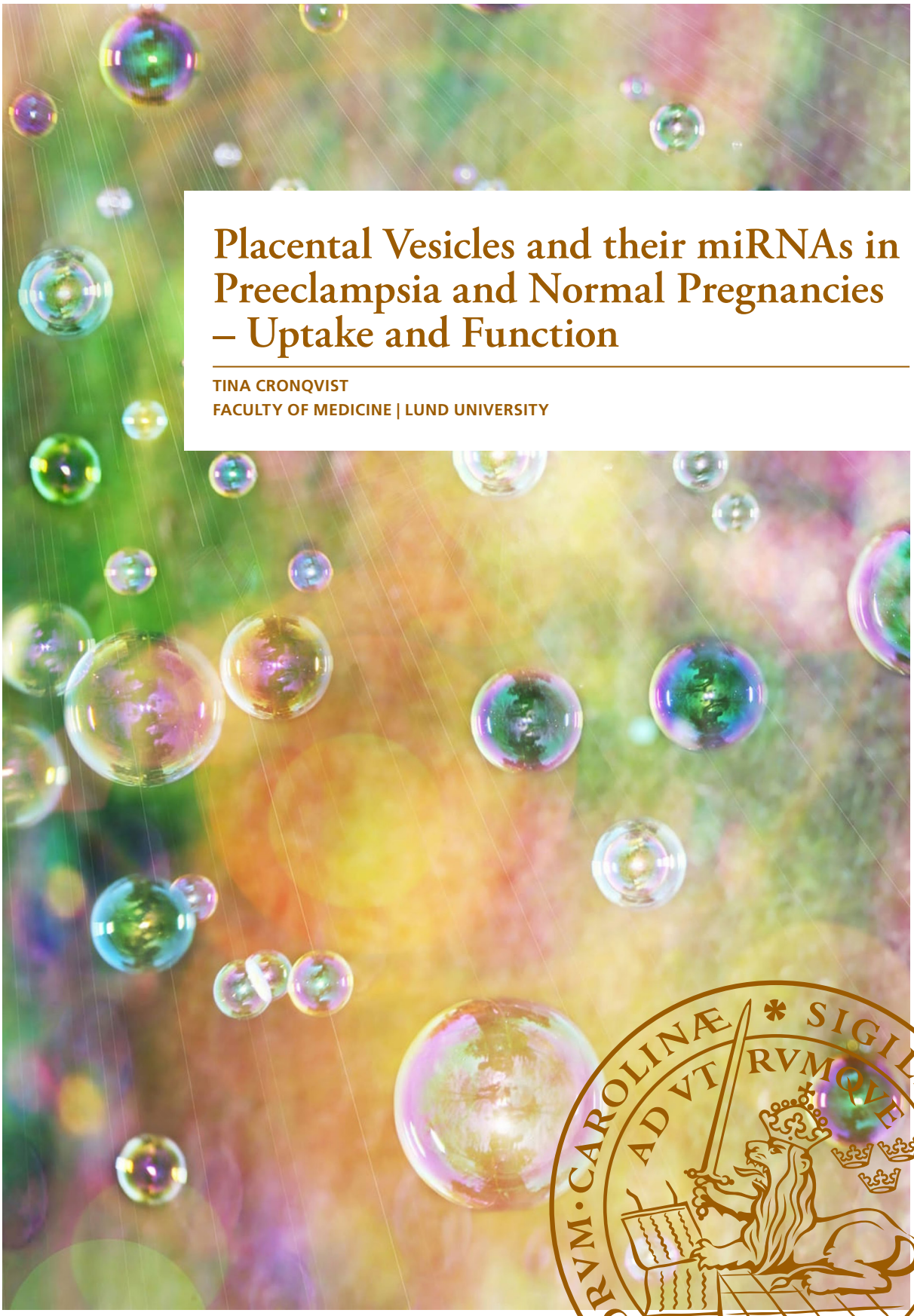
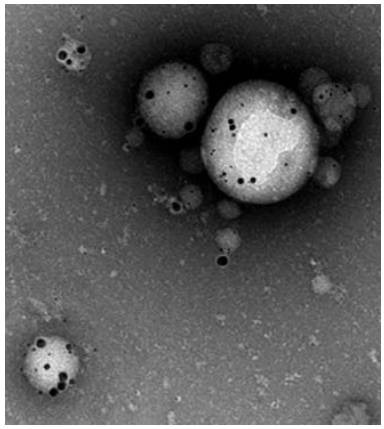


Placental Vesicles and their miRNAs in Preeclampsia and Normal Pregnancies – Uptake and Function

TINA CRONQVIST
FACULTY OF MEDICINE | LUND UNIVERSITY



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.

Printed by Media-Tryck, Lund 2020 NORDIC SWAN ECOLABEL 3041 0903

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FACULTY OF MEDICINE

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2020:10
ISBN 978-91-7619-870-4
ISSN 1652-8220

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Tina Cronqvist

LUND
UNIVERSITY

DOCTORAL DISSERTATION
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To be defended at Segerfalksalen, BMC A1005, Sölvegatan 17, Lund.

Friday January 31st 2020 at 1 pm.

FACULTY OPPONENT
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Department of Women's and Reproductive Health
University of Oxford, UK

Organization LUND UNIVERSITY Department of Obstetrics and Gynecology Institution: Clinical Sciences in Lund	Document name: DOCTORAL DISSERTATION
	Date of issue: January 31st 2020
Author: Tina Cronqvist	Sponsoring organization

Title and subtitle: Placental Vesicles and their miRNAs in Preeclampsia and Normal Pregnancies – Uptake and Function

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

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title: 1652-8220		ISBN: 978-91-7619-870-4
Recipient's notes	Number of pages: 76	Price
	Security classification	

Signature  Date 2019-12-12

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Coverphoto: artwork depicting soap bubbles, adapted from pxfuel.com

Lund University, Faculty of Medicine
Department of Obstetrics and Gynaecology
Doctoral Dissertation Series 2020:10

ISBN 978-91-7619-870-4
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2020

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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).

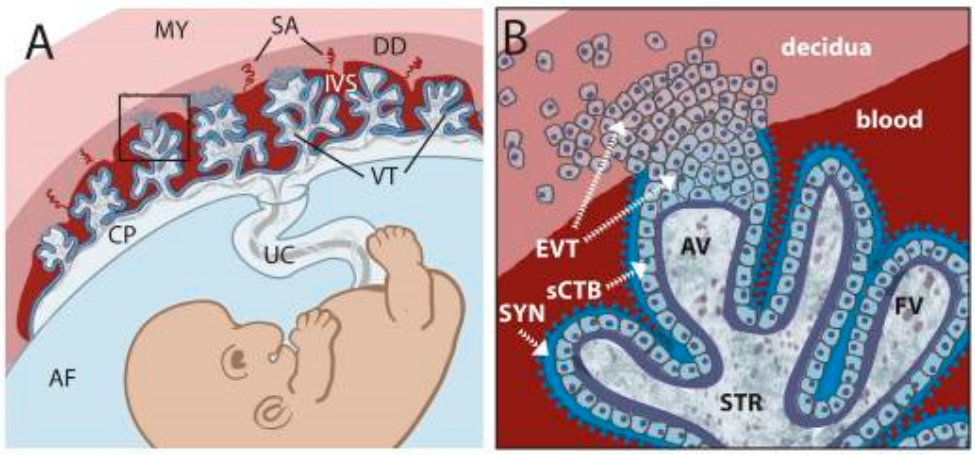

Figure 1. Overview of the placental structure.

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).

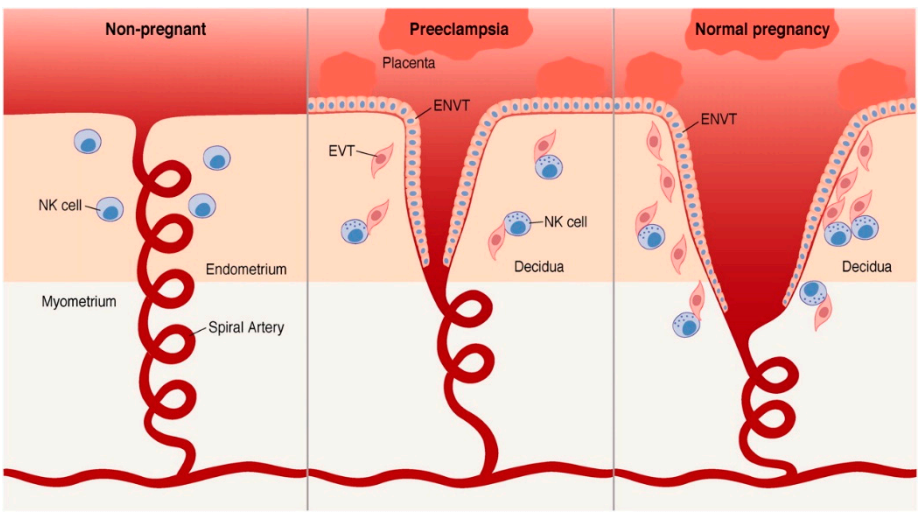

Figure 2. Spiral artery remodelling in normal and PE pregnancy.
The left panel shows a spiral artery in the non-pregnant uterus. The right panel shows spiral artery remodelling in normal pregnancy, with EVT invasion and ENVT lining of the artery, as well as interactions with uNK cells. The middle panel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

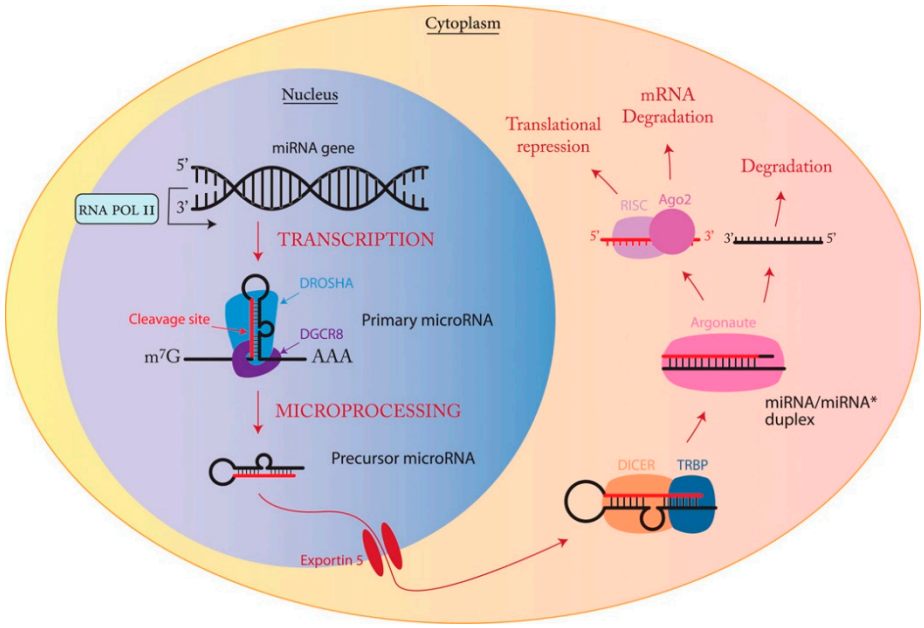

Figure 3. miRNA maturation
An overview of miRNA maturation. The miRNAs are transcribed to pri-miRNA, converted to pre-miRNA and exported out of the nucleus,where they are further modified until a mature miRNA is present. Adapted from Hemmatzadeh 2019 (33).

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).

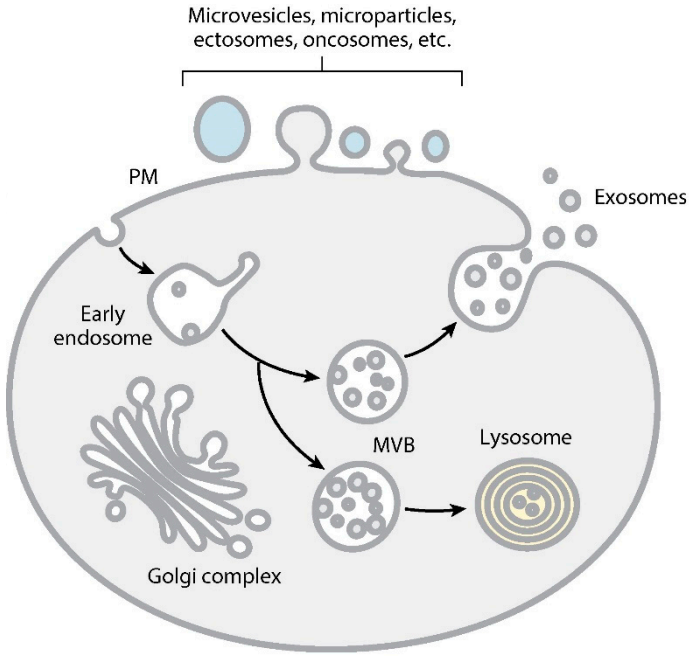

Figure 4. Extracellular vesicle subtypes
Extracellular vesicles from different cellular origins. The microvesicles are released from the cell of origin by budding off from the cell plasma membrane The exosomes are formed inside endosomal compartments and multivesicular bodies (MVB) and released through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

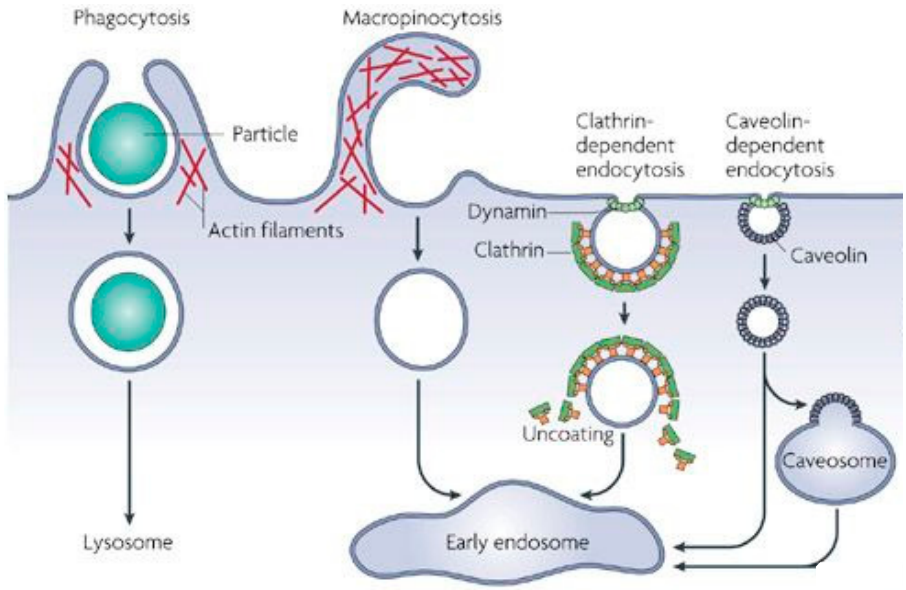

Figure 5. Endocytic uptake pathways

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).

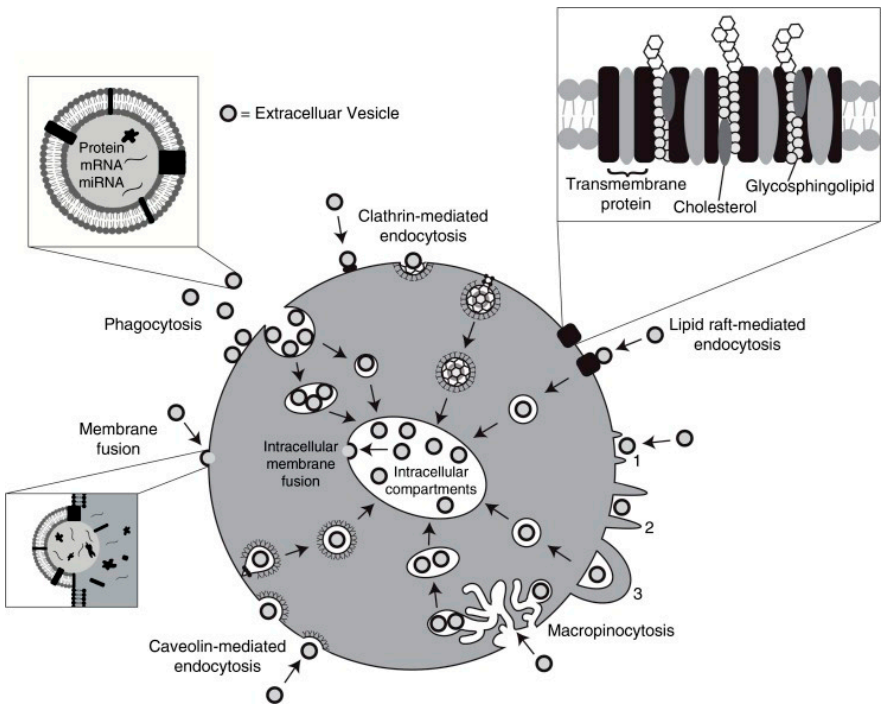

Figure 6. Uptake pathways of extracellular vesicles
Extracellular vesicles can be taken up by the recipient cells by many different pathways. Described in a review by Mulcahy et al. are; clathrin-mediated endocytosis, as well as clathrin-independent but lipid raft-mediated, endocytosis, which includes 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.

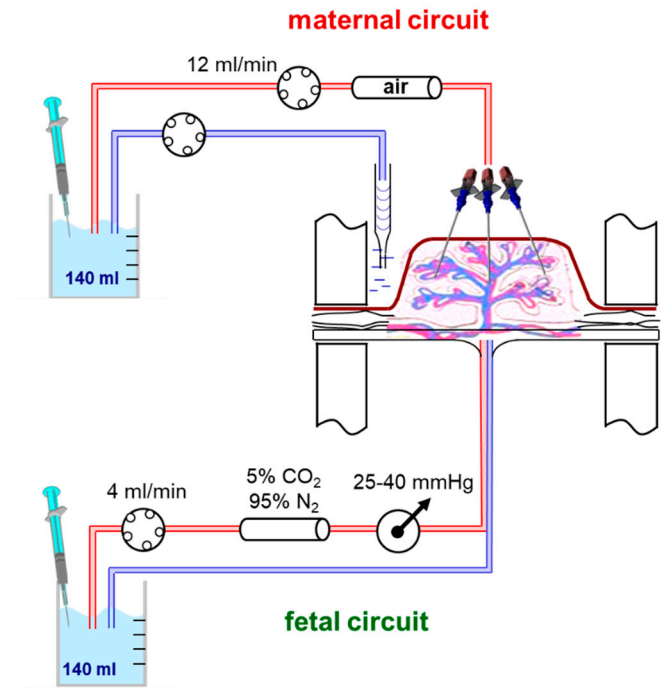

Figure 7. The placental perfusion model
Schematic overview of the *ex vivo* placental perfusion system. A placental cotyledon is placed in the perfusion chamber. A maternal and foetal circulation is established and perfused continuously during the experiment. It is possible to add drugs, or in our case 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.

