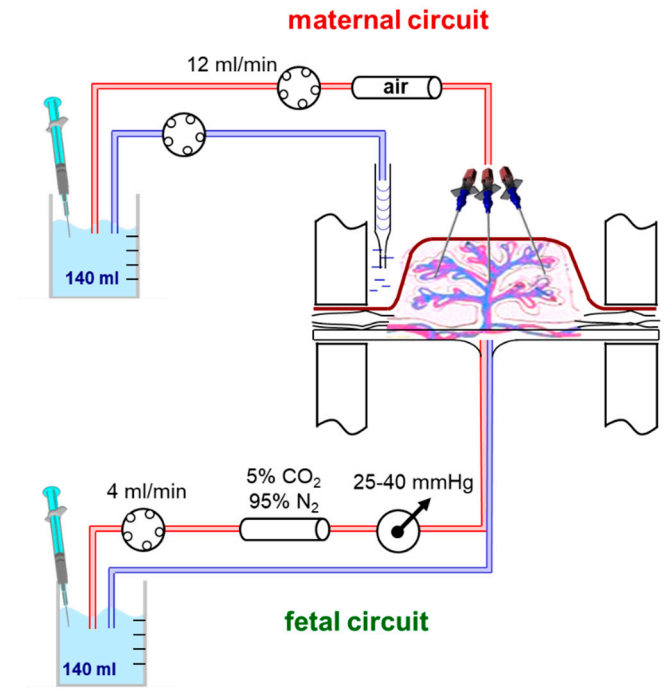

Figure 7. The placental perfusion model
Schematic overview of the *ex vivo* placental perfusion system. A placental cotyledon is placed in the perfusion chamber. A maternal and foetal circulation is established and perfused continuously during the experiment. It is possible to add drugs, or in our case Hb, to either circulation and measure the transfer over the placenta. Adapted from May et al. 2011 (192).

Pros and cons of placental perfusion

But why use such a complicated method to analyse STBEVs? Many different methods and sources of placental vesicles have been used but the perfusion-derived STBEVs are described to have properties most resembling the *in vivo* situation, as compared to STBEVs from other sources. Different sources of placental vesicles are explained in the previous section “STBEVs and endothelial cells”.

The pros and cons of placental perfusion have also been discussed by Hutson et al. 2011 (186). Together with our own observations and experiences, there follows a list of considerations.

Disadvantages of placental perfusion:

- Time- and resource-consuming. A success rate that varies greatly between "perfusionists", from as low as 15%, to generally between 30-75% (based on our own experiments as well as Conings et al. 2017 (193)). The PE placentas are reportedly the most difficult to perfuse.
- Close collaboration with the maternal ward is required and perfusions must start as quickly as possible following delivery.
- Risk of contamination from maternal blood. (However, an equilibration phase is normally used where maternal blood is rinsed and discarded.)
- Risk of foetal-maternal leakage, which together with several other parameters is closely monitored.
- Term placentas are most commonly used, which limits the conclusions about early pregnancy.
- Quantification of STBEV release is not reliable since cotyledons vary greatly in size even within a single placenta. This quantification would require a normalisation to the perfused cotyledon STB surface area.

Advantages of placental perfusion:

- Non-invasive tissue collection, few ethical considerations.
- Closely resembles the *in vivo* situation, with maintained placental structure of both normal and PE placentas. An advantage when studying transfer over the placenta.
- Provides a high yield of STBEVs, both normal and PE, which was essential in the experiments reported here. According to Dragovic et al. (194), this method supplies STBEV fractions with very little contamination of other cell EVs.
- Provides a unique situation where STBEVs can be collected from the causative organ. The possibility to study STBEVs from STBs in PE placentas (195).
- Probably the most physiological release of STBEVs compared to other methods.

Results

Paper I

In placental development and pathology, a common injury is that the placenta becomes hypoperfused, leading to cellular hypoxia. The role of miRNAs and their effect on placental gene expression as well as function is of great interest. Previously, the placenta-abundant miR-424 was identified as one of few miRNAs being down-regulated in hypoxic placentas. Due to the known role of miR-424 in important cellular functions, this study aimed to gain a deeper understanding of its regulatory ability.

Results in short

- The expression of miR-424 increases in primary human trophoblasts during differentiation, although this increase is halted when the cells are cultured in hypoxic conditions.

- The effect of hypoxia on miR-424 levels is specifically due to impaired trophoblast differentiation.

- The silencing of FGFR1 by miR-424 is mediated by a conserved 3'UTR binding site. During hypoxic conditions, when miR-424 is decreased, FGFR1 expression levels increase.

- Members of the miR15/16 family share a seed element with miR-424 and also modulate FGF1 expression, although their expression levels are not dependent on hypoxia.

Paper II

Studies from our group have previously suggested HbF as a possible link between the first and second stages of PE, causing extensive damage and oxidative stress to the placenta as well as increased cell blebbing. Increased release of STBEVs has also been suggested as a causative factor for the endothelial dysfunction of PE. We aimed to study the release of STBEVs after perfusion of human placentas with free Hb, as well as potential alterations in their miRNA content. Of specific interest were miRNAs connected to PE, as well as miRNAs belonging to the C19MC and miRNAs affected by hypoxic conditions. The STBEVs in this paper were isolated by differential centrifugation at either 10,000 xg or 150,000 xg and are referred to in the paper as 10K STBMs or 150K STBMs.

Results in short

- Both 10K and 150K STBMs carried the surface markers CD63 and TF, with no difference between the STBM fractions. Perfusion with Hb had no effect on CD63 or TF expression.

- The 10K STBMs had a slightly larger median size compared to the 150K STBMs.

- All nine analysed miRNAs were present in both 10K and 150K STBMs.

- Hb perfusions led to down-regulation of three miRNAs (miR-517a, miR-141 and miR-517b) in 10K STBMs, while no effect was seen in 150K STBMs.

- The 10K and 150K STBMs appeared to be able to carry Hb.

Paper III

The effect of placenta-released STBEVs on the endothelium and their role in the endothelial dysfunction of PE was investigated in paper III. Here, perfusions of both normal and PE placentas were performed and the isolated STBEVs compared in terms of cellular uptake, miRNA transfer and effect on gene expression. Since Hb appeared to be carried by STBEVs in paper II, this was also a focus of this study.

Results in short

- The STBEVs from both normal and PE placentas were internalised by primary ECs in a time-dependent manner.

- The STBEVs transferred their miRNA content to the ECs, where it was further directed to the ER and mitochondria after STBEV degradation. The PE STBEVs deposited their miRNA to a higher degree in the ER and normal STBEVs in the mitochondria. Co-treatment of normal STBEVs and HbF led to redirection of miRNA deposition towards the ER.

- Both normal and PE STBEVs caused down-regulation of several predicted and previously validated target genes.

- The PE STBEVs caused extensive cell membrane ruffling, not seen after treatment with normal STBEVs. HbF treatment caused similar cell membrane ruffling as after PE STBEV treatment.

- HbF appeared to be carried by PE STBEVs but not normal STBEVs.

Paper IV

Previous studies had led us to the hypothesis that normal and PE STBEVs might have different uptake routes. This was studied by inhibiting different endocytic pathways before STBEV uptake. In paper III we showed an effect on miRNA target gene expression, but in this study we aimed to focus on genes specifically related to EC biology and function. The STBEV effect on EC activation as well as the specific interaction with ASA was also investigated.

Results in short

- The uptake of both normal and PE STBEVs was partially blocked by the uptake inhibitor methyl-beta-cyclodextrin (MβCD) and almost completely blocked by chlorpromazine, dynasore and wortmannin. ASA had no effect on STBEV uptake

- Normal STBEVs down-regulated ICAM-1 surface expression on the ECs, while co-treatment with ASA had no additional effect. The PE STBEVs showed a slight up-regulatory effect on ICAM-1 expression, while co-treatment with ASA instead resulted in an up-regulation

- Neither STBEVs nor ASA had any significant effects on EC gene expression.