Acknowledgments

A big thank you to everyone who made this thesis possible. It has been quite a journey with almost "everything" in life happening alongside. I contacted Stefan in 2007 and started as a summer student, the PhD started officially in 2012 and 2020 appears to be the year it will actually be finished. Many people have been involved by answering questions, lending a helping hand or time, supported and given feedback.

First of all; thank you thank you thank you **Stefan**, my supervisor. Thank you for being my exact opposite in personality, giving me extra courage and making me believe in myself and my results. You are always positive, optimistic and see the possibility in everything.

My co-supervisor **Lena** who has been a scientific as well as a moral support for many years. Even though I "drop in" and do research irregularly you have always been there to answer questions and talk about life.

To **Dionne**. A true vesicle and placental inspiration, always kind and helpful. Thank you for all your help and answers throughout the years. Thank you to **Mary**, for help, inspiration and knowledge.

One of the very first persons to introduce me to lab life – **Karen**. I am so grateful that you were a guest researcher in our lab when I spent my very first summer figuring out what a placenta was. Even though the lab practically bathed in blood I kept coming back. Thank you for always being happy and kind and staying late with me, eating pizza and talking while the vessels bathed.

Thank you to **Yoel Sadovsky** for having me in your lab in Pittsburgh, teaching me the miRNA basics and letting me get a taste of US research. Thank you to **Elena**, for teaching me methods, showing me around town and taking care of me.

A thank you to the colleagues at the lab, who have shifted over the course of the years I have been there; **Iréne** (my un-official supervisor, friend, supporter**), Eva, Katja, Åsa, Maya, Caroline, Magnus, Ulrik, Zuzana, Zahra, Vera** and many more. To our close collaborators **Bo** and **Magnus**.

A special thank you to the gang at C14, for coffee breaks, fredagsfrukostar and science discussions, to **Ida, Anneli, Ann-Charlotte, Kalle, Annie, Mina** and many more.

Stort tack till **psykiatriska kliniken i Lund** såväl som i **Halmstad**, för att ha gett mig möjligheten att kombinera klinisk tjänstgöring med forskning. Tack till **ALF** och **medicinska fakulteten i Lund** för tilldelade medel så jag kunnat bedriva forskningen.

Ett stort tack till **Yvonne** och **Aleks Giwercman**. Ni gav mig mod och inspiration att satsa på såväl läkarkarriär som forskning.

Mina fina vänner ”Läkarbrudarna”, tack för fantastisk Lundatid, fantastiska ”brudhelger” (vem använder ordet brud egentligen?!) och välbehövliga, mer eller mindre betydelselösa (fast egentligen extremt betydelsefulla) updates på messenger. Fina älskade **Lotta, Bitte, Sofia, Emelie** och **Camilla**.

Och de strålande biomedicinarna **Madde, Jennifer, Sara, Sara, Mia, Ida, Helena, Hanna** och **Ewa** – som inspirerat och satt ribban bara liite för högt för hur en avhandling ska gå till. Äntligen joinar jag er i doktorsskaran och tackar för häng, socialt nörderi och vänskap.

Ett stort tack till min familj, mina föräldrar – älskade mamma **Kirsten** och pappa **Bruno** som gett mig tillräckligt med hybris för att hitta på något sånt här. Mamma tack för allt stöd och alla hejarop! Pappa, du hade varit så odrägligt stolt. Jag saknar dig varje dag. Min lillebror **Daniel** och hans fina **Martina**. Och **Voltaire**! Tack för att ni finns!

Ett stort tack till min extended men close family – svärisarna **Annika** och **Lars** som stöttat och passat och hejat på. Svärsyskon med respektive **Cissi, Oskar, Anna** och **Tobias**. **Olle, Bruno** och **Heffner**… kul att ni varit med också, en dag kanske jag vågar leka med er, woff!

Sander och **Asker** – mina älskade små hjältar. I hela era liv har jag varit doktorand, nu är det dags för ett nytt kapitel. **Johan**, min skat, i nästan hela vårt gemensamma liv också, tack för allt stöd och all anpassning! Hela livet har hänt och det hade inte gått utan dig. Älskar dig och våra små gollungar mest i hela världen!

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Paper I

BIOLOGY OF REPRODUCTION (2013) **89**(2):25, 1–9
Published online before print 26 June 2013.
DOI 10.1095/biolreprod.113.110049

The Unique Expression and Function of miR-424 in Human Placental Trophoblasts[1]

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ABSTRACT

Placental hypoperfusion causes cellular hypoxia and is associated with fetal growth restriction and preeclampsia. In response to hypoxia, the repertoire of genes expressed in placental trophoblasts changes, which influences key cellular processes such as differentiation and fusion. Diverse miRNAs were recently found to modulate the cellular response to hypoxia. Here we show that miR-424, which was previously shown to be upregulated by hypoxia in nontrophoblastic cell types, is uniquely downregulated in primary human trophoblasts by hypoxia or chemicals known to hinder cell differentiation. We also identify FGFR1 as a direct target of miR-424 in human trophoblasts. This effect is unique to miR-424 and is not seen with other members of this miRNA family that are expressed in trophoblasts, such as miR-15 and miR-16. Our findings establish a unique role for miR-424 during differentiation of human trophoblasts.

FGFR1, hypoxia, microRNA, miR-424, trophoblasts

INTRODUCTION

Hypoperfusion, and the consequent cellular hypoxia, is one of the most common injuries to the placenta during human pregnancy [1, 2]. Hypoxia affects many molecular and cellular pathways, including the differentiation and function of placental trophoblasts. Thus, hypoxia may impact the maternal-fetal exchange interface, adversely influencing fetal growth.

As a part of our ongoing effort to better define the molecular mechanisms underlying trophoblast adaptation to injury, we recently focused on the expression of microRNAs (miRNAs) in placental trophoblasts exposed to hypoxia. MicroRNAs are small, noncoding RNAs that regulate gene expression at the posttranscriptional level through binding to partially complementary sequences in the 3′UTR of protein-coding mRNAs, causing translation inhibition and mRNA decay (review in [3]). MicroRNA pathways have emerged as important regulators of many cellular processes, and their aberrant expression has been associated with numerous pathological states [4–8]. High-throughput miRNA surveys have shown that placental trophoblasts express unique patterns of miRNAs [9–13]. The function of most of these trophoblastic miRNAs remains unknown, with only a few miRNAs associated with abnormal placentas derived from complicated pregnancies [14–17].

We recently identified a set of differentially regulated miRNAs in human primary trophoblasts that were exposed to hypoxic stress [18]. Among these miRNAs, we found that miR-424 is one of the only species that was downregulated in hypoxia. MicroR-424 is a mammalian-specific miRNA that is particularly abundant in placental trophoblasts [11, 12], and recent studies indicate that abundance is a critical determinant of the biological activity of miRNAs [19, 20]. MicroR-424 is known to regulate important cellular functions, including differentiation, proliferation, cell cycle, and angiogenesis [21–26]. Interestingly, it was recently shown that hypoxia upregulates the expression of miR-424 in endothelial cells [23]. Three other miRNA species—miR-15a, miR-15b, and miR-16—that share the same seed sequence with miR-424 are also highly expressed in placental trophoblasts. In this study, we investigated the expression of miR-424 and other related miRNAs in placental trophoblasts. We show that the reduced expression of miR-424 is not unique to hypoxia but is associated with hindered trophoblast differentiation. This effect was not observed in other miR-424 family members. We also show that miR-424 directly regulates the expression of FGFR1 in human trophoblasts through a discrete 3′UTR site. While miR-15/16 species are also expressed in trophoblasts and capable of targeting and silencing FGFR1, their expression level is not affected by hypoxia or the differentiation state of the cells.

MATERIALS AND METHODS

Cell Culture

Primary human trophoblasts (PHT) were prepared from normal term placentas using the trypsin-deoxyribonuclease-dispase/Percoll method as described by Kliman [27], with previously published modifications [28]. All placentas were obtained after term delivery using a protocol approved by the Institutional Review Board at the University of Pittsburgh.

Cultures were plated at a density of 350 000 cells/cm^2 and maintained in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Hyclone) and antibiotics at 37°C in a 5% carbon dioxide-air atmosphere. After 4 h, designed to allow cell attachment, the culture plates were allocated to either standard (O_2 = 20%) or hypoxic (O_2 < 1%) environments [18]. Differentiation was routinely monitored by medium human chorionic gonadotropin (hCG) levels using ELISA (DRG International), showing a characteristic increase in medium hCG as cytotrophoblasts differentiated into syncytiotrophoblasts, with attenuation of this process in hypoxic or undifferentiated cells [28, 29].

Immortalized, human, first-trimester, extravillous trophoblast cells (HTR8/SVneo), used as a convenient system for molecular assessment of miRNA-target interaction, were provided by C.H. Graham (Kingston, ON [30]) and were cultured in RPMI-1640 (Cellgro) supplemented with 5% bovine growth

[1]Supported by grants to J.F.M. and T.C. from the State of Pennsylvania Department of Health Formula Research Funds, to R.B.D. by a postdoctoral research fellowship from Magee Womens Research Institute, and National Institutes of Health R01HD065893 and R21HD071707, both to Y.S.
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Received: 15 April 2013.
First decision: 9 May 2013.
Accepted: 19 June 2013.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

serum (HyClone) and antibiotics. JEG-3 human choriocarcinoma cells were maintained in DMEM containing 10% FBS and antibiotics as described [18]. Human umbilical vein endothelial cells (HUVEC; Lonza) were cultured in endothelial basal medium, phenol red-free (Lonza), supplemented with supplier-recommended concentrations of growth factors—human recombinant epidermal growth factor (hEGF), human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), ascorbic acid, hydrocortisone, and recombinant insulin-like growth factor (R3-IGF)—and 10% FBS (Lonza). Endothelial colony-forming cells (ECFC) were prepared as described in [31] and were maintained in endothelial basal medium, phenol red-free, supplemented with supplier-recommended concentrations of hEGF, hFGF, VEGF, ascorbic acid, hydrocortisone, and R3-IGF, with 10% FBS at 5×107 cells per well on collagen-coated six-well plates (BD Biosciences). HUVEC and ECFC were used at passage 3 and plated at a density of 48 000 cells per well in a six-well culture plate for incubations in a hypoxic chamber and at 96 000 cells per well in a six-well culture plate for incubations at standard oxygen concentration.

RNA Isolation, Reverse Transcription, and Real-Time Quantitative PCR

Total cellular RNA was purified using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. For mRNA analysis, reverse transcription (RT) was performed using High Capacity RNA-to-cDNA RMaster Mix (Applied Biosystems) in a 20-μl reaction mix at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. The RT product was used for real-time PCR as described previously [32]. Table 1 lists primer sequences used for the study. All primer sequences were BLAST-checked (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) for specificity. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. Control samples of H_2O were included in PCR reactions in each experiment. Samples were normalized to parallel reactions using primers specific for the trophoblast housekeeping gene YWHAZ [33]. The fold increase relative to control samples was determined by the 2-ΔΔCT method [34].

For miRNA analysis, RT and quantitative PCR (RT-qPCR) of duplicate samples was performed using the miScript PCR system (Qiagen) following the manufacturer's instructions. Primers (miScript) were used to detect expression of miR-15a, miR-15b, miR-16, miR-424, and RNU6B. Total RNA input was normalized using RNU6B RNA as an endogenous control. Dissociation curves and control samples were included, as described above.

Small RNA Sequencing

Small RNA of 18–28 nucleotides in length was gel-purified from 10 μg of total RNA isolated from PHT cells cultured in standard or hypoxic conditions for 48 h. Small RNA libraries were prepared and sequenced at the Genome Sequencing & Analysis Core Resource at Duke University using the Genome Analyzer II (Illumina). For analysis of the sequencing data, the FASTQ files were preprocessed to remove the adapter sequences. The trimmed reads, along with the trimmed quality scores, were aligned to human reference genome using the short-read aligner Bowtie [35]. Only perfect alignments were allowed. Ensembl annotation (http://uswest.ensembl.org/index.html) of all mRNA and noncoding RNAs were used to annotate the aligned reads. The annotated reads were summarized to derive each miRNA count. All miRNA counts from all libraries were further normalized using the quantile normalization method.

Plasmids, Mutagenesis, Transfection, and Luciferase Assay

MicroRNA expression vectors were engineered by cloning an approximately 500-bp fragment of genomic DNA that harbored the miRNA precursor along with its flanking sequences into a pcDNA3 vector (Invitrogen). For each miRNA, we designed an miRNA sensor construct by cloning a synthetic fragment that contained three perfectly matching miRNA-responsive elements (MRE) into psiCHECK2 (Promega). For 3′UTR of putative miRNA target genes, we PCR-amplified the relevant 3′UTR sequences using human genomic DNA as a template and inserted them into psiCHECK2 at the *Xho*I/*Not*I sites. Mutations in putative MREs were performed using a site-directed, ligase-independent mutagenesis [36] and included deletion of 10–20 nucleotides that included the MRE's seed sequences within the target 3′UTR. To reduce the risk of unintended mutations, each mutated insert was confirmed by sequencing and then subcloned back into a native psiCHECK2 vector that was not subject to mutagenesis.

Luciferase reporter constructs, along with the miRNA expression constructs, were cotransfected using polyethylenimine-mediated transfection [37]. HTR-8/SVneo cells, plated in 12-well plates, were transfected with 10 ng of reporter construct along with increasing concentrations of CMV-based vectors (pcDNA3) expressing relevant miRNAs. At 48 h after transfection, the cells were lysed with passive lysis buffer (Promega), and firefly and Renilla luciferase activities were measured consecutively with the Dual Luciferase Reporter system (Promega). Renilla luciferase activity was normalized to the firefly luciferase control. The miRCURY LNA microRNA Power Inhibitor, targeting miR-424, and a nontargeting control LNA oligonucleotide were obtained from Exiqon, Inc. HTR-8/SVneo cells were cotransfected with LNA inhibitors and a luciferase reporter plasmid using DharmaFECT Duo transfection reagent (Thermo Scientific). For transfection of PHT cells after 4–8 h, designed to allow PHT attachment to culture plates, cells were incubated with 25 nM and up to 100 nM of miRNA LNA inhibitor with DharmaFECT-1 transfection reagent in OPTI-MEM I (Invitrogen) for 24 h. Cotransfections of 25 nM antagomir with miRNA luciferase reporter construct were performed using DharmaFECT Duo transfection reagent in OPTI-MEM I for 24 h. After 24 h, the medium was changed to DMEM containing 10% FBS and antibiotics, and cells and media were harvested 48 h or 72 h after the start of transfection.

TABLE 1. List of primers used in this study with their sequences.

Name	Sequence
Mutagenic primers	
TM-FGFR1-F1	AGGTCCCTCAATAAAAATGCTTCATTTATCTATGGGCTG
M-FGFR1-F1	GCTTCATTTATCTATGGGCTG
TM-FGFR1-R1	ATTTTTATTGAGGGACCTAAACTGAAAATAGGTTTAGAA
M-FGFR1-R1	AAACTGAAAATAGGTTTAGAA
TM-FGFR1-F2	TATGTTTTCATTTCTGTAGGTTTCTGAGCTAGGGATTTTTTGG
M-FGFR1-F2	TTTCTGAGCTAGGGATTTTTTGG
TM-FGFR1-R2	CCTACAGAAATGAAAACATATTGAACTTTCTTTTGTATTTAGCAGTA
M-FGFR1-R2	TTGAACTTTCTTTTGTATTTAGCAGTA
TM-MAP2K1-F1	GCAGTGCATGTGAAGCATTGAAAATGAGCATCAGAGAGTGT
M-MAP2K1-F1	TGAAAATGAGCATCAGAGAGTGT
TM-MAP2K1-R1	ATGCTTCACATGCACTGCCTGTGAAGGATCTCACAAGGC
M-MAP2K1K1-F1	CTGTGAAGGATCTCACAAGGC
Primers for miRNA cloning	
M424-1F	GATCGGATCCGCAGCTCCTGGAAATCAAAT
M424-1R	GATCGGATCCCCCAGCCTAGCCAGGAATAC
M1516-1F	GATCGGATCCGGGCACAGAATGGACTTCAG
M1516-1R	GATCCTCGAGTTGATGGCATTCAATACAATTATTA
M1516-2F	GATCGGATCCAGAACGGCCTGCAGAGATAA
M1516-2R	GATCCTCGAGTGCTTAGGTAAATCAAACACCAA
Real-time PCR primers	
FGFR1-F	GTGACTTCCACAGCCAGATG
FGFR1-R	TTCATGGATGCACTGGAGTC

Correlation Studies

PHT cells from four placentas were cultured under standard and hypoxic conditions. Total RNA samples were extracted at time 0, 6, 12, 24, 48, and 72 h and hybridized to a microarray (Agilent SurePrint G3 Human GE 8x60K; Agilent). Expression of miR-424 was measured using Agilent microRNA microarray (Human miRNA Microarray Release 16.0, 8x60K). Data represent the average of log2 expression of the FGFR1 variants and miR-424 over the samples from four placentas. The Pearson correlation between the averaged log2 expression of FGFR1 variant 1 and the averaged log2 expression of miR424 is −0.7446, with a P value of 0.008591. The Pearson correlation between the averaged log2 expression of FGFR1 variant 6 and averaged log2 expression of miR424 is −0.7626, with a P value of 0.006341.