Discussion

Since the first discovery of EVs, researchers have tried to divide the vesicles into sub-categories in order to evaluate potential differences and biological functions. Commonly used techniques for EV isolation are differential centrifugation or ultra-centrifugation, with or without the combination of filtration steps or sucrose gradients. Also commonly used is immunoaffinity isolation, where antibodies to specific EV markers are used to trap wanted EV populations. The EV isolation field is rapidly expanding and there are also many commercial kits available today for isolating EVs (196-198).

Using immunoaffinity assays is tempting and something we considered initially. It requires known EV markers that can reliably distinguish between exosomes and microvesicles if one wishes to separate the sub-populations. Common surface markers for exosomes and microvesicles have been reviewed extensively (197, 199). Initially, CD63 was suggested as a specific exosome marker (199) but in paper II we show CD63 on vesicles of all sizes, probably also microvesicles. This confirms the difficulties in separating the sub-populations of EVs. A general STBEV marker is PLAP, which has been used in papers II and III. This would be a suitable marker for isolating placental vesicles from the circulation, separating them from other EV types. In our studies, it has not been necessary to add this extra step due to the fact that our STBEV source is a perfused placenta and it has been shown that most EVs isolated from the perfusate are of placental origin (194).

One of the most common techniques for isolating EVs is differential centrifugation (198), with or without size filtration. This usually involves at least two steps; the first being a low-speed centrifugation or filtration to exclude larger sized vesicles, attempting to separate the larger microvesicles from the preparation. In the second step, ultra-centrifugation is performed to pellet the smaller exosomes. It is known that during centrifugation, the EVs do not sediment merely based on size but depending on cargo and density. A common problem is aggregation of EVs, which also interferes with the separation (196). In paper II we intended to separate exosomes and microvesicles by differential centrifugation steps; 10,000 xg and 150,000 xg. Even though there was a shift in median size, where the 10,000 xg STBEVs were larger, suggestive of more microvesicles, they overlapped to a large extent. The two preparations also overlapped in terms of surface markers. We drew the conclusion that separation was not possible using this method and decided to continue investigating the effect of "all" placental vesicles in the up-coming studies. In papers III-IV the STBEVs were isolated with one ultra-centrifugation step at 110,000 xg. Since ultra-centrifugation is known to sediment extravesicular proteins as well, a way to perfect the isolation procedure in future experiments would be to use a sucrose gradient (196). It is worth noting that every step will impact the results,

from choosing the STBEV source to the isolation of STBEVs, and later on the isolation of RNAs or other analysis steps. As has already been discussed, STBEVs from different sources impact ECs in different ways. Different EV/STBEV isolation techniques will affect yield, sub-population and contaminating factors such as extravesicular proteins (197, 200). Furthermore, it has been shown that different RNA isolation techniques affect the EV RNA profile (201). Regardless of the secretion mechanisms, different EV subtypes cannot be fully separated according to size or density because of overlapping physical characteristics (90). Consistency is key in order to compare and reproduce results, which is why, in paper IV, we decided to continue isolating STBEVs in the same manner as in paper III. The International Society for Extracellular Vesicles (ISEV) continuously provides new and insightful studies regarding everything from nomenclature to methodological considerations.