Western Immunoblotting

Cells were lysed in Cell Culture Lysis Reagent (Promega) supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific). Lysates were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes using standard procedures. Membranes were immunoblotted with a mouse monoclonal anti-FGFR1 antibody (MAB658; 0.5 mg/ml used at 1:500 dilution; R&D Systems) that recognizes most human FGFR1 isoforms. A goat anti-mouse conjugated with horseradish peroxidase (115-035-146; Jackson ImmunoResearch) was used as a secondary antibody. For normalization, the same membrane was immunoblotted with anti-actin antibody (MAB1501; EMD Millipore). The blots were washed and processed for chemiluminescence using SuperSignal West Dura (Thermo Scientific) and densitometrically quantified with VisionWorks LS software (version 6.6a; UVP BioImaging).

Statistics

All experiments were repeated at least three times. All data were analyzed using linear mixed-effect models, with the factor of interest as the fixed effect, and a single or nested random effect to represent the clustered structures. For each experiment, one or a few comparisons were preplanned to address a priori biological questions and tested by applying the Student t-test to the corresponding contrasts of the estimated coefficients of the fixed effects of the relevant linear mixed-effect models. No post hoc tests were performed. Significance level for each comparison was set at 0.05. For RT-qPCR data, the statistical analyses were done on the Ct values. For the microarray data, the robust multiarray average method [38], as employed in R-package AgiMicroRna [39], was used to obtain the summarized and normalized miRNA expression level. The statistical analyses of the miRNA and gene expression microarray data were done on the log2-transformed expression levels.

RESULTS

Expression of miR-424 in Hypoxic Human Trophoblasts

Previous analysis of miRNA profiles in PHT cultured in standard conditions ($O_2 = 20\%$) or hypoxic conditions ($O_2 < 1\%$) suggested reduced expression of miR-424 in response to 48 h of hypoxia [18]. Because the effect of hypoxia on miR-424 seemed inconsistent among various cell types [23], we analyzed the expression of trophoblastic miR-424 using deep sequencing of miRNAs in PHTs cultured in standard or hypoxic conditions and confirmed the reduced accumulation of miR-424 in hypoxic trophoblasts (Fig. 1A). To further detail miR-424 expression patterns, we used RT-qPCR to assess its expression over a period of 72 h in both standard conditions and hypoxia. FGFR1 is present as multiple splice variants that are not entirely characterized [40]. Therefore, primer pairs for FGFR1 were designed to anneal within common regions of the multiple transcripts of FGFR1. We found that miR-424 expression was relatively stable in PHTs during the first 24 h of culture; however, it was upregulated after 48 h of culture in standard conditions but not in hypoxia (Fig. 1B). To assess whether the effect of hypoxia was observed in other cell types, we examined miR-424 expression in other cells exposed to hypoxic conditions, including a choriocarcinoma cell line (JEG-3) and two models of endothelial cells: ECFC and HUVEC. Unlike PHT cells, these cells exhibited increased expression of miR-424 in hypoxia (Fig. 1C), consistent with a previous study reporting the induction of miR-424 by hypoxia in endothelial cells [23]. Thus, the relative reduction in miR-424 expression upon exposure to hypoxia seems to be a unique feature of placental PHTs.

We next sought to determine whether miRNA genes that are localized near miR-424 on chromosome X—genes that may constitute a unique polycistronic cluster (miR-503, mir-542-5p, mir-542-3p, mir-450a, mir-450b-5p, and mir-450b-3p) [21]—exhibit the same expression pattern as miR-424 in trophoblasts. Because some of these miRNA genes express two mature miRNAs, we report the results of the most abundant strand from each stem loop. We found that the expression pattern of miR-503 in hypoxia was similar to that of miR-424 (Fig. 1D), likely reflecting the fact that they are both derived from a unique common polycistronic precursor on the X-chromosome [21]. The other tested miRNAs were expressed at a markedly lower level and exhibited a pattern that was clearly distinct from that of miR-424 (Fig. 1D). The most distal species in this region, miR-450b, was below detection levels in PHT cells.

Expression of miR-424 During Trophoblast Differentiation

PHT cells isolated from term third-trimester placentas are mostly cytotrophoblasts, and they differentiate into syncytiotrophoblasts in vitro within 48–72 h in standard culture conditions. This differentiation is severely impaired when cells are cultured in low oxygen conditions [28, 41, 42]. To determine whether an altered differentiation state, even without hypoxia, reduces miR-424 expression, we measured the expression of miR-424 in primary trophoblasts grown in the presence of 1.5% dimethyl sulfoxide (DMSO), which is known to hinder trophoblast differentiation [43, 44]. A medium level of hCG in DMSO-exposed cells confirmed the attenuation of differentiation by DMSO (Fig. 2). We observed a substantial reduction of miR-424 expression in DMSO-exposed cells as compared to that of cells exposed to vehicle alone. No further decrease of miR-424 expression was observed when cells were exposed to hypoxia in addition to DMSO, supporting the conclusion that the reduction of miR-424 levels in hypoxic trophoblasts is to be attributed to abrogated differentiation of PHT cells in these cultures' conditions.

Targeting of MAP2K1 and FGFR1 by miR-424 in Trophoblasts

In our previous study, we reported FGFR1 as a potential target of miR-424 [18]. The TargetScan algorithm predicts three MREs within the 3′UTR of FGFR1. The two 5′-proximal sites overlap and were, therefore, considered as one site in subsequent experiments (Fig. 3A). We found that inactivation of the proximal conserved binding site (labeled 1 and 1′ in Fig. 3A) completely abolished the silencing by miR-424, while mutation in the 3′-end nonconserved element (site 2) had no effect on the reporter gene activity (Fig. 3B). These data confirmed the functional interaction between FGFR1 and miR-424 and indicated that the silencing of FGFR1 by miR-424 in HTR8/SVneo cells is entirely mediated by the conserved element. Interestingly, our data previously suggested that MAP2K1 might also be a target for miR-424. However, we noticed several sequence variations in the MAP2K1-3′UTR cDNA (Fig. 3C). Sequencing of additional MAP2K1 clones revealed two types of sequences, with one type corresponding to the true MAP2K1 and the other type showing a perfect match with MAP2K1P1, a pseudogene that is likely to have arisen by retrotransposition of a Map2k1 transcript [45]. Surprisingly, miR-424 had a weak yet consistent repressive

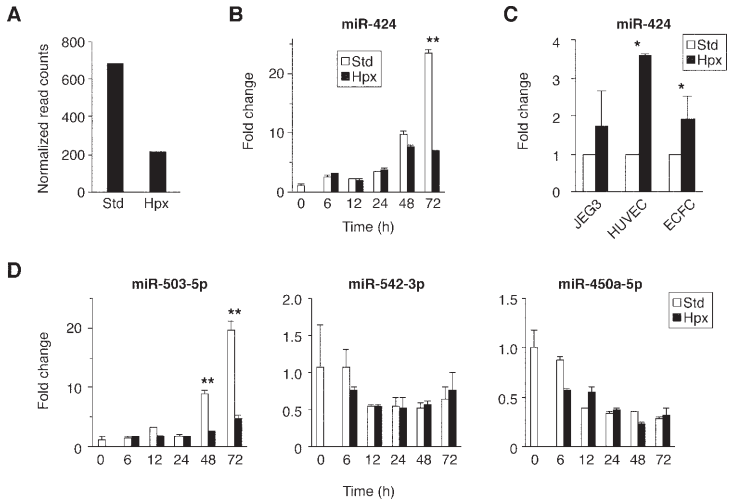

FIG. 1. MicroRNA expression in PHTs cultured in standard condition (Std) and hypoxia (Hpx). **A**) Expression level of miR-424-5p based on read counts from high-throughput sequencing (n = 1). Cells were cultured for 48 h in normoxia or hypoxia before RNA extraction. Two libraries of small RNAs were derived from these samples and deep-sequenced using an Illumina GAII analyzer. **B**) Time course of miR-424 expression in PHT cells cultured for 72 h in standard conditions compared to hypoxia and based on RT-qPCR. One representative of four experiments is shown. Error bars indicate the variability between two technical replicates. Data were normalized using U6 small nuclear RNA as an endogenous control. Statistical analysis was performed based on all independent experiments. $^{**}P < 0.01$ compared with the standard group. Note that miR-424 expression levels in standard conditions were also significantly ($P < 0.01$) elevated at 72 h compared to t = 0. **C**) Expression levels of miR-424 in JEG3, HUVEC, or ECFC cells. Data are mean ± SEM of three independent experiments, and each is measured in duplicate. Statistical analysis was performed on three independent experiments and is shown on a representative graph. $^{**}P < 0.01$ compared with standard conditions. **D**) Expression levels of the miR-424-clustered miRNAs in PHT cells cultured for 72 h in standard conditions compared to hypoxia. $^{*}P < 0.05$ compared with standard conditions.

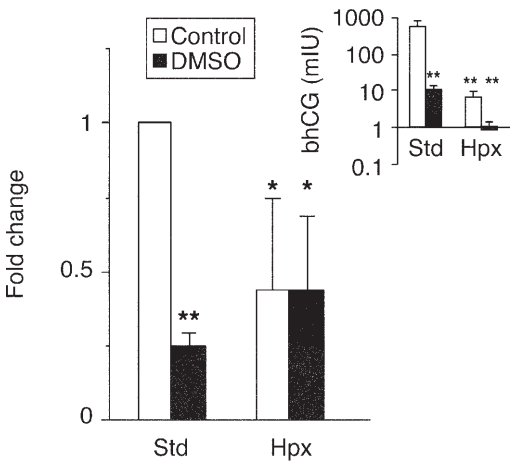

FIG. 2. Trophoblast differentiation enhances the expression of miR-424. RT-qPCR analysis of miR-424 expression in PHT cells cultured for 48 h in standard conditions (Std) or hypoxia (Hpx) and in the absence or presence of DMSO. Data were normalized using U6 small nuclear RNA as an endogenous control. Inset: the concentration of hCG released in the medium, measured by ELISA as described in *Materials and Methods*. Data represent the geometric mean of three independent experiments, each performed in duplicate and plotted on a logarithmic scale. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control in the standard conditions group.

effect on the true MAP2K1 sequence in HTR8/SVneo cells (Fig. 3D, left panel). In contrast, whereas MAP2K1P1 was repressed by miR-424 at a level comparable to other validated targets (Fig. 3D, right panel), a mutation of the putative miR-424 element on MAP2K1P1 did not abrogate the repressive effect (Fig. 3D), suggesting that a further downstream perfect seed match element may mediate the silencing of our MAP2K1P1 reporter.

Because expressed pseudogenes have the potential to act as miRNA decoys [46], we investigated the expression status of MAP2K1P1 in PHT cells. Using RT-qPCR, we detected only the expression of MAP2K1 but not the pseudogene transcripts, indicating that the pseudogene MAP2K1P1 is not expressed in these cells. To further confirm that miR-424 can target FGFR1, we used a locked nucleic acid-based miR-424 inhibitor to repress endogenous miR-424 levels in the human trophoblast cell line HTR8/SVneo. As shown in Figure 4, luciferase activity from an FGFR1 luciferase reporter plasmid was enhanced proportionally to the amount of transfected miR-424 inhibitor, with no effect by the control (scrambled) oligonucleotide.

Lastly, we tested whether members of the miR15/16 family, which share a seed element with miR-424, can also regulate the FGFR1 reporter. Although miR-15 and miR-16 silenced FGFR1 luciferase reporter similarly to miR-424, and this effect was also primarily mediated by the conserved proximal element of FGFR1 3′UTR (Fig. 5A), we found that the expression of miR-15/16 species, while abundant in trophoblasts, is not altered by hypoxia (Fig. 5B). We noted a modest increase in luciferase activity of FGFR-Δ2 mutant when co-transfected with miR-15b/16.2 (Fig. 5A, right panel). This

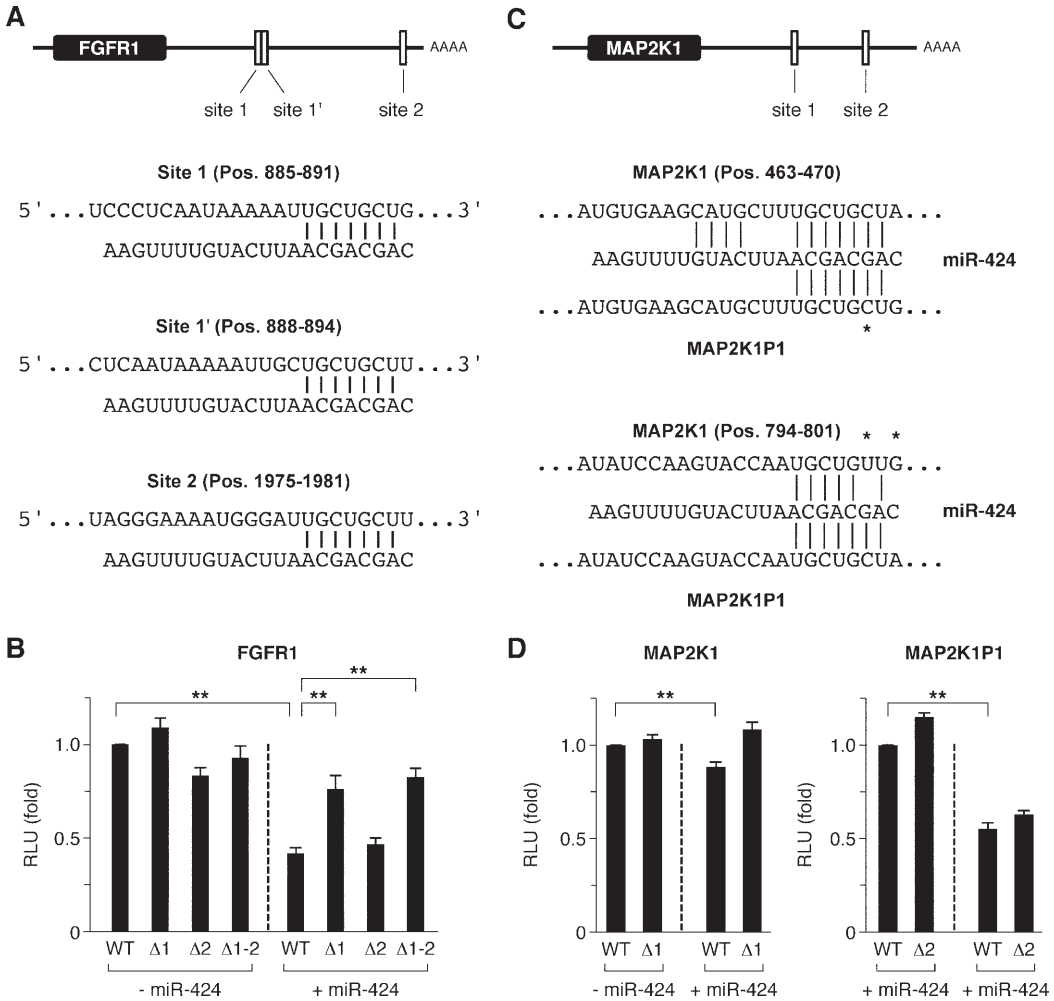

FIG. 3. MicroR-424 targets a discrete 3′UTR sequence in FGFR1 and MAP2K1 genes. **A**) Schematic representation of the predicted miR-424 MREs within the 3′UTR of the FGFR1 mRNA. Alignments between the miR-424 binding sites and miR-424 are shown. **B**) Deletion analysis of FGFR1 3′UTR. HTR8/SVneo cells were transfected with the wild-type FGFR1-3′UTR reporter (WT) or with a promoter that harbors a mutation in each of the two miR-424 binding sites, alone (Δ1 or Δ2) or in combination (Δ1,2). Each reporter was transfected along with an empty vector (pcDNA3) or a vector expressing miR-424. **C**) Schematic representation of a conserved miR-424 MRE within MAP2K1 (site 1) and of a putative miR-424 8-mer site (site 2) in the sequence of MAP2K1P1. Alignments between miR-424 and MAP2K1 and MAP2K1P1 are shown. Asterisks indicate the diverging nucleotides in the seed region between the sequences of MAP2K1 and MAP2K1P1. **D**) Luciferase reporter assay using MAP2K1-3′UTR and MAP2K1P1 reporter that harbors a mutation in each of putative binding sites (Δ1 or Δ2) or wild-type (WT) MRE. Relative luciferase unit (RLU) activity from each reporter, normalized to firefly luciferase activity, was determined 48 h later. Experiments using MAP2K1 (left panel) were performed seven times ($n = 7$), and statistical significance was determined using a linear mixed-effects model with the experiment batch as the random effect. Using this method, MAP2K1 WT was repressed in the presence of transfected miR-424 by about 17.45%, with $P = 3.28 \times 10^{-14}$ (**). Data derived from the MAP2K1P1 construct are geometric means of three independent experiments performed in duplicate. Statistical significance was determined by the Student *t*-test (SEM). $*P < 0.01$.

effect was modest, yet may represent a weak synergistic action of miR-15/16 activity on that site. Together, these data suggest that miR-424 or the miR15/16 family has the ability to modulate the expression of FGFR1 in PHT cells, but only miR-424 controls FGFR1 expression in altered differentiation or in hypoxia.

FGFR1 Expression Is Increased in Hypoxic Trophoblasts

Having confirmed the functional interaction between FGFR1 reporter and miR-424 in a trophoblast cell line, we sought to interrogate this interaction in PHT cells. As predicted, both hypoxia and exposure to DMSO increased in

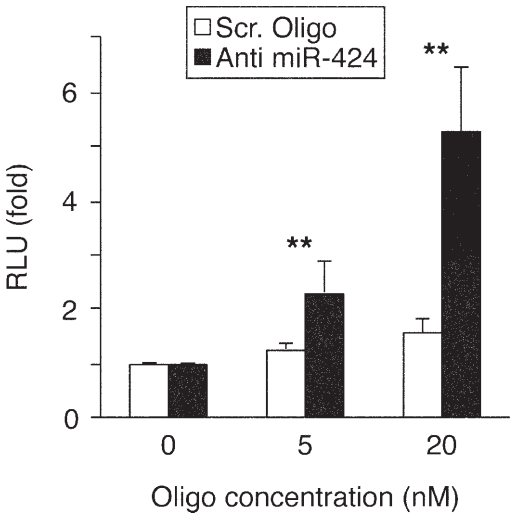

FIG. 4. Inhibition of miR-424 derepresses FGFR1 expression. Luciferase assay of HTR8/SVneo cells cotransfected with FGFR1-luciferase reporter and either anti-miR-424 or a scrambled-sequence oligonucleotide as control. RLU activity from each reporter construct, normalized to firefly luciferase activity, was determined 48 h later. Data are geometric means of three independent experiments performed in duplicate. $^{**}P < 0.01$ compared with the control group (scrambled [Scr.] oligo).

the accumulation of FGFR1 transcripts (Fig. 6A). Similarly, we observed a twofold increase of FGFR1 protein in PHT cells exposed to hypoxia or DMSO (Fig. 6B). To further document the inverse relationship between miR-424 and FGFR1 in trophoblasts, we performed an analysis of the correlation between expression levels of miR-15/16/424 and several isoforms of FGFR1. Using microarray data from experiments in which we analyzed four sets of PHTs cultured in standard conditions or hypoxia across different time points (0, 12, 24, 48, and 72 h), we found a statistically strong negative correlation between FGFR1 transcripts and miR-424, but not miR-15/16 (Fig. 7).

DISCUSSION

Placental trophoblasts express common, as well as unique, patterns of miRNAs. Although not restricted to trophoblasts, miR-424 is highly abundant in the placenta, as are with several members of the miR-16 family of miRNAs. In this study, we found that the expression of miR-424 directly correlates with the differentiation of trophoblasts. The upregulation of miR-424 as cells differentiate in vitro is completely abolished when differentiation into syncytiotrophoblasts is abrogated by hypoxia or exposure to DMSO. In addition, we found that downregulation of miR-424 in cells exposed to hypoxia is specific to primary trophoblasts and was not observed in a trophoblast cell line (JEG-3) or in either of two models of endothelial cells (HUVEC and ECFC), in which miR-424 was upregulated, as shown by us and others [23]. Interestingly, we recently reported that miR-424 was upregulated in the plasma of pregnant women with fetal growth restriction, compared to

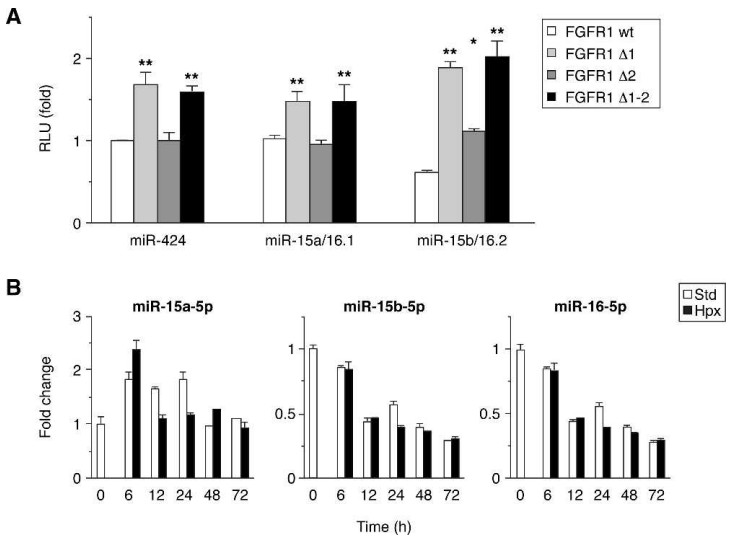

FIG. 5. Silencing of FGFR1 by members of the miR-15/16 family. **A**) Activity of wild-type and mutant FGFR1-3′UTR reporter genes in response to expression of members of the miR-15/16 family of miRNAs. HTR8/SVneo cells were transfected with FGFR1 reporters along with an miR-424 or miR-15/16 expression vector. RLU activity from each reporter construct, normalized to firefly luciferase activity, was determined 48 h later. Data are geometric means of three independent experiments performed in duplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group for each miRNA expression vector (FGFR1 wt). **B**) Expression levels of some members of the miR-424 family in PHT cells cultured for 72 h in standard conditions (Std) compared to hypoxia (Hpx). One representative of four experiments is shown, with error bars indicating the variability between two technical replicates. Data were normalized using U6 small nuclear RNA as an endogenous control.

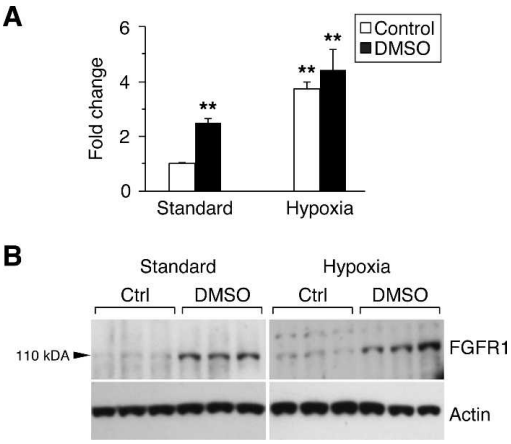

FIG. 6. The effect of the differentiation state on the expression of FGFR1 in PHT cells. **A**) RT-qPCR analysis of FGFR1 expression in PHT cells cultured in hypoxia and exposed to DMSO for 72 h. Data represent the geometric mean of three independent experiments performed in duplicate. **B**) Impaired trophoblast differentiation by hypoxia or DMSO enhances FGFR1 expression. Western blot analysis of FGFR1 in three sets of PHT cells that were cultured for 72 h in standard conditions, in hypoxia in complete medium (Ctrl), or in complete medium containing 1.5% of DMSO. $^{**}P < 0.01$ compared with the control group in standard conditions.

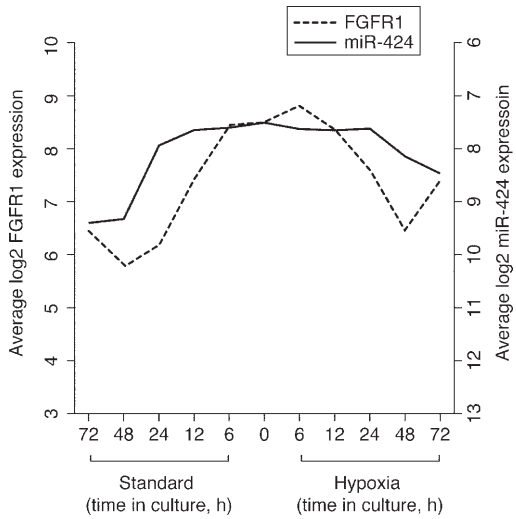

FIG. 7. Inverse correlation between FGFR1 mRNA and miR-424 in PHT cells. FGFR1 (most abundant transcript) or miR-424 was detected using microarray, and log2-transformed expression was plotted against the log2-transformed expression of miR-424 (note the inverted axes, designed to illustrate the expression pattern). Data represent the average of log2 expression of the FGFR1 variants and miR-424 for samples derived from four placentas. The Pearson correlation between the averaged log2 expression of FGFR1 and the averaged log2 expression of miR424 is -0.7446 ($P < 0.01$).

controls [16]. This observation was unexpected, because fetal growth restriction is commonly associated with placental insufficiency and cellular hypoxia. However, our past observation could be explained by changes in maternal endothelial cells. It will be interesting to dissect the molecular mechanisms underlying the difference in miRNA response to hypoxia in PHT and endothelial cells.

We also found that the pattern of miR-424 expression in trophoblasts is specific to miR-424 and its clustered partner miR-503. Analysis of several miRNAs located in close genomic proximity, including miR-542 and members of the miR-450 family, did not reveal significant changes when cells were exposed to hypoxia. Similarly, miRNAs that share the same seed sequence of the miR-424 family, including miR-16 and miR-15, were unchanged by hypoxia, while miR-195 and miR-497 were almost undetected in trophoblasts.

We defined the molecular interaction of miR-424 with 3′UTR sites within the FGFR1 gene, showing that only the conserved binding site proximal to the stop codon mediates the silencing activity of miR-424. The distal, less conserved site did not play a role in miR-424 action. Interestingly, the proximal site also mediated the suppressive activity of miR-16 and miR-15. Two recent studies have also reported the targeting of FGFR1 by miR-424 [47, 48], further confirming FGFR1 as a target of miR-424. Expression of FGF receptors, including FGFR1, has been confirmed in the human placenta, where it is postulated to play a role in villous development [49, 50].

FGFR1 has a complex genomic organization and has been implicated throughout development in many signaling pathways controlling cellular proliferation, differentiation, survival, and angiogenesis [51]. Deregulated FGF signaling is also common in many types of cancers, and FGFR1 amplification has also been reported in breast cancers [52], oral squamous carcinoma [53], ovarian cancer [54], bladder cancer [55], and rhabdomyosarcoma [56]. To date we have not been able to silence FGFR1 expression in PHTs, likely reflecting suboptimal silencing efficiency in PHTs. Additionally, FGFR1 is highly regulated posttranscriptionally, resulting in many splicing variants [57], and the precise genomic context of the miR-424 binding elements(s) remains to be validated. In the mouse, deletion of FGFR1 results in embryonic lethality around Day 7.5 to 9.5 (E7.5 to E9.5), preventing a detailed analysis of its role in the placenta [58, 59]. In humans, inactivating mutations in the FGFR1 gene are associated with the Kallmann syndrome, a rare genetic disorder characterized by hypogonadism, infertility, and anosmia [60]. Kim et al. [48] recently showed that miR-424 and miR-503 regulate FGF signaling in the lungs by targeting FGFR1 and the ligand FGF2. We also tested the 3′UTR of FGF2 in our system and observed a repression of the luciferase activity in cells cotransfected with miR-424 (data not shown). Together, these observations support the relevance of miR-424 to FGF signaling, which probably plays a role in placental development and adaptation to stress.

Lastly, we noted a weak effect of miR-424 on MAP2K1 reporter, with a more potent effect on the pseudogene MAP2K1P1. It is unclear whether this low repression effect results from suboptimal experimental conditions or is a feature of the pair MAP2K1-miR-424 in trophoblasts. It was recently shown that MAP2K1 is a target of miR-424 in senile hemangioma [61] and that MAP2K1 can be silenced by miR-497, which harbors the same seed sequence as miR-424 [62]. Altogether, these findings strongly suggest that MAP2K1 is

probably a genuine target of this family of miRNA, yet is unlikely to be relevant to trophoblastic miR-424.

An interesting finding that will need further investigation is the potential interplay between miR-424 and other members of the family, particularly miR-15 and miR-16. We showed that these miRNAs have the ability to repress an FGFR1 reporter. These miRNAs are normally abundant in trophoblasts, but their levels did not change in the conditions tested; therefore, it is unlikely that they affect FGFR1 during the process of differentiation or response to hypoxia. However, we cannot rule out the possibility that, in other circumstances, miR-15/16 could play a role in the regulation of FGFR1.

ACKNOWLEDGMENT

The authors thank Dr. C.H. Graham (Queen's University, Kingston, ON) for the HTR-8/SVneo cells and Dr. C.A. Hubel (University of Pittsburgh, Pittsburgh, PA) for HUVEC and ECFC cells. The authors also thank Judith Ziegler and Elena Sadovsky for technical assistance, and Lori Rideout and Bruce Campbell for assistance during preparation of the manuscript.

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Paper II

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Syncytiotrophoblast Vesicles Show Altered micro-RNA and Haemoglobin Content after *Ex-vivo* Perfusion of Placentas with Haemoglobin to Mimic Preeclampsia

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Abstract

Background: Cell-free foetal haemoglobin (HbF) has been shown to play a role in the pathology of preeclampsia (PE). In the present study, we aimed to further characterize the harmful effects of extracellular free haemoglobin (Hb) on the placenta. In particular, we investigated whether cell-free Hb affects the release of placental syncytiotrophoblast vesicles (STBMs) and their micro-RNA content.

Methods: The dual *ex-vivo* perfusion system was used to perfuse isolated cotyledons from human placenta, with medium alone (control) or supplemented with cell-free Hb. Perfusion medium from the maternal side of the placenta was collected at the end of all perfusion phases. The STBMs were isolated using ultra-centrifugation, at 10,000×g and 150,000×g (referred to as 10K and 150K STBMs). The STBMs were characterized using the nanoparticle tracking analysis, identification of surface markers and transmission electron microscopy. RNA was extracted and nine different micro-RNAs, related to hypoxia, PE and Hb synthesis, were selected for analysis by quantitative PCR.

Results: All micro-RNAs investigated were present in the STBMs. Mir-517a, mir-141 and mir-517b were down regulated after Hb perfusion in the 10K STBMs. Furthermore, Hb was shown to be carried by the STBMs.

Conclusion: This study showed that Hb perfusion can alter the micro-RNA content of released STBMs. Of particular interest is the alteration of two placenta specific micro-RNAs; mir-517a and mir-517b. We have also seen that STBMs may function as carriers of Hb into the maternal circulation.

Citation: Cronqvist T, Saljé K, Familari M, Guller S, Schneider H, et al. (2014) Syncytiotrophoblast Vesicles Show Altered micro-RNA and Haemoglobin Content after *Ex-vivo* Perfusion of Placentas with Haemoglobin to Mimic Preeclampsia. PLoS ONE 9(2): e90020. doi:10.1371/journal.pone.0090020

Editor: Ana Claudia Zenclussen, Medical Faculty, Otto-von-Guericke University Magdeburg, Medical Faculty, Germany

Received October 30, 2013; **Accepted** January 30, 2014; **Published** February 27, 2014

Funding: This work was supported by The Swedish Research council (TC); the Marianne & Marcus Wallenberg foundation (TC); the Torsten Söderbergsfoundation (TC); the Maggie Stephens foundation (TC); and the Wellcome Trust Technology Development Grant Ref GR087730 (CG). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: CWR is a consultant for A1M Pharma AB. BÅ, MG, SRH are co-founders and owners of A1M Pharma AB. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Preeclampsia (PE) is a disorder that affects 2–7% of all pregnancies [1] and is characterized by new onset hypertension and proteinuria [2]. There is no method to predict the disorder [1]. To date, the only cure is delivery and the treatment is purely symptomatic [3]. PE is thought to develop in two stages; the first is initiated by defective placentation resulting in inadequate formation of the utero-placental circulation. This results in an inadequately perfused placenta, which causes reperfusion injury, oxidative stress and formation of reactive oxygen species (ROS). As a result of this, in the second stage, placenta derived factors are released into the maternal circulation where they are believed to cause systemic inflammation, endothelial damage and organ failure [4].

Recently, cell-free foetal haemoglobin (HbF) was described to be an important placenta derived factor, potentially linking the first and second stage of PE. Analysis of placental gene expression by Centlow et al. revealed that the haemoglobin (Hb) chains, Hbα2, Hbγ and Hbβ, were significantly up regulated in PE, and an accumulation of HbF in the vascular lumen of PE placentas [5]. Also, perfusion of the placenta with cell-free Hb was shown to induce structural damage similar to that seen in PE [6]. As both HbF and its down-stream metabolites methaemoglobin, free heme and ROS are potent redox agents that can cause tissue damage

[7], it may be hypothesized that cell-free HbF plays a role in the aetiology of PE by inducing oxidative damage to the blood-placenta barrier [6]. Placenta derived factors, including HbF, then leak into the maternal circulation where they are able to contribute to oxidative stress, endothelial damage, inflammation, hypertension and proteinuria [8,9]. Clinical studies have shown that HbF leaks into the maternal circulation as early as the first trimester and is increased in women that will subsequently develop PE [10]. Furthermore, the levels of HbF correlates with the blood pressure, i.e. the severity of the disease, in term pregnancies [8].

Programmed cell death leads to cell blebbing, causing apoptotic debris, and extracellular vesicles (EVs) to be released [11]. Circulating EVs are often divided into apoptotic debris, micro-vesicles (>100 nm) and exosomes (<100 nm), which in addition to their size, differ regarding their membrane proteins and mode of release [12–15]. The EVs are recognized as a form of cell-to-cell communication that can transport proteins, DNA, RNA and micro-RNA (miRNA) from one cell to another and alter the phenotype and response of target cells [16–22].

The placental syncytiotrophoblast cells release EVs, named syncytiotrophoblast vesicles or STBMs (previously called syncytiotrophoblast microparticles). They have been suggested to be important for the foetal-maternal cross talk, allowing the maternal immune system to adapt to the on-going pregnancy [14,23]. The role of STBMs in the aetiology of PE is an emerging field of interest. The number of STBMs in maternal plasma increases significantly in PE compared to normal pregnancies [9,16,24]. Placental perfusion with cell-free Hb increases blebbing of the cell membranes suggesting an increased vesicle release [6]. Release of apoptotic material into the maternal circulation has been suggested to contribute to the endothelial dysfunction seen in PE and increased numbers of STBMs to be involved in the characteristic maternal inflammatory response [11,25–27]. For example, studies have shown that STBMs isolated from perfused placentas, when incubated with cultured monocytes, up regulate CD54 and down regulate CD11a expression [26], and STBMs incubated with cultured human umbilical vein endothelial cells activate peripheral blood leukocytes [28], including both monocytes [23] and neutrophils [29].

Micro-RNA (miRNA) are small non-coding RNA molecules predicted to regulate approximately 30% of all human genes [30]. Gene expression is generally down regulated by miRNA, either by degradation of the target mRNA or by preventing its translation [31]. The miRNAs are important for the development and function of the placenta. There is an abundance of miRNAs in the placenta, originating from a large, primate-specific, genomic cluster commonly referred to as the chromosome 19 miRNA cluster (C19MC) [32,33]. C19MC miRNAs are differentially expressed in trophoblastic cells, as well as placental tissue when comparing first and third trimester placentas [34,35]. Placental trophoblasts have been shown to release exosomes containing miRNAs in general and to be enriched in miRNAs belonging to C19MC in particular [32,33,36]. Both pregnancy specific [37] and placenta-specific miRNA have been detected in maternal plasma [36]. Many groups have also reported differentially expressed miRNAs in PE and hypoxic placentas [38–41].