As described, miRNAs play an important role in placental development. In paper I we studied the placenta-abundant miR-424 and its role in regulating specific genes, as well as how hypoxic conditions affect the miR-424 levels. Under normal conditions, it was shown that trophoblast differentiation correlates with increasing miR-424 levels. When the trophoblast cells are subjected to hypoxia, the miR-424 is no longer up-regulated. The same response is seen when subjecting the cells to DMSO, which is known to halt differentiation. It was interesting that other miRNAs, sharing the same seed element, were not affected by hypoxia. This would mean that external stress, such as hypoxia or maybe even increases in HbF as our group showed, could affect very specific miRNAs and pathways. In other studies, it has been shown that miR-424 levels are also decreased in placentas from women who suffered severe PE (69). There is a stronger connection between early onset PE and FGR in terms of placental dysfunction, which might also be connected to the miR-424 levels. In plasma from pregnant women, the miR-424 levels are increased compared to non-pregnant women (61), which is not surprising since miR-424 is abundantly expressed in the normal placenta as well and probably released into the circulation in for STBEVs, although, surprisingly, the miR-424 levels increase to even higher levels in FGR pregnancies compared to uncomplicated pregnancies (56). Even though this might simply be explained by differences in experimental setup, it leads to speculation involving how miRNAs are sorted into vesicles. One theory is that a stressed placenta releases more EVs containing miRNAs. With increased release there might be a depletion of certain miRNAs in the placental cells. Another question would be whether the sorting of miRNAs into vesicles is organised or random depending on which miRNAs are present in the proximity of the vesicle formation. As described in paper I, not all miRNAs were down-regulated by hypoxia, in fact, most were up-regulated. The up- and down-regulation can be explained by the placenta adapting its transcription to a stressful environment.

In paper II, the stress of Hb perfusions led the placenta to release STBEVs with altered miRNA content. The stress of Hb did not lead to changes specifically for miR-424 but changed the content of C19MC miRNAs. Overall, it seems as if the miRNA expression is sensitive to outside stressors and there might be changes to specific miRNAs. When sending out placenta-specific miRNAs, the placenta communicates with the maternal system. By altering the miRNA content in the STBEVs, different signals can be sent to the receiving cells. The changes seen in STBEV miRNA content might also be reflective of a shift in the exosome/microvesicle balance. Not only is there an increase in STBEVs in PE, there is also a shift towards more (harmful) microvesicles. Since exosomes are released by exocytosis and microvesicles by blebbing of the cell surface, it is likely that they load different miRNAs.

In paper II we showed that STBEVs could carry both Hb and miRNAs. The possibility for EVs from the placenta to carry Hb gives rise to many new questions. One is whether the increases in HbF during PE pregnancies, as described by our group, could be present in STBEVs. Another question is whether this is a way for HbF to be sheltered from degradation in the circulation. In paper III we showed that HbF was in fact carried by PE STBEVs but not by normal STBEVs, which is consistent with the HbF increase in PE. Furthermore, HbF was shown to be deposited into primary ECs where it could be harmful and toxic to the cell. This could be one of the ways that HbF exhibits its harmful effects in PE and could also explain one of the negative differences between normal and PE STBEVs.

As we and other groups have shown, STBEVs consist mainly of C19MC miRNAs, which are not present in other cell types. The fact that in paper III we could show the transfer of these placenta-specific miRNAs to primary ECs was very exciting. The introduction of completely new material, from a unique and temporary organ, could alter the target cells' gene expression in new ways. A study by Delorme-Axford et al. in 2013 (141) showed how C19MC miRNAs can confer viral resistance to target cells. In paper III we showed that the uptake of STBEVs into primary ECs led to down-regulation of the FLT1 gene, which is an exciting PE connection. We believe that STBEVs from placental perfusions best reflect the *in vivo* situation. And as described above, different preparations affect ECs differentially. The EC gene expression can be affected by trophoblastic debris, but no effect has been shown by mSTBEV or by circulating vesicles. In papers III and IV, we studied the effect of perfusion-derived STBEVs on primary ECs. In paper III, we chose miRNA target genes using prediction algorithms. In paper IV we chose an array analysing EC biology-related genes and none were significantly affected. Even if a single miRNA can regulate hundreds of genes, there can also be great specificity, as was shown in paper I, both in terms of what genes the miRNA regulates but also how the miRNA itself is affected by different conditions. In PE, and in the interaction between STBEVs and ECs, certain specific miRNAs might be the ones responsible for the

endothelial dysfunction of PE. It has been shown that miRNAs can play an important role in EC function and angiogenesis (202).