Previous work has described placental *ex-vivo* perfusion with cell-free Hb as a model for PE [6]. The aim of this study was to further investigate the harmful mechanisms of extracellular Hb, and to examine the characteristics of STBMs released from placentas perfused with cell-free Hb. In particular, we investigated the miRNA content of released STBMs, following perfusion with cell-free Hb. Nine interesting miRNAs were chosen for the study. We selected mir-222, mir-16 and mir-210 based on previous studies showing their involvement in the regulation of HbF expression [42–44] as well as their involvement in PE [38–41]. Mir-517a, mir-517b and mir-518b were chosen because of their placenta specificity [36] and mir-518b being dys-regulated in PE [41]. Mir-424 and mir-205 are altered in hypoxia [45] and mir-141 in PE [39].

Materials and Methods

Ethics statement

The ethical review committee at Lund University approved the study and all mothers gave their written informed consent before delivery.

Placental perfusion and sample collection

Sample collection and dual *ex-vivo* perfusion of isolated human placental cotyledons was performed as previously described by May et al [6]. Briefly, the perfusion experiment consisted of three perfusion phases lasting 120 minutes each, with medium exchange between the phases. Perfusion medium was supplemented with cell-free adult Hb (HbA) in the foetal circulation to mimic the PE condition, during phase II (3 mg/ml HbA, n = 6) and medium only in phase I and III. Control experiments were performed using medium alone for all phases (n = 6). The perfusate was collected from the maternal side at the end of all phases and used for isolation and analysis of STBMs.

Isolation of STBMs from the perfusion medium

The STBM isolation was performed according to established protocols [46] from phase I and II. Thirty five ml of the maternal perfusate was centrifuged twice at 1500×g for 10 minutes in order to remove cellular debris. Ten ml of the supernatant was then further centrifuged for 30 minutes at 10,000×g at 4°C (pellet referred to as 10K STBM). The supernatant was ultra-centrifuged for 2 hours at 150,000×g at 4°C (pellet referred to as 150K STBM). The 10K and 150K STBM pellets were washed once with 1xPhosphate Buffered Saline (PBS) and re-suspended in 150 µl and 50 µl PBS respectively, aliquoted and stored at −80°C.

Characterization of STBMs in the perfusion medium

Protein concentration of STBMs. The STBM protein concentrations were determined spectrophotometrically using a NanoDrop Spectrophotometer ND-1000 (NanoDrop technologies, Wilmington, USA).

Transmission electron microscopy (TEM) of STBMs. Transmission electron microscopy (TEM) was performed in two sets of preparations, first with antibodies against the human proteins tissue factor (TF), CD 63 and hsa-mir-222, a micro-RNA Assay primer (Applied Biosystems Inc., Foster City, CA, USA), labelled with colloidal gold (30, 15 and 5 nm in diameter, BBI International) as previously described [47]. In the second preparation, antibodies against the human adult Hb (HbA) protein was labelled with colloidal gold. The STBMs from both the 10K and 150K fraction were mixed with gold-labelled conjugates for 20 minutes at room temperature and then processed for negative staining, as previously described [48]. It is known that vesicles are permeabilized during TEM preparation, allowing antibodies and probes to label targets both on the surface and inside the STBMs. TEM was carried out three times in different specimens for both control and Hb perfused STBMs.

Nanoparticle Tracking Analysis. Nanoparticle tracking analysis (NTA) was performed using the NanoSight NS500 instrument (NanoSight, Amesbury, UK)[49]. This instrument passes a focused 488 nm laser beam through a suspension of the

particles of interest and collects the scattered light using conventional microscope optics aligned at 90° to the beam axis. An electron multiplying charge coupled device captures a video of the field of view at 30 frames per second. The NTA program identifies and tracks Brownian motion of each particle from frame to frame, thus enabling the calculation of the hydrodynamic diameter via the Stokes-Einstein equation.

Samples analysed with NTA were from the different phase II STBM preparations; 10K control STBM preparations (n = 6), 10K Hb (n = 6), 150K control (n = 6) and 150K Hb (n = 6). The STBM preparations were diluted in sterile filtered PBS at 1:500 or 1:1000 prior to analysis, in order to give vesicle counts of 1.5–9.0×10^8/ml. The diluted sample was introduced into the sample chamber and ten 20-second videos were recorded (shutter speed of 600; camera gain of 250). Fresh sample was introduced automatically between each video recording to eliminate settling and reduce sampling error. The videos were processed using optimised instrument settings (detection threshold 10; blur automatic; and minimum particle size 100 nm).

Analysis of miRNA in STBMs

RNA isolation. Small RNA was isolated from the10K and 150K STBMs using mirVana™ miRNA Isolation Kit (Applied Biosystems, Carlsbad, USA) according to manufacturer's instruction. Briefly, the RNA extraction procedure consists of a step using Acid-Phenol:Chloroform, separating RNA in an upper organic phase from RNA and proteins which partitions in a lower aqueous phase. This prevents the Hb protein from being present in the RNA preparations and interacting in the subsequent PCR procedure. All RNA sample concentrations were spectrophotometrically determined using a NanoDrop Spectrophotometer ND-1000 (NanoDrop technologies, Wilmington, USA). RNA quality and miRNA content was assessed with an Agilent 2100 Bioanalyzer, using the Small RNA assay (Agilent Technologies, Palo Alto, USA).

cDNA synthesis and real-time quantitative PCR. RNA was transcribed using TaqMan® MicroRNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems Inc., Foster City, CA, USA). 10 ng RNA was used for the10K STBMs. For the 150K STBMs, 5 ng RNA was used because of a lower RNA yield. The following nine miRNAs were analysed using pre-designed TaqMan® MicroRNA assays (Applied Biosystems): homo sapiens-microRNA-517b (hsa-mir-517b), hsa-mir-518b, hsa-mir-222, hsa-mir-424, hsa-mir-210, hsa-mir-16, hsa-mir-141, hsa-mir-205, hsa-mir-517a, Rnu6b. Sequence for the hsa-mir-517b assay corresponds to ppy-mir-517b (pongo pygmaeus-microRNA, according to the miRNA database mirbase.org), which differs two nucleotides from the human hsa-mir-517b-3p.

Quantitative PCR (qPCR) was performed using standard protocol supplied by manufacturer for TaqMan® MicroRNA Assays on an ABI PRISM 7000 sequence detection system (Applied Biosystems). Primers and probes as described above. Each reaction was run in duplicate. Negative controls with no template as well as no reverse transcriptase controls were included for every miRNA primer pair. Data were normalized to Rnu6b, commonly used in miRNA PCR procedures. The fold-change values were calculated by normalizing against control samples from control perfused placentas placentas.

Statistical analysis

All statistical analysis was performed using Origin 9 software (Microcal, Northampton, MA, USA). Mann-Whitney *U*-test was used and p-value <0.05 was considered statistically significant.

Results

Protein concentration and RNA content of the STBMs

After isolating the 10K and 150K STBMs, protein concentration was determined (Table 1). Protein concentration in the 10K STBM controls was significantly higher than in the 150K STBM controls (p = 0.0022). There was a slight difference, however not significant, between 10K and 150K STBMs from the Hb perfusions (p = 0.0649). No difference was found between control and Hb perfusion in the 10K (p = 0.1320) or 150K STBMs (p = 0.5887).

Analysis of the small RNA, with Agilent 2100 Bioanalyzer, confirmed the presence of miRNA in all samples. The small RNA concentrations (Table 1) in the two STBM fractions were expressed either as RNA concentration (ng/μl) or amount of RNA per mg of protein (RNA/STBM ratio) (Table 1). Clearly, there was more RNA in the 10K STBM pellets compared to the 150K STBM pellets (p = 0.0022). No difference was seen between the control and Hb perfusions within the same centrifugation group. After calculating the RNA/STBM ratio, adjusting for size and amount of STBMs, the levels of RNA appeared to increase after Hb perfusion (Table 1). The RNA/STBM ratio was also more variable after Hb perfusions compared to controls. However, these results never reached statistical significance, p = 0.0649 for 10K and p = 0.1320 for 150K.

Characterization of STBMs

Transmission electron microscopy of STBMs. In order to distinguish between different sizes and types of vesicles, STBMs were investigated with transmission electron microscopy (TEM) using CD63 as a marker for exosomes [14] and TF as a general STBM marker [50] (Figure 1). Samples from both the 10K and 150K fractions as well as samples from both control and Hb perfusions, contained vesicles that were marked with CD63, TF and both (Table 2, and Figure 1B and 1C). There were no

Table 1. RNA and protein concentrations, as well as RNA/STBM ratio.

	Control	Hb
Small RNA concentration (ng/μl)		
Ph I 10K STBM	6,35±6,42	1,63±1,14
Ph II 10K STBM	17,3±6,48	16,85±1,41
Ph I 150K STBM	*	*
Ph II 150K STBM	4,14±3,01	7,41±3,32
Protein concentration (μg/μl)		
Ph I 10K STBM	3,17±2,11	1,34±0,91
Ph II 10K STBM	2,96±1,55	1,77±1,65
Ph I 150K STBM	0,76±0,39	0,67±0,21
Ph II 150K STBM	0,55±0,33	0,62±0,33
RNA/STBM ratio		
Ph I 10K STBM	1,19±1,32	2,40±3,05
PhII 10K STBM	6,52±2,30	15,92±9,9
Ph I 150K STBM	-	-
Ph II 150K STBM	8,04±3,88	14,09±7,28

Values are expressed as mean ± standard deviation.
* = immeasurable concentration by NanoDrop, RNA/STBM ratio not possible to calculate.
doi:10.1371/journal.pone.0090020.t001

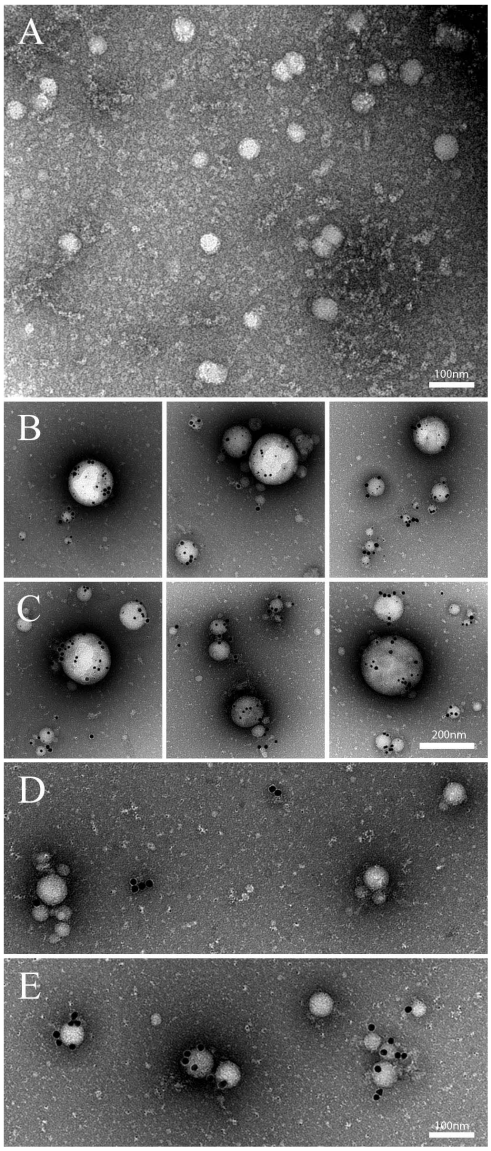

Figure 1. Transmission electron micrographs of isolated STBMs. The TEM panel (A) shows a selection of the 150K control STBMs, prior to gold-labelling. The STBMs were mixed with gold-labelled antibodies, as described in the Materials and Methods section, in two preparations. In the first preparation, STBMs shown in panels (B, control) and (C, Hb perfusions), were treated with antibodies against TF, CD63 and hsa-mir-222 labelled with colloidal gold of different sizes; CD63 (30 nm colloidal gold), TF (15 nm) and mir-222 (5 nm). Panel (B) shows the 150K control STBMs which are similar to the 150K Hb STBMs (C) in regards to the number of vesicles and their size. The 150K control STBMs (B) contain more mir-222 than 150K Hb STBMs (C). For the second preparation, STBMs shown in panels (D, control) and (E, Hb perfusions), were treated only with antibodies against Hb, labelled with colloidal gold. Control STBMs (D) carried small amounts of Hb, whereas STBMs from the Hb perfused placentas (E) showed higher labelling for Hb on particles of all sizes.
doi:10.1371/journal.pone.0090020.g001

significant differences between the 10K and 150K fraction when comparing the four markers CD63, TF, Hb and mir-222. The CD63 and TF markers did not differ between controls and Hb STBMs. However, the analysis of the gold signal for mir-222 displayed a down regulation after Hb perfusion (Table 2, and Figure 1B and 1C). Also, mir-222 labelling was found in vesicles expressing both CD63 and TF. Interestingly, in a subsequent preparation with gold-labelling of Hb antibodies, TEM revealed that STBMs carried Hb, and vesicles isolated from Hb perfusions carried more Hb than vesicles from the control perfusions (Table 2, and Figure 1D and 1E).

Nanoparticle Tracking Analysis. To determine the vesicle count and size distribution of vesicles in the 10K and 150K STBMs, we used the NTA methodology. Vesicle count broadly reflected the protein concentration of the 10K STBM preparations ($r = 0.71$) with stronger correlation in the 150K preparations ($r = 0.95$). This was probably due to the greater homogeneity observed in the 150K preparations. The size distribution for 10K and 150K STBMs ranged between 50–560 nm and 50–500 nm respectively (Figure 2A and 2B) The size range suggests that both STBM preparations contain both exosomes and microvesicles. There was no difference in this range between the controls and the Hb perfusions. The median size for 10K control was 184 nm and for Hb STBMs 187 nm (Figure 2A). For 150K control and Hb STBMs the median size was 171 nm and 166 nm respectively (Figure 2B). The median size was significantly larger for the 10K STBMs (186 nm) compared to the 150K STBMs (168 nm), shown in Figure 2C. This suggests that the 10K STBMs contain more STBMs in the microvesicle size range and fewer in the exosomes size range.

Analysis of miRNAs in STBMs

All miRNAs (mir-517a, mir-517b, mir-518b, mir-205, mir-210, mir-222, mir-141, mir-16 and mir-424) analysed in this study were present in both 10K and 150K STBMs. After Hb perfusion, mir-517a ($p = 0.03671$), mir-141 ($p = 0.01219$) and mir-517b ($p = 0.03671$) were significantly down regulated in 10K STBMs (Figure 3). To confirm that the differences obtained between the groups were dependent on Hb perfusion, mir-141 and mir-517a were also analysed in phase I, before addition of Hb. There was no significant difference ($p = 0.27034$ and 0.17791 respectively) in phase I. In contrast, the 150K STBMs showed a general trend towards up-regulation of miRNA after Hb perfusion, although none were significant (data not shown).

Discussion

Extracellular HbF has been suggested as a potential link between the first and second stage of PE [5,10]. *Ex-vivo* studies have shown that cell-free Hb induces placental damage similar to that seen in PE placentas, and therefore might provide an experimental *ex-vivo* model for PE. Electron microscopy showed that cell-free Hb causes oxidative stress, apoptosis and extensive membrane damage to perfused placentas [6]. In this study, we have further investigated the perfusion medium from these experiments in order to see how cell-free Hb affects the release of STBMs and their miRNA content. The data show that Hb

Table 2. Transmission electron microscopy (TEM) data, showing the amount of gold labels per square micrometre.

	10K STBM Control	10K STBM Hb	150K STBM Control	150K STBM Hb
TF	46±16	58±15	52±13	63±15
CD63	31±12	44±15	28±9	41±18
mir-222	98±14	52±17	96±15	63±16
Hb	9±3	49±17	10±3	51±17

Values are expressed as mean ± standard deviation.
doi:10.1371/journal.pone.0090020.t002

perfusion indeed does alter the content of miRNAs in STBMs. Since perfusion with Hb leads to increased cell blebbing [6] we also hypothesized that the placenta released more STBMs, as described in PE [9,16,24]. However the NTA analysis could not confirm this hypothesis. An unexpected and interesting finding was however that the STBMs carried Hb, possibly inside but also on the surface.

The different STBM fractions, showed a decreased median vesicle size in the 150K STBMs compared to the 10K STBMs, confirming earlier results [46]. This suggests more vesicles in the exosome size range (<100 nm) and fewer of microvesicle size range (>100 nm) after higher centrifugation speed. The two surface markers TF and CD63 used in TEM, showed a similar distribution between the two centrifugation fractions. In contrast to the NTA analysis, this finding suggest that there is no major differences between the types of vesicles isolated at different centrifugation speeds. However, even though the surface marker CD63 has been suggested to be a specific exosomes marker [14], it has also been found on the syncytiotrophoblast surface [51], indicating that it is likely to be present on the microvesicles released by syncytiotrophoblasts. The TEM showed that STBMs, larger than 100 nm, were positive for CD63, confirming this. The TF marker was also shown on vesicles of all sizes confirming previous findings [50].

Although there were no obvious differences in the STBM characteristics between control and Hb perfused placentas, we found significant differences in their miRNA content. The nine selected miRNAs were related to the C19MC cluster, hypoxia, PE or Hb synthesis [36,38–45].

Three miRNAs were down regulated in the 10K STBMs after Hb treatment; mir-517a, mir-141 and mir-517b. Mir-141 is one of the most abundant miRNAs in the placenta and found in high levels in maternal plasma during pregnancy [52,53]. Mir-517a and mir-517b belong to the C19MC cluster [32]. The C19MC miRNAs have previously been shown to be transported by trophoblast exosomes [32,36]. Recently it has been shown that mir-517b can be transported by trophoblast exosomes to recipient cells, normally not expressing C19MC miRNAs, and inhibit viral infections [54]. By sending out placenta specific miRNAs, the placentas may communicate to the maternal system. By altering the miRNA content in the STBMs, different signals can be sent to the receiving cells.