The vast differences in techniques and studies, as discussed above, make it hard to compare results. Every step towards simplifying pathways or reactions in order to elucidate the details includes a risk of losing important interplays between different cell types, vesicles and other factors.

The purpose of investigating the uptake pathways of STBEVs in paper IV was to understand if PE STBEVs are re-routed and their miRNA deposited in different compartments compared to normal STBEVs. In paper III we showed that normal STBEVs deposited more miRNA to mitochondria and PE STBEVs deposited their miRNA in a higher degree to the ER. Furthermore, we showed an extensive membrane ruffling caused by PE STBEVs, as well as when normal STBEVs were combined with HbF. We hypothesised that the effect on the cell membrane suggests destruction of the cytoskeleton, leading to disruption in intracellular pathways. An endocytic uptake pathway was suggested due to the STBEVs appearing in endosomes. The deposition of C19MC miRNAs in close proximity to the ER could probably affect gene expression as well as cause ER stress. Another purpose of paper IV was to further elucidate the molecular mechanisms and processes in both normal and PE pregnancies, and to show how the STBEVs interact with ECs. We could not see any significant differences between uptake of normal and PE STBEVs although the inhibitors consistently inhibited the PE STBEVs to a lower degree than normal STBEVs.

Conclusion and future perspectives

The current work provides evidence of the involvement of miRNAs and STBEVs in normal and PE pregnancies. We have shown how the placenta-abundant miR-424 is connected to trophoblast differentiation and affected by hypoxic conditions. We and others have shown that the miRNA composition of placental vesicles or STBEVs is primarily C19MC miRNAs. Furthermore, we have shown that STBEVs are taken up by primary ECs, transfer miRNA content, and affect target cell gene expression, and also affect the ECs' ICAM-1 surface expression. The STBEVs can also carry Hb and HbF as well as transfer this toxic molecule to target cells. It appears from our studies that normal and PE STBEVs deposit their content in different compartments of the cell with the possibility of altering which genes or processes they target. The PE STBEVs also affect target cells negatively by ruffling the cell plasma membrane.

We conclude that specific miRNAs might play an important role in placental development, pathology as well as systemic communication with the maternal endothelium. Stressors such as hypoxia and Hb treatment affect the miRNA profile of both the placenta and released STBEVs, which leads to altered communication and an effect on gene expression in target cells. Since miRNAs in general have great impact on gene expression, and the C19MC miRNAs specifically have been shown to transfer viral resistance, it is of great interest to elucidate the systemic effects of placental miRNAs. It is not unlikely that STBEV interactions with target cells can cause activation and alterations in surface molecules, further changing uptake pathways and re-directing endocytosed material. It has been shown that PE STBEVs carry different surface markers compared to normal STBEVs, but the evidence concerning their ability to activate ECs is somewhat conflicting due to the use of different experimental setups and definitions of STBEVs over the years.

There is evidence indicating that STBEVs are involved in both normal pregnant physiology as well as the PE pathophysiology, but it has not been clarified exactly how these processes and interactions occur. Therefore, it is of great importance to investigate further the molecular mechanisms and interactions between vesicles and target cells, with great care being taken regarding the isolation of placental vesicles and in choosing target cells that are representative of the *in vivo* environment. It is, of course, impossible to mimic real life completely in an experimental setting but interactions between different cell types, vesicles and other factors must be taken into account. The lack of activation by STBEVs on one cell type might simply be due to the setup being too simplified. On the other hand, making a setup complicated makes it harder to distinguish which molecules interact with each other and what roles they have.