The TEM analysis showed that mir-222 was down regulated in both 10K and 150K STBMs. This was not confirmed by quantitative PCR, possibly due to small groups. There is an interesting connection between mir-222 and PE, it has been shown to be up-regulated in the PE placenta [38–41] but also present in circulating EVs from healthy controls [55]. To our knowledge, no previous studies have described mir-222 in STBMs. The mir-222 regulates fms-like tyrosine kinase-1 (Flt1) [43], which is an anti-angiogenic factor, well described in PE [4]. Furthermore, mir-222 plays a role in the human haemoglobin switch, i.e. when the newborn baby switches from HbF to HbA production, which takes place during the peri/post-natal period [43]. Since previous data have shown an increased production of HbF in PE placentas [5],

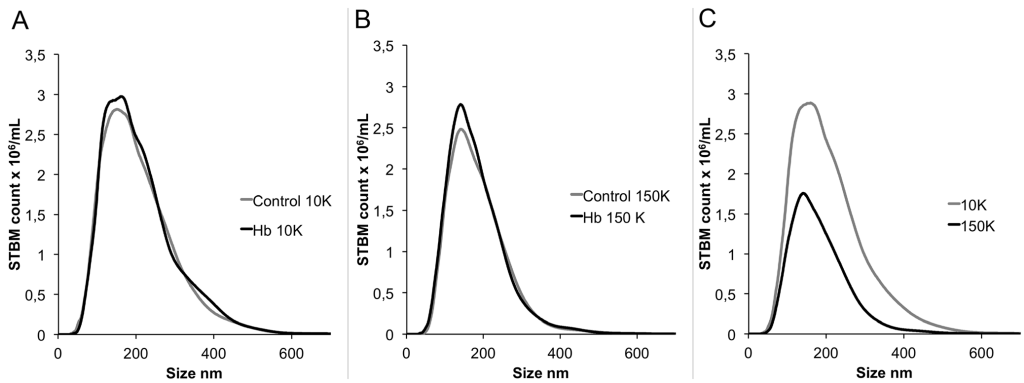

Figure 2. NTA analysis of vesicle size distribution in the 10K and 150K fraction. Nanoparticle tracking analysis (NTA) size distribution profiles for STBMs; comparing effect of Hb perfusion for 10K (A) and 150K (B) STBMs. In (C) the effect of centrifugation speed, 10K vs 150K, is compared. 10K STBMs had a significantly larger median size than 150K STBMs.
doi:10.1371/journal.pone.0090020.g002

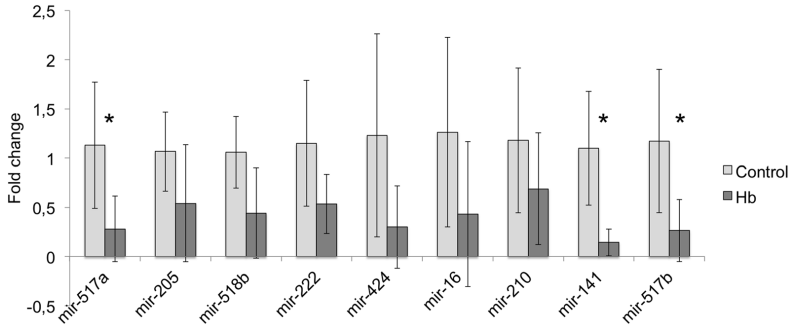

Figure 3. Bar charts showing miRNA fold change in STBMs from control and Hb perfusions. Nine selected miRNAs were analysed using quantitative PCR, as described in the Materials and methods section. The miRNA expression was normalized against Rnu6b and given as fold change. The fold change values were calculated by normalizing against control samples from control perfused placentas. Results are presented as mean ±SD. Differences between the respective control and Hb perfusions were analysed using Mann-Whitney *U*-test. * $p<0.05$. In the 10K STBMs mir-517a, mir-141 and mir -517b were significantly down regulated in Hb perfusions compared to controls. No significant differences were detected in the 150K STBMs (data not shown).
doi:10.1371/journal.pone.0090020.g003

and elevated levels of s-Flt [4], the role of mir-222 needs further exploration.

The 150K STBMs did not show any significant changes in the miRNA content for the nine miRNAs studied. Data from the NTA analysis suggest that the 150K fractions contain smaller STBMs, and possibly therefore more exosomes. Since exosomes are released by exocytosis and microvesicles by blebbing of the cell surface [12], it may be likely that they load RNA and miRNA in different ways. It has been suggested that exosomes are beneficial to normal pregnancy whereas microvesicles may be harmful [56]. When comparing different trophoblastic cells and cell lines, a previous study has shown great variation of miRNA expression, in particular C19MC miRNAs, which may account for the differences in behaviour between these cells [34]. The differences in miRNA expression between the 10K and 150K fractions might be part of the explanation of why exosomes and microvesicles play different roles in normal pregnancies and in PE. Even though Hb treatment does not alter the shedding of vesicles from the human placenta, the data shows that miRNA content can be significantly altered. This could suggest that Hb has an effect at the level of gene expression. On the other hand, there was a trend towards a generally impoverished miRNA content in 10K STBMs and enriched miRNA content in 150K STBMs. Rather than having an effect on a transcriptional level in the cells, Hb could be changing the profile of STBMs carrying miRNAs where exosomes increase their miRNA content during stress and microvesicles decrease theirs.

The TEM results indicated that cell-free Hb was accumulated inside or on the surface of the STBMs. The small amount of Hb present in the control STBMs, may be due to the natural Hb metabolism occurring in the placenta. During Hb perfusion there is a high Hb concentration outside the vesicles, which may cause binding to the surface. It is important to note that the Hb antibody used for the specific TEM, targets the alpha-chain and therefore measures total Hb, both the Hb added to the perfusion as well as the endogenous foetal production in the placenta. These findings suggest a novel way for Hb to be transferred into the maternal circulation from the placenta, and will be subject for future studies.

In summary, the data in this study suggest that Hb perfusion of the placenta significantly affects the content of some miRNAs in released STBMs. The increased amount of STBMs in PE may be potentially loaded with Hb and differentially expressed miRNAs, which will have negative effects on target cells such as endothelial cells and lymphocytes. It may contribute to the endothelial dysfunction and inflammation seen in PE [4]. STBMs may be important for communicating the status of the placenta systemically. Accumulation of Hb in STBMs may prevent Hb from being degraded. Upon fusion with other cell types, a direct intracellular deposit of Hb may cause toxic damage. Accumulated Hb may also oxidize the STBMs content, modifying RNA and proteins, which could have further impact on the vesicle-to-cell signalling.

Acknowledgments

We thank Irene Larsson, Maria Baumgarten and Pia Chassale for valuable technical assistance. We also thank Professor Yoel Sadovsky and Professor Lucia Mincheva-Nilsson for helpful suggestions during the course of this work and preparation of the manuscript.

Author Contributions

Conceived and designed the experiments: TC KS MF SG HS CG ILS CWR MM BÅ MG SRH. Performed the experiments: TC KS SG CG MM SRH. Analyzed the data: TC SG CG MM BÅ MG SRH. Contributed reagents/materials/analysis tools: SG CG ILS CWR MM BÅ SRH. Wrote the paper: TC SRH.

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Paper III

SCIENTIFIC REPORTS

OPEN

Syncytiotrophoblast derived extracellular vesicles transfer functional placental miRNAs to primary human endothelial cells

Received: 5 January 2017
Accepted: 2 May 2017
Published online: 04 July 2017

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During the pregnancy associated syndrome preeclampsia (PE), there is increased release of placental syncytiotrophoblast extracellular vesicles (STBEVs) and free foetal haemoglobin (HbF) into the maternal circulation. In the present study we investigated the uptake of normal and PE STBEVs by primary human coronary artery endothelial cells (HCAEC) and the effects of free HbF on this uptake. Our results show internalization of STBEVs into primary HCAEC, and transfer of placenta specific miRNAs from STBEVs into the endoplasmic reticulum and mitochondria of these recipient cells. Further, the transferred miRNAs were functional, causing a down regulation of specific target genes, including the PE associated gene fms related tyrosine kinase 1 (FLT1). When co-treating normal STBEVs with HbF, the miRNA deposition is altered from the mitochondria to the ER and the cell membrane becomes ruffled, as was also seen with PE STBEVs. These findings suggest that STBEVs may cause endothelial damage and contribute to the endothelial dysfunction typical for PE. The miRNA mediated effects on gene expression may contribute to the oxidative and endoplasmic reticulum stress described in PE, as well as endothelial reprogramming that may underlay the increased risk of cardiovascular disease reported for women with PE later in life.

Preeclampsia (PE) is a complex and severe pregnancy associated disorder and is diagnosed on the presence of newly developed hypertension and proteinuria from 20 weeks of gestation[1]. It is generally agreed that PE develops in two stages. During the first stage, the placenta is inadequately implanted which causes reduced placental perfusion, oxidative stress, increased placental apoptosis and excessive shedding of trophoblast debris. In the second stage, these components together with the anti-angiogenic factor, soluble receptor vascular endothelial growth factor (s-Flt), are released into the maternal circulation, where they cause systemic inflammation, endothelial dysfunction and organ failure[2,3]. These factors, although not unique for PE, are present in excessive amounts during this disorder[4,5]. Risk factors for PE include diabetes, obesity, previous PE pregnancies and chronic hypertension amongst others[6].

Several placental factors have been suggested to link the first and second stage of PE. Studies from our research group suggest free foetal haemoglobin (HbF) may be an important factor in this transition[7] as increased synthesis and accumulation of free HbF has been shown in PE placentas[8]. Further, leakage of HbF, from the damaged placenta, into the maternal circulation has been demonstrated both *ex vivo*[9] and in different cohort studies[10,11]. The negative effects of free HbF and its metabolite heme on placental function have been confirmed in several animal studies[12–14]. By perfusing human normal placentae *ex vivo* with free Hb, an increased perfusion pressure and induction of PE-like pathological changes, as well as increased placental cell blebbing and formation of apoptotic

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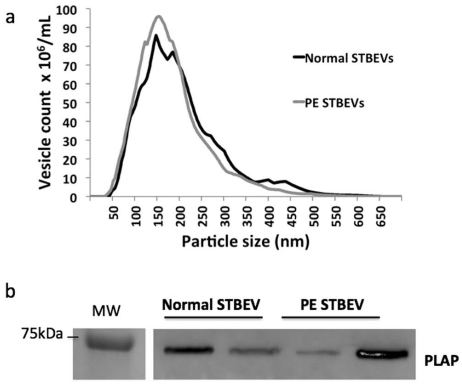

Figure 1. Characterisation of STBEVs using NTA and western blotting. Using Nanoparticle Tracking Analysis (NTA), vesicle size distribution and concentration was determined. The NTA showed STBEV vesicle sizes ranging between 50–500 nm (**a**) for both normal and PE STBEVs. Both normal and PE STBEV samples were PLAP positive by western blotting (**b**), shown by using the primary antibody NDOG2 against the STBEV surface marker PLAP (60 kDa). Each lane represents one individual sample. Blots have been cropped and inverted, see Supplementary Information S5 for full-length blots. Please note that the molecular marker shown in this figure is from a different blot run simultaneously, also shown in Supplementary Information S5.

vesicles, was shown[9,15]. Based on these studies, we hypothesized that free HbF may be responsible for inducing the increased shedding of syncytiotrophoblast extracellular vesicles (STBEVs) evident in PE[16,17].

Extracellular vesicles (EVs) are membrane vesicles released by all cells studied to date, and divided into exosomes and microvesicles based on size and site of formation in the cell. Exosomes are released by exocytosis from multivesicular bodies and have a size ranging between approximately 30–100 nm. Microvesicles are shed directly from the plasma membrane and range from 100nm-1μm in diameter. The placenta also releases syncytial nuclear aggregates (20–500 μm) as well as apoptotic bodies (1–4 μm)[18,19]. Placental EVs are often referred to as STBEVs due to their syncytiotrophoblast cell of origin, and they are believed to play an important role both in normal and dysfunctional pregnancies. It has been suggested that STBEVs adapt the maternal immune system to the on-going normal pregnancy[20,21]. In PE, the plasma level of STBEVs is increased and PE STBEVs show different characteristics compared to normal STBEVs. For example, PE STBEVs show increased expression of Tissue Factor (TF)[18,22], which may trigger the immune system to be more active and/or damaging[16,17]. It is also known that PE STBEVs are larger in size compared to normal STBEVs[23]. The STBEVs are believed to be one factor that causes the endothelial dysfunction seen in PE[24].

STBEVs carry[15,25] and transfer miRNAs to recipient cells[26–28]. miRNAs are short non-coding RNAs, which affect gene expression either by degrading mRNA, or by inhibiting mRNA translation[29]. The chromosome 19 miRNA cluster (C19MC) has been shown to be almost exclusively expressed in the placenta[30–32] and C19MC miRNAs are found in placenta released STBEVs[25,26,28,33]. C19MC miRNAs have been characterized in the maternal circulation and their expression profile is altered in PE[34,35]. We have reported that Hb perfusion of human placentas alter the miRNA content of released STBEVs from normal placentas[15].

Several studies have shown increased arterial stiffness – an indicator of endothelial dysfunction, in women with PE – correlating with PE severity[24]. Women with PE have an increased risk of cardiovascular disease later in life, possibly due to PE-induced endothelial and vascular dysfunction[36,37]. We hypothesize that the increased levels of STBEVs together with free HbF play an important role in endothelial dysfunction, by inducing oxidative stress and membrane damage. We also hypothesize that STBEVs transfer potentially functional miRNAs, to endothelial cells to alter gene expression, leading to cellular alterations that cause long term changes in the endothelium. In the present study, we investigated the uptake of STBEVs, isolated from perfused placentas, from normal and PE pregnancies, by primary human coronary artery endothelial cells (HCAECs). To mimic the double hit seen in PE, with altered STBEVs as well as increased HbF, the role of free HbF on the STBEV uptake was specifically studied. We also investigated the transfer of placental miRNAs and their effects on endothelial cell target gene expression.

Results

Characterisation of STBEVs. *Nanoparticle Tracking Analysis.* To determine vesicle count and size distributions of normal and PE STBEVs, we used the Nanoparticle Tracking Analysis (NTA) methodology. Mean vesicle sizes were 206 ± 20 nm and 195 ± 27 nm for normal and PE STBEVs respectively (Fig. 1a). No significant differences were observed in terms of vesicle count or size ranges between groups.

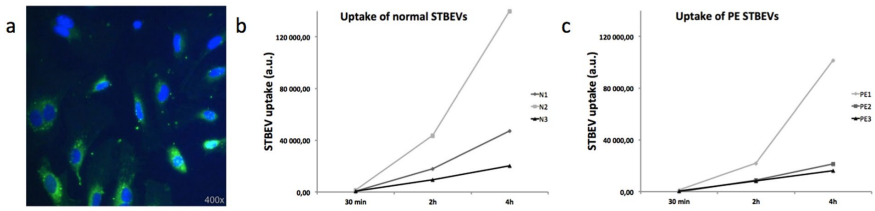

Figure 2. Time course assessment of STBEV uptake. HCAEC uptake of PKH-labelled STBEVs was visualised using fluorescent microscopy (**a**). A representative image of HCAECs following 6 hours of incubation with PKH-labelled STBEVs. The cell nuclei are labelled blue and PKH-labelled STBEVs are seen as green. Time-dependent uptake of normal (b, N1-N3) and PE (c, PE1-PE3) STBEVs was quantified using flow cytometry. Although variable between samples, all samples showed a time-dependent uptake. The horizontal axis shows mean fluorescent values; a.u., arbitrary units.

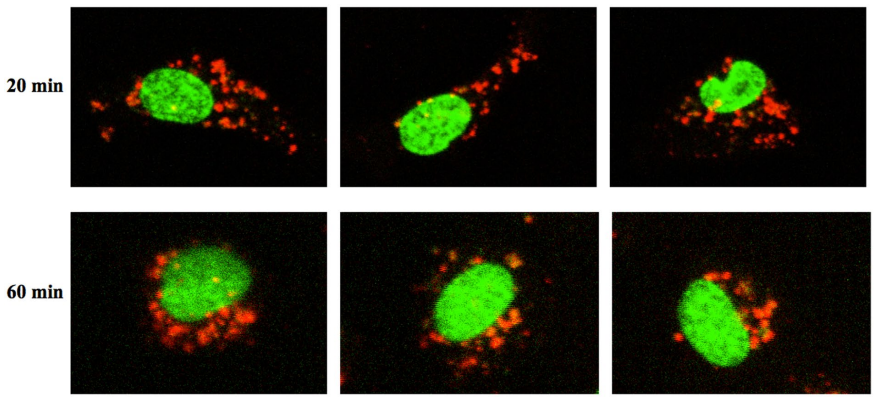

Figure 3. Live confocal microscopy visualizing STBEV uptake and intracellular transport. Uptake of PKH-labelled STBEVs into HCAECs was followed by live confocal microscopy. Three representative cells are shown after 20 and 60 min of incubation. After 20 min the STBEVs are clearly internalised and widely distributed in the cell cytoplasm. After 60 min the STBEVs appear to be closer to the cell nuclei. Cell nuclei are pseudocolored in green, and PKH-labelled STBEVs in red. The figures are from live imaging, explaining the lower intensity at 60 minutes due to bleaching.

Western Blot. Western blot analysis showed that both normal and PE STBEV samples were PLAP positive, consistent with their placental syncytiotrophoblast origin (Fig. 1b).

STBEV uptake in primary human coronary artery endothelial cells. *Time course assessment of STBEV uptake.* The uptake of PKH-labelled STBEVs in primary HCAECs was visualized by fluorescence microscopy (Fig. 2a) and quantified over time using flow cytometry (Fig. 2b). There was no obvious uptake after 15 minutes incubation, but uptake was detected after 30 minutes and increased throughout the 24 hours time course. At 24 hours, all visualised cells appeared positive for PKH-stained STBEVs. There were no apparent differences in uptake pattern between normal and PE derived STBEVs, with respect to time-course or number of PKH-STBEV positive cells.

Confocal microscopy. PKH-labelled STBEV internalization was confirmed by confocal microscopy, clearly showing that the STBEVs were not located on the cell surface (Fig. 3). The dynamics of internalized STBEVs could be further visualized by real-time confocal imaging (Supplementary information S1: video). Confocal imaging was more sensitive than flow cytometry analysis, and this analysis clearly demonstrates internalised STBEVs at 20 min. At 60 min of internalisation STBEVs were not as widely distributed in the cell cytoplasm, but appeared closer to the cell nucleus (Fig. 3).