In conclusion, the present work provides a small piece of the PE puzzle by showing the importance of specific miRNAs in both trophoblast differentiation and in re-programming of target cells. The strength of our studies is that we have used STBEVs resembling the *in vivo* situation, and combined with primary ECs. In future studies it would be very interesting to dive deeper into the regulatory role of specific miRNAs by transfecting ECs with certain C19MC miRNAs and investigating the effect on genes as well as on a protein level. The methods from paper I would be very suitable to implement in the experimental setup used in papers III and IV. Since miR-424 and other miRNAs are abundant in the placenta, it will be interesting to find out why this abundance is not seen in released STBEVs. The fact that STBEVs introduce completely new material to ECs, i.e. miRNAs that are not normally expressed, is very exciting. These results should be followed up by looking at STBEV release and specifically the sorting of miRNAs into the vesicles, to determine whether this is regulated or completely reflects the cytoplasmic levels of miRNAs. It would be very interesting to compare placental tissue and STBEVs from the same patient, as well as to analyse which miRNAs are sorted into the recipient cell.

When studying the current literature, it seems clear that there is a difference between normal and PE STBEVs. However, we found no overwhelming differences in terms of miRNA content and effect on target cell gene expression. Other studies have shown that PE STBEVs differ from normal STBEVs in terms of surface markers, interactions with target cells and effects on vasoconstriction in functional studies. Our group has recently started functional studies as well, to investigate how STBEVs affect vascular contractility, and the preliminary results have been exciting. There are certainly differences between normal and PE STBEVs but one cannot rule out that much of the STBEV effect might be due to the higher STBEV levels in PE, making the effect of PE STBEVs dose-dependent. This could quite easily be investigated in an experimental setting. One major difference between almost all experimental setups and the *in vivo* situation, is the fact that a pregnancy is on-going for up to nine months, with continuous shedding of STBEVs into the circulation. The experimental situation gives an indication of the acute situation. Furthermore, the differences between normal and PE STBEVs in uptake pathways, which we have not been able to show thus far, should be further looked into by examining any receptor interaction with the target cells. We have seen that normal and PE STBEVs deposit their miRNA content in somewhat different compartments, which should be confirmed and examined more closely. It would be interesting to investigate whether there are specific miRNAs that are more likely to be deposited in target cells and whether they are active, as well as measuring the direct effect on ER and mitochondrial stress.

Finally, it could be a good idea to focus not only on what harmful effects the PE STBEVs have, but also on what beneficial effects the normal STBEVs might have,

and to determine if these effects are not apparent in the case of PE. In paper IV we showed that normal STBEVs appear to down-regulate ICAM-1 expression, which is indicative of decreased activation. This could suggest a more protective role of the normal STBEVs that is not found with PE STBEVs. A previous study showed that apoptotic debris from the placenta protected ECs from activation by necrotic placental debris (157).

As a final remark – these studies add a mechanistic piece to the puzzle as well as raising many new questions. The world of miRNAs and STBEVs in PE is very exciting and needs further investigation. I am still amazed at how a temporary organ can communicate systemically, send out unique information, and affect maternal cells. The placenta is not only a conveyer of nutrients, oxygen and hormones, but serves as a communication centre between mother and foetus.

Populärvetenskaplig sammanfattning

En graviditet är någonting som många av oss går igenom, mer eller mindre medvetna om riskerna. Vi är inte heller alltid medvetna om de riskfaktorer vi själva tar med oss in i en graviditet. Och frågan är om den vetskapen skulle ändra någonting. Att bli gravid och skaffa barn är inte bara en otroligt stark drift utan även kritiskt för människans överlevnad. Jag är dock övertygad om att kunskap alltid är av godo och ju mer vi vet om hur graviditeter fungerar desto bättre förutsättningar kan vi skaffa oss för att klara av dem på bästa sätt.