STBEV transfer of miRNAs to primary human coronary artery endothelial cells. *Micro-RNA analysis.* Total RNA was extracted from HCAEC cultures treated with normal or PE STBEVs. The miRNAs belonging to the C19 miRNA cluster (C19MC) and the 371–373 cluster, as well as four commonly expressed miRNAs (mir-210; mir-222; mir-16; mir-141*) were analysed using a custom TaqMan® miRNA array card from Applied Biosystems (The full list of miRNA sequences are listed in Supplementary information S2). The miRNAs showing expression levels at the cut-off value Ct <35 in all triplicates for one or more samples were included for further analysis. Out of the 52 C19MC miRNAs examined, 12 miRNAs fulfilled these criterion (marked with # in S2, Ct values presented in Supplementary information Table S3). Of special interest were the three C19MC miRNAs mir-517c, mir-517a and mir-519a, which were expressed in a majority of the samples but not in the controls, i.e. untreated cells. None of the 371–373-cluster miRNAs, fulfilled the cut-off criteria. Mir-210, mir-222 and mir-16, were expressed in all samples including the controls. Mir-141* was not detected in any samples. In the array analysis, there was significantly lower expression of mir-210 in the PE STBEV treated cells compared to cells treated with STBEVs from normal pregnancies ($p = 0.022$, Table S3). The mir-222 showed significantly higher expression in the cells treated with normal STBEVs compared to the untreated control cells ($p = 0.031$, Table S3). These differences however could not be confirmed using real time quantitative PCR (RTqPCR). There was no significant difference between normal and PE STBEV treatments (Table S3). The endogenous control U6 snRNA was stably expressed throughout all samples.

Based on the array results, four miRNAs (mir-517a, mir-517c, mir-519a and mir-210) were selected for further analysis using RTqPCR. Control cells, i.e. not treated with STBEV, did not express these three C19MC miRNAs. The analysis confirmed the presence of the three C19MC miRNAs in cells treated with either normal or PE STBEVs (Fig. 4a). After treatment with normal STBEVs, the levels of mir-517a, mir-517c and mir-519a increased significantly ($p = 0.00164$, $p = 0.0002$ and $p = 0.00164$ respectively). Treatment with PE STBEVs also caused a significant increase ($p = 0.00124$, $p = 0.00012$ and $p = 0.00124$ respectively). The mir-210 expression levels remained stable in the cells regardless of treatment group (Fig. 4b), most likely reflecting the cells endogenous mir-210 expression. This also suggests that the STBEVs contain very low levels of mir-210, consistent with previous studies showing that trophoblast cells release vesicles mainly containing C19MC miRNAs[25].

Micro-RNA target genes. The strategy for predicting miRNA targets is detailed in the Methods section. Briefly, by using three different prediction algorithms, as well as the TarBase database for experimentally validated miRNA targets, we identified putative target genes for the miRNAs mir-517a, mir-517c and mir-519a (Fig. 5). The following genes were analysed by RTqPCR; fms related tyrosine kinase (FLT1), transforming growth factor beta receptor 2 (TGFBR2), platelet derived growth factor D (PDGFD), very low density lipoprotein receptor (VLDLR), estrogen receptor 1 (ESR1) and programmed cell death 1 ligand 2 (PDCD1LG2). As an indicator of cell stress, heme oxygenase 1 (HMOX1) was analysed. The VLDLR mRNA expression remained unchanged after both normal ($p > 0.05$) and PE ($p = 0.039$, fold change <1.5) STBEV treatment. The ESR1 mRNA expression was not detectable in our samples. Normal STBEV treatment led to significant down regulation of the genes FLT1 ($p = 0.0014$), TGFBR2 ($p = 0.0074$), PDGFD ($p = 0.0074$), PDCD1LG2 ($p = 0.0033$) and HMOX1 ($p = 0.0096$), In the same manner, PE STBEV treatment lead to significant down regulation of FLT1 ($p = 0.0005$), TGFBR2 ($p = 0.0021$), PDGFD ($p = 0.0012$), PDCD1LG2 ($p = 0.0012$) and HMOX1 ($p = 0.0071$). There was no statistically significant difference comparing normal and PE STBEV treatment (Figs 4c and 5).

Transmission Electron Microscopy. *STBEV characterisation.* The placental marker PLAP was visualized on vesicles of all sizes using Transmission Electron Microscopy (TEM) (Supplementary Figure S4). The estimated size range was 10–400 nm in both normal and PE (Supplementary Figure S4a) samples, which was in good agreement with the NTA data in Fig. 1a. By using a gold-labelled miRNA primer (hsa-mir-517c), mir-517c could be visualized inside both PLAP positive and PLAP negative STBEVs, the PE STBEVs shown in Supplementary Figure S4b. Furthermore, mir-517c was also localized outside of the vesicles, in what appeared to be protein complexes. HbF was visualized with HbF specific antibodies inside both PLAP positive and PLAP negative PE STBEVs of all sizes (Supplementary Figure S4c). Normal STBEVs did not contain HbF (Supplementary Figure S4d).

Cell analysis. Uptake of STBEVs into HCAECs was visualized using TEM. The placental marker PLAP was located on vesicles outside the cells, bound to the cell surface, as well as inside the endothelial cells in different compartments (Fig. 6). The PLAP marker was also found to be recycled to the cell membrane (Fig. 6g). Both normal and PE STBEVs were positive for PLAP and mir-517c. The mir-517c remained co-localized with PLAP positive vesicles until the STBEVs reached the endosomes. Hereafter, the STBEVs appeared to be degraded, whereby PLAP was possibly recycled to the cell surface, and mir-517c re-located to either the endoplasmic reticulum (ER) or the mitochondria. The normal STBEVs appeared to deposit their miRNA content in higher quantity to the mitochondria (Fig. 6j, Table 1). In contrast, the PE STBEVs were deposited in a higher degree to the ER (Fig. 6e, Table 1). With TEM, the intracellular distribution could be followed, although it is worth mentioning that these results are primarily descriptive and not established statistically. Furthermore, treatment with PE STBEVs caused extensive damage to the endothelial cell membrane inducing membrane ruffling (Fig. 6b), which was not seen after treatment with normal STBEVs (Fig. 6g). The primary endothelial cells were also exposed to a combination of HbF and normal STBEVs (Fig. 6), to evaluate the potential role of the HbF found in the PE STBEVs. A severe membrane ruffling occurred (Fig. 7b and c), similar to that seen in cells treated with PE STBEVs. The normal STBEVs were taken up into endosomes (Fig. 7d), but the co-treatment with HbF resulted in miRNA deposition shifting towards the ER (Fig. 7e and f), as was seen for PE STBEVs.

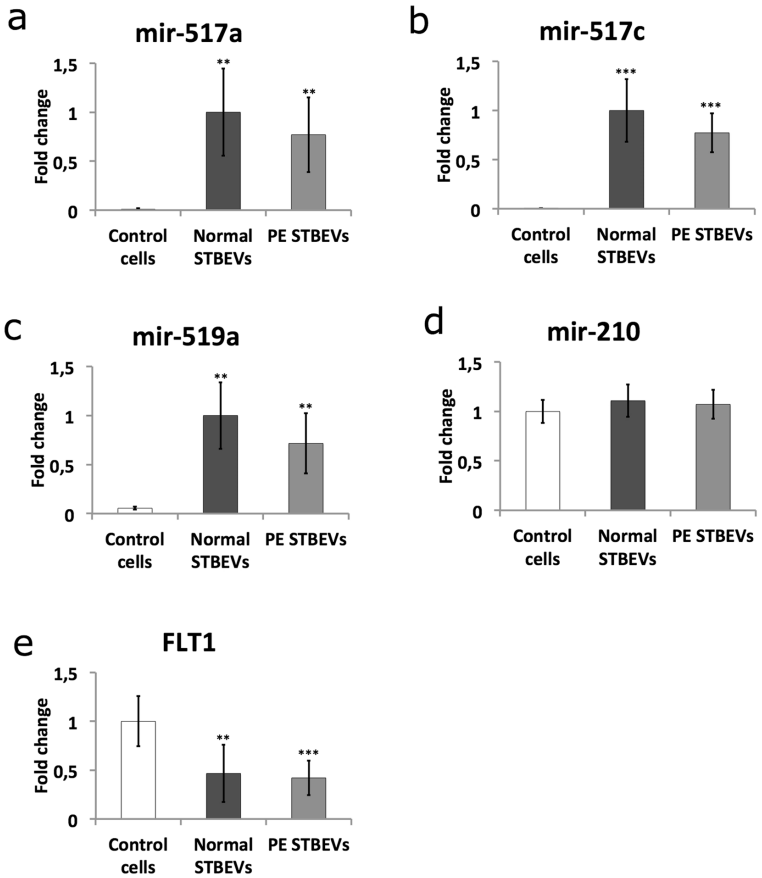

Figure 4. Micro-RNA and target gene expression in primary human coronary artery endothelial cells following STBEV treatment. The three placental C19MC miRNAs, mir-517a (**a**), mir-517c (**b**) and mir-519a (**c**), were analysed using RTqPCR. None of the three C19MC miRNAs were present in un-treated control cells. After treatment with normal (n = 5) or PE (n = 5) STBEVs, the levels of all three miRNAs increased significantly compared to control cells. No significant difference was seen in miRNA expression when comparing normal and PE STBEV treatments for any of the three C19MC miRNAs. The mir-210 (**d**) was present in controls cells and unaffected by STBEV treatment. The miRNA expression is expressed as fold change (±SD), which were calculated by normalizing against cells treated with normal STBEVs. Expression level of the predicted target gene FLT1 (**e**) was down regulated after normal (n = 5) and PE (n = 5) STBEV treatment. FLT1 is shown here as representative of the four genes; FLT1, TGFBR2, PDGFD and PDCD1LG2, all affected similarly by STBEV treatment. See also Fig. 5. The expression of predicted target genes was calculated as fold change (±SD) by normalizing against control samples, i.e. untreated control cells. Differences between control and normal STBEV treatments, as well as between control and PE STBEV groups, were analysed using Mann-Whitney *U*-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

The present study provides evidence that STBEVs are internalized into HCAECs, and that STBEV cargo, placenta specific miRNAs, is transferred into recipient cells. To our knowledge, no previous uptake studies have been performed on HCAECs using STBEVs from perfused placentas. Previous studies have visualized the uptake of mechanically derived placental or cell-culture derived syncytiotrophoblast vesicles into cells such as vascular smooth muscle cells[38], BeWo cells or primary villous cytotrophoblasts[39].

	Control cells	Normal STBEVs	PE STBEVs
HMOX1	1.00	0.62	0.57
PDCD1LG2	1.00	0.54	0.50
VLDLR	1.00	0.74	0.74
PDGFD	1.00	0.53	0.49
FLT1	1.00	0.47	0.42
TGFBR2	1.00	0.64	0.56
ESR1	N.D.	N.D.	N.D.

Figure 5. Target gene fold changes in HCAECs, after treatment with normal or PE STBEVs compared to controls. Heat-map showing fold change for each gene relative to control cells. A deviation from 1.0 indicates down-regulation of gene expression. N.D (not detected) in HCAEC cells.

The results show that STBEVs are internalized with increased uptake after longer incubation time, lending support to the notion that uptake of STBEVs into HCAECs is active and involves a specific uptake mechanism. The evidence for STBEV internalization was obtained both indirectly by flow cytometry, as well as directly by confocal microscopy live imaging and TEM. The flow cytometry analysis was also used to quantify the time-dependent uptake observed *in vitro*. In our hands, the STBEV uptake was significant after 15–30 minutes, with increasing uptake throughout the 24-hour time course. Prior to internalization, the STBEVs appeared to bind to the cell surface. Using TEM, we showed that the STBEVs were transported to endosomes, where it is possible that the vesicles were degraded and re-cycled to the cell-surface, as appears to be the case for PLAP labelled vesicles. The uptake into endosomes suggests that the STBEVs are, at least in part, taken up by endocytosis, but the presence of PLAP on the plasma membrane, could also suggest a partial uptake through membrane fusion. The exact uptake mechanisms and whether these differ between EV subgroups of exosomes and microvesicles, remains to be elucidated[18].

The miRNA content (specifically mir-517c) was, after vesicle degradation, transported primarily to the rough endoplasmic reticulum (ER) or the mitochondria. Localization of miRNAs to the ER suggests that the miRNAs are actively involved in regulating gene expression at the translational level. Data on target gene expression further supports the idea that STBEV miRNAs are functional after uptake, since several C19MC miRNA predicted or validated target genes were down regulated. Previous studies have also shown vesicles affecting target gene expression in recipient cells. For example, human umbilical vein endothelial cells (HUVECs) were shown to take up plasma-EVs derived from pregnant women, and several studies have reported that STBEV had effects on both gene expression and cellular functions[40–42]. In addition there were different effects depending on whether the EVs were derived from normal or PE patients[43]. In the present study we saw no difference in target gene down-regulation when comparing normal and PE STBEVs. The miRNA expression data suggests that normal and PE STBEVs contain the same amount of C19MC miRNAs, and the cells were treated with equivalent concentrations of STBEVs, which could explain why the effect on gene expression is similar. Another possible explanation is

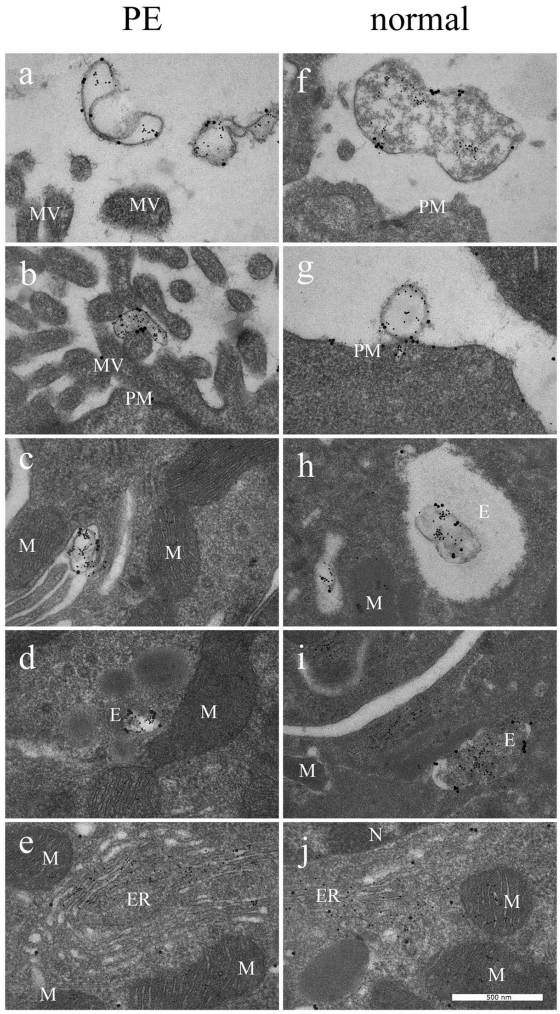

Figure 6. STBEV uptake and miRNA transfer visualised by transmission electron microscopy. By using TEM, PE STBEVs (**a–e**) and normal STBEVs (**f–j**) were visualized in HCAECs. In panel (a), PE STBEVs approach the HCAEC. In (**b**) and (**c**), PE STBEV appears to be binding to the ruffled plasma membrane. The STBEV is seen inside the cells in what appears to be an endosome (**d**), in close proximity to the mitochondria, endoplasmic reticulum and cell nucleus. The mir-517c from PE STBEVs appears to be in larger number in the ER (**e**) compared to the mitochondria. PLAP appears to stay in the endosomes and is possibly recycled to the cell membrane. The normal STBEVs, which also carry mir-517c are found outside the cells (**f–g**) and can be found in endosomes (**h,j**). In contrast to PE STBEVs, mir-517c from normal STBEVs are localized in a higher degree to mitochondria and in lower quantity to the ER (**j**). The STBEV and miRNA intracellular distribution are also described in Table 1. Abbreviations; E endosome, ER endoplasmic reticulum, MV microvilli, M mitochondria, N nucleus, PM plasma membrane. PLAP labelled with 20 nm and mir-517c labelled with 5 nm colloidal gold.

	Normal STBEVs (n = 100)	PE STBEVs (n = 100)	Normal STBEVs+HbF (n = 100)
Plasma membrane	43 ± 10	69 ± 15	81 ± 18
Cytoplasm	39 ± 11	34 ± 12	36 ± 11
Endosomes	54 ± 9	66 ± 13	71 ± 12
Mitochondria	42 ± 6	28 ± 8	31 ± 10
ER	21 ± 4	39 ± 8	41 ± 9

Table 1. Semi-quantification of miRNA gold label per square micrometre in Transmission Electron Microscopy data.

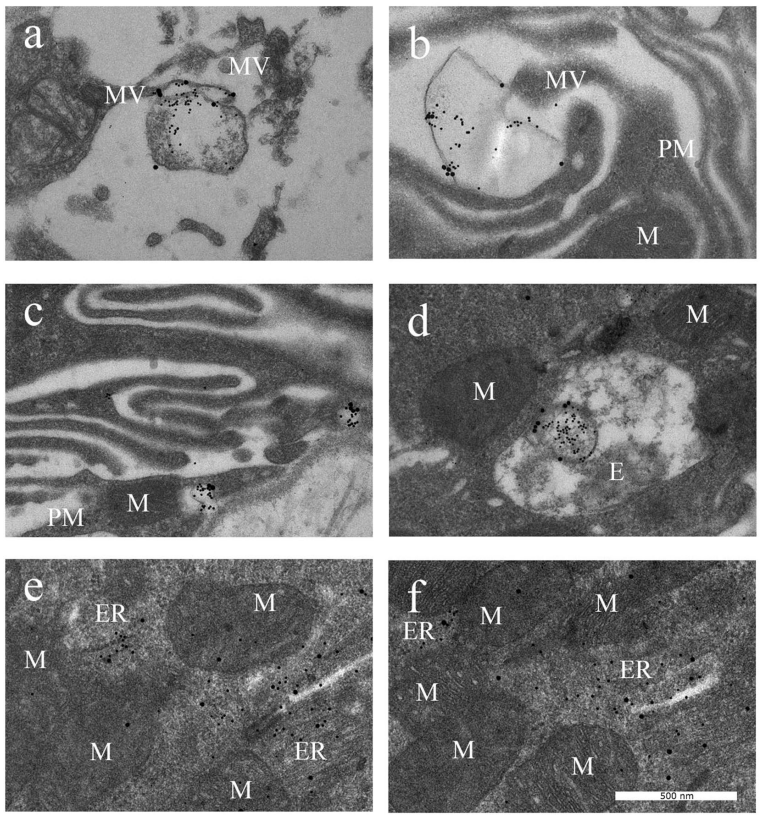

Figure 7. STBEV uptake and effect of free HbF visualised by transmission electron microscopy. The normal STBEVs, co-treated with HbF, appear to bind the plasma membrane (**a**), which is ruffled (**b,c**) as was also seen in cells treated with PE STBEVs (see Fig. 6b). The STBEVs were internalised into endosomes (**d**) and transported to intracellular compartments (**e,f**). The mir-517c is localised predominantly into the ER than in the mitochondria (**e,f**), also seen in cells treated with PE STBEVs (see Fig. 6e). Abbreviations; E endosome, ER endoplasmic reticulum, MV microvilli, M mitochondria, N nucleus, PM plasma membrane. PLAP labelled with 20 nm and mir-517c labelled with 5 nm colloidal gold.

that there could be long-term effects after several months' treatment, as is the case in pregnant women. The ruffled plasma membrane caused by PE STBEVs may lead to cellular dysfunction over time.