Den forskning som presenterats här behandlar en av de vanligaste graviditetsrelaterade komplikationerna, nämligen havandeskapsförgiftning (preeklampsi). Detta syndrom drabbar 3-8% av alla gravida och orsakar årligen omkring 76000 kvinnors död över hela världen. Man diagnosticerar vanligen havandeskapsförgiftning genom att den gravida har ett nytillkommet högt blodtryck efter 20e graviditetsveckan samt har tecken på organskada, exempelvis läckage av proteiner i urinen som tecken på njurpåverkan. Utöver de livshotande konsekvenserna tillkommer ett stort lidande, risk för förtidig födsel, risk för tillväxthämning av fostret och inte minst en ökad risk för hjärt-kärlsjukdom senare i livet. Kvinnor med kroniskt högt blodtryck, diabetes och BMI över 30 löper ökad risk. En riskökning finns även förknippad med om graviditeten är resultat av IVF eller om det finns mer än ett foster. Många av riskfaktorerna är så kallat kardiovaskulära (hjärta-kärl) och det är denna kärlpåverkan som knyter ihop såväl riskfaktorer som själva havandeskapsförgiftningen och långtidskonsekvenserna.

Mekanismerna bakom havandeskapsförgiftning är fortfarande inte helt kända, vilket har till följd att man inte kan förutsäga eller bota de som får havandeskapsförgiftning. All behandling är rent symptomatisk. Vad man hittills har kunnat förstå avseende detta komplexa och mångfacetterade syndrom, är att det utvecklas i två stadier. Vid en okomplicerad graviditet kommer det befruktade ägget att fästa in i livmodervägen och under utveckling av moderkakan sker ett samspel mellan specialiserade moderkaksceller och mammans celler i livmodern. I moderkakan ska fostrets kärl komma i nära kontakt med mammans blod, för utbyte av näringsämnen och syre. Vid havandeskapsförgiftnings första stadie har man sett att samspelet och utvecklingen av kärlen i moderkakan sker på ett ofördelaktigt sätt. Detta leder i sin tur till att moderkakan och dess celler stressas och utsöndrar olika faktorer och så kallade vesikler till mammans blodomlopp. Det är dessa faktorer

som man tror bidrar till det andra stadiet i havandeskapsförgiftningen, nämligen skada på mammans endotel, cellerna som utgör ytskiktet av alla kärl, och därigenom orsakar de kliniska symptomen.

I placentan finns små RNA-molekyler som kallas mikro-RNA. Deras uppgift är att reglera gener. I moderkakan finns det speciella mikro-RNA som uttrycks i högre utsträckning där jämfört med andra celler och organ. Vissa mikro-RNA har kopplats till moderkakans normala utveckling såväl som till havandeskapsförgiftning. Man har funnit mikro-RNA från moderkakan även i blodet hos gravida kvinnor.

En av de faktorer som man tror kopplar ihop första och andra stadiet av havandeskapsförgiftning är som sagt vesikler. Vesikler är ett slags farkoster som kan bära med sig bland annat mikro-RNA från moderkakan och skicka dem till andra celler i kroppen. När det gäller moderkakas-vesikler, innebär detta att helt nya mikro-RNA, som bara uttrycks i moderkakan, kan överföras till exempelvis endotelceller som helt ny information och helt ny reglering av generna, och därmed potentiellt förändrad produktion av proteiner och förändring av cellernas funktion. Vid havandeskapsförgiftning har man sett att moderkakan frisätter ett ökat antal vesikler samt att de har annorlunda egenskaper jämfört med normala.

I detta arbete har vi detaljerat studerat hur ett mikro-RNA kan påverka genuttryck. Vi har även visat att moderkaks-vesikler innehåller mikro-RNA, att de kan tas upp av mänskliga endotelceller, lämna moderkakas-specifika mikro-RNA inuti cellerna och påverka genuttrycket. Vidare har vi studerat detaljerna kring hur vesiklerna tas upp. Vi har med detta arbete gjort ett försök att lägga en pusselbit till förståelsen av havandeskapsförgiftning. Vi tror att en djupare förståelse kring mekanismerna i detta komplicerade syndrom i förlängningen kan leda till bättre sätt att förutsäga sjukdomen, behandla symptom och motverka långtidskonsekvenserna.

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