Interestingly, by semi-quantifying the gold labelled STBEVs with TEM, we saw that normal STBEV miRNAs were deposited at a higher degree in the mitochondria, while PE STBEV miRNAs primarily were deposited to the ER. We speculate that this could be due to different molecules or tags on the EV surface, directing the intra-cellular transport of vesicles to different subcellular compartments. In previous studies, HbF has been shown to affect both mitochondria and ER[14] and PE placentas are subject to high levels of both oxidative and ER stress[44].

This led us to the hypothesis that the HbF inside PE STBEVs cause oxidative stress and damage to the mitochondria or ER, thereby re-directing the STBEVs and their miRNA cargo. The extensive membrane ruffling, seen after treatment with PE STBEVs as well as with normal STBEVs co-treated with free HbF, suggests destruction of the cytoskeleton, which also disrupts intracellular pathways. A functioning cytoskeleton is required for EV uptake through endocytosis[45]. The re-direction of STBEV cargo and shifted balance of STBEV miRNA deposition from the mitochondria to the ER could increase the ER stress further. This could possibly affect the translation of various proteins important for normal pregnancy adaptation. As shown here, even normal STBEVs transfer unique information in the form of placenta specific miRNAs. And this may be important for placenta-maternal communication.

Our TEM studies showed that PE STBEVs carried HbF. Since free HbF is a toxic molecule[3] this could have damaging effects on endothelial cell structure and function, and may play an important role in causing the endothelial dysfunction characteristic of PE[4]. By co-treating normal STBEVs with HbF, similar cell membrane ruffling was seen, as with PE STBEVs alone, lending support to the notion that HbF is at least one of the harmful differences between normal and PE STBEVs. Various conditions have previously been shown to affect different EV uptake pathways. For example, blocking specific receptors with heparin or Annexin-V, inhibits endocytic uptake via protein-protein interactions. Further, acidic conditions increase uptake through membrane fusion, whereas low temperature (4 °C) inhibits the energy-requiring uptake through endocytosis[45]. We speculate that HbF may enhance or inhibit certain uptake pathways or intra-cellular transport mechanisms, for example by disrupting the cytoskeleton or inducing oxidative stress.

Regarding STBEV characterisation, the NTA data showed no difference in vesicle count or size distribution, between normal and PE samples. It is well documented that STBEVs increase significantly in the plasma of women with PE[22]. However, at present it is not possible to normalize the surface area of a perfused placenta, and generally only one cotyledon is perfused, and these vary in size even within a single placenta. Therefore the number of STBEV released per perfused cotyledon is variable. Further, the vesicle size can be affected by freezing[46], which may explain the differences between the groups. We therefore draw no conclusions in this study regarding the STBEV count in plasma from pregnant women.

In conclusion, the results show STBEV uptake and internalization by HCAECs, and transfer of placenta specific miRNAs, which in turn affect predicted and validated target gene expression. Normal and PE STBEVs deposited their miRNA content in different cellular compartments and co-treatment with free HbF and normal STBEVs, re-directed the miRNA deposition to a pattern similar to that seen in cells treated with PE STBEVs. Also, PE STBEVs caused extensive cell membrane damage, not seen with normal STBEVs. In PE, there is a higher plasma concentration of both STBEVs and free HbF, which may lead to re-programming of endothelial cells that may cause detrimental cellular functions such as arterial stiffness[24]. This may explain the long-term cardiovascular consequences seen in women who have suffered PE during pregnancy[37].

Methods

Ethics statement. The perfusion studies were approved by the Oxfordshire Research Ethics Committee C at Oxford University, and informed written consent was obtained from all participants. All experiments were performed in accordance with relevant guidelines and regulations.

Placental perfusion and sample collection. Dual *ex-vivo* perfusion of isolated human placental cotyledons from normal (n = 5) and PE (n = 5) pregnancies was performed as previously described[47]. Briefly, the perfusion experiment consisted of one equilibration phase of 30 minutes, from which the maternal perfusate was discarded. After equilibration, the maternal circuit was closed and the placenta was perfused for 3 hours. Only perfusions with a foetal return rate of >80% were included. The perfusate was collected from the maternal side at the end of the 3 hours perfusion and used for isolation and analysis of STBEVs. The placental perfusion method was chosen for collecting normal and PE STBEVs in this study, due to the large vesicle yield that is available using this method, and the possibility to study STBEVs from syncytiotrophoblasts in PE placentas[48].

Isolation of STBEVs. The STBEV isolation was performed using sequential centrifugation steps. After perfusion, the maternal perfusate was centrifuged at 600 × g or 1500 × g to remove red blood cells, and the supernatant was frozen at −80 °C until further analysis. The supernatant was centrifuged for 30 minutes at 3,500 × g and 4 °C to remove cellular debris. The 3,500 × g supernatant was further ultra-centrifuged for 3 hours at 110,000 × g at 4 °C, and the pelleted material used for further study and referred to as the STBEVs. The STBEV protein concentrations were determined using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, USA), or using the Pierce™ BCA Protein Assay (Thermo Scientific, Rockford, USA). The STBEVs were re-suspended in 1xPhosphate Buffered Saline (PBS), and stored at −80 °C.

STBEV characterisation using Nanoparticle Tracking Analysis. For quantification and determination of size distribution, the STBEVs were analysed using NTA, with the NanoSight LM10 (Nanosight Ltd., Amesbury, UK) equipped with 635 nm laser. The STBEV preparations from normal (n = 5) and PE (n = 5)

placentas, isolated by ultra centrifugation, were immediately diluted 1:1000 in 1xPBS and frozen at −80 °C until analysis. The diluted samples was introduced into the sample chamber at room temperature (22.7 ± 0.23 °C) using 1 mL syringes, and the chamber was washed between samples with PBS. For each sample, a 30-second video was recorded, using a camera gain of 400. The video was analysed using the NTA 2.3 software (NTA 2.3 build 0356 from Nanosight Ltd). Camera level was set to 14 and detection threshold set to 10. Automatic settings were used for all other parameters.

STBEV characterisation using Western Blot. For identification of the STBEV surface marker placental alkaline phosphatase (PLAP), western blotting was carried out on STBEV lysates. Briefly, 10ug/well per sample of total protein from normal STBEVs (n = 2) and PE STBEVs (n = 2) were separated on 4–20% bis-tris gradient gel (Bio-Rad, Hercules, CA, USA), using molecular weight standard (Precision Plus All Blue, Bio-Rad). Separated proteins were transferred onto PVDF membranes, and nonspecific binding blocked with 5% Blotting-Grade Blocker (Nonfat Dry Milk, Bio-Rad) in PBS-Tween. The membranes were incubated with the primary antibody NDOG2 against PLAP (made in-house and provided by Prof Sargent) overnight at 4 °C. Membranes were washed prior to incubation with the secondary antibody Alexa Fluor 647 goat anti-mouse IgG (Life Technologies, Carlsbad, CA, USA), washed again and thereafter the bands were detected in a ChemiDoc XRS unit (Bio-Rad).

Cell culture. Human coronary artery endothelial cells (HCAECs, Lonza Walkersville, Inc., MD, USA) were cultured in endothelial cell basal medium-2 supplemented with human epidermal growth factor (hEGF), hydrocortisone, human recombinant fibroblast growth factor-beta (hFGF-b), vascular endothelial growth factor (VEGF), insulin-like growth factor (R3-IGF-1), ascorbic acid, fetal bovine serum (FBS) and gentamicin/amphotericin-B (GA). The cells were cultured in a humidified incubator with a gas supply of air and 5% CO_2 at 37 °C.

STBEV uptake in primary human coronary artery endothelial cells. *Fluorescent labelling of STBEVs.* Using the PKH67 Fluorescent Cell Linker Kit (Sigma-Aldrich, St. Louis, MO, USA), the STBEV membranes were labelled with PKH67 dye according to manufacturer's instructions. Briefly, the STBEV sample was mixed with diluent C and dye for 5–10 minutes, and the reaction stopped by adding FBS. For the initial time course assessment, using conventional fluorescence microscopy, the dyed STBEVs were recovered by centrifuging the solution for 30 minutes at 20,000 × g, the pellet washed once with 1xPBS and repeating the centrifugation step for an additional 30 minutes. The final pellet was resuspended in 1xPBS. For confocal microscopy and flow cytometry analysis, the dyed STBEVs were washed and recovered by centrifuging for 3 hours at 100,000 × g twice before resuspension in 1xPBS.

Time course assessment using fluorescence microscopy. The HCAECs were plated on gelatine (1%) coated cover slips and incubated overnight. The following day, the medium was changed and PKH-labelled STBEVs (20ug/mL) added to the cultured cells, or vehicle only for controls (no STBEV treatment). The experiment was ended at specific time points; 15 minutes, 30, 45, 60, 90 and 120 minutes, and 6 or 24 hours. The media was removed and 2 mL Hoechst 33342 (1 μg/mL, Life Technologies), nuclear stain diluted in 1xPBS, was added for 5 minutes. The cells were washed with 1xPBS before adding 2 mL 4% paraformaldehyde (PFA) (HistoLab Products AB, Gothenburg, Sweden) for 10 minutes. The cells were once again washed with 1xPBS, the cover slips removed, tapped dry and mounted on to a microscope slide using Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich). The cells were visualized using a Zeiss Axiostar plus microscope at 100x and 400x magnification.

Time course assessment using flow cytometry. The HCAECs were plated out in 24 well plates overnight, and the following day, treated with PKH-labelled normal (n = 3) or PE (n = 3) STBEVs (5ug/mL) in serum free medium for 30 minutes, 2 hours or 4 hours. All samples were analysed in duplicates or triplicates. Cells were detached by trypsinization and washed and resuspended in PBS before cell-associated fluorescence was measured using an Accuri C6 Flow cytometer and analysed using Accuri C6 software (BD Biosciences).

Confocal microscopy. To confirm the uptake and internalization of STBEVs into HCAECs, live cell confocal laser scanning microscopy was performed following treatment with normal or PE STBEVs. Cells were grown in glass bottom chamber slides, and 10–20 μg/ml of PKH-labeled STBEVs added to subconfluent cells in phenol red-free and serum-free conditions and incubated for 1 hour. Surface-bound STBEVs were removed by extensive washing with 1 M NaCl and serum-free medium, followed by live cell imaging of intracellular STBEV in phenol-red free medium. The acquisition of images was performed using Zeiss LSM 710 confocal scanning microscope equipped with excitation laser wavelengths of 405, 488, and 633 nm, and a C-Apochromat 63X/1.20 W korr M27 water or Plan-Apochromat 63X/1.40 DIC M27 oil immersion objective. Images were acquired using Zen 2011 software (Carl Zeiss). For live imaging, cells were transferred to a humidified 5% CO2, 21% O2 atmosphere incubator integrated with the confocal scanning equipment. Images were collected during a time series of approximately 10 min with 10 s cycle time.

STBEV transfer of miRNAs to primary human coronary artery endothelial cells. *Cell treatment.* The HCAECs were seeded into 6 well plates at a concentration of 0.2×10^6 cells per well. After reaching 80% confluency, media was changed and normal (n = 5) or PE (n = 5) STBEVs added (20ug/mL). After 6 hours incubation, medium was removed and the cells washed with 1xPBS before addition of lysis buffer. The lysate was transferred to eppendorf tubes and frozen at −80 °C. As controls (n = 6), PBS or no vehicle/treatment were used.

RNA extraction and quality assessment. Total RNA was extracted using mirVana™ miRNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instruction. RNA quality and miRNA

content was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) using the Nano Assay to evaluate RNA integrity number (RIN) value and total RNA quality, as well as the Small RNA Assay to investigate miRNA percentage and quality. A RIN value of 10 corresponds to intact RNA, and 1 to totally degraded RNA. The analysis confirmed the presence of high quality miRNA and RNA, which showed RIN values above 8.3 in all samples.

Profiling of placental miRNAs using a custom made miRNA array. To investigate the transfer of miRNAs from placental STBEVs, we designed a custom miRNA TaqMan® miRNA array card (Applied Biosystems). See Supplementary information Table S2, for a complete list of mature miRNA sequences from chromosome 19 miRNA cluster (C19MC). The cluster gives rise to 52 individual mature miRNAs accoding to the miRBase.org database. The 371–373 cluster gives rise to 8 individual mature miRNAs. Included on the array were also four well known miRNAs, which are widely expressed and relevant in placenta research; hsa-miR-141*, hsa-miR-210, hsa-miR-16[49] and hsa-miR-222[50].

Using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), total RNA was transcribed to cDNA according to manufacturer's instructions. The cDNA was mixed with TaqMan® Universal PCR Master Mix before addition to the array cards. The custom miRNA TaqMan® miRNA array cards were of format 64, which included 63 target assays (described above) and one endogenous control (U6 snRNA). Each card analysed two unique samples. qPCR was performed using standard protocol supplied by manufacturer for TaqMan® Array Micro Fluidic Cards on a QuantStudio 7 Flex (Applied Biosystems). Data were normalised to the endogenous control U6 snRNA. Using the Thermo Fisher Cloud software (Thermo Fisher Scientific, 81 Wyman Street Waltham, MA USA 02451), the Comparative C_T Method ($\Delta\Delta$ C_T Method, Applied Biosystems) was used, according to manufacturer's instructions, to calculate fold differences relative to samples treated with normal STBEVs.

Real time quantitative PCR for miRNA analysis. Following miRNA array analysis, four miRNAs were selected based on changes in expression; mir-517c, mir-517a, mir-519a and mir-210. These specific miRNAs were verified and analysed with RTqPCR in cells treated with normal or PE STBEVs.

Total RNA was extracted using the mirVana™ miRNA Isolation Kit, as described above. Ten ng RNA was transcribed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. The following pre-designed TaqMan® MicroRNA assays (Applied Biosystems) were analysed: homo sapiens-microRNA-517c (hsa-mir-517c), hsa-mir-517a, hsa-mir-519a, hsa-mir-210 and U6 snRNA (sequences according to Supplementary information S2).

qPCR was performed using the standard protocol supplied by the manufacturer for TaqMan® MicroRNA Assays on an ABI PRISM 7000 sequence detection system (Applied Biosystems). Primers and probes were as described above. Each reaction was run in duplicate. Negative controls with no template as well as no reverse transcriptase controls were included for every miRNA primer pair. Data were normalized to U6 snRNA. Using the $\Delta\Delta C_T$ method described by Livak *et al.*[51], fold differences were calculated relative to samples treated with normal STBEVs for the C19MC miRNAs, since these miRNAs were not detected in control samples. However, for mir-210, fold differences were calculated relative to control cells, i.e. untreated cells, for mir-210. A p-value < 0.05 was considered statistically significant. A fold change of >1.5 was defined as an increased miRNA content, while a fold change <1.5 was defined as a decrease.

Predicted miRNA target genes. *Rationale.* Current understanding of miRNA binding to target genes suggest that the 5′ end of the miRNA, via the seed sequence of 7–8 nucleotides is important and is conserved between species. Positions 1–10 are important for binding to Agonaute (Ago) in the RNA induced silencing complex (RISC) and bases 2–6 interact with mRNA. Binding to Ago requires a helical confirmation involving energy considerations. Another consideration is how much 'imperfection' in base-pairing can be tolerated in miRNA-mRNA interactions given that miRNA can bind to multiple places on one mRNA, and many different miRNA can bind to one mRNA. The miRNA binding is considered to take place on the mRNA 3′ end but the coding region is also possible. Most computational prediction tools for miRNA target genes are considered to provide 30% precision[52–54]. All predicted targets need validation. Taking these considerations into account, the strategy used in this study was based on using predication models that focused on seed sequence stringent pairing, thermodynamic considerations, conserved seed sequences and where possible experimentally validated miRNA-mRNA interactions. Thus, the prediction programs chosen used multiple criteria. Unfortunately C19MC miRNA are primate-specific and therefore conservation criteria are limited, although TargetScan has *Pan troglodytes* data. Target genes, if retrieved by several different computational prediction tools, were selected for further analysis via literature searching for interactions in the placenta or cardiovascular function.

Prediction algorithms. Using the prediction algorithms DIANA-microT-CDS (diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index)[55], TargetScan (v7.0; targetscan.org)[56] and miRmap (mirmap.ezlab.org)[57], we identified potential miRNA target genes for the C19MC miRNAs mir-517a, mir-517c and mir-519a. By combining the gene lists from the three prediction algorithms, we found 33 genes for mir-517a and mir-517c as well as 872 genes for mir-519a. Using TarBase (v7.0; diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index)[58], experimentally supported miRNA targets were identified. Tarbase provided one (1) experimentally supported gene for mir-517a, no genes for mir-517c and four (4) genes for mir-519a. The literature was searched for possible gene functions. The following genes: FLT1, TGFBR2, PDGFD, ESR1, VLDLR and PDCD1LG2 (Fig. 5), were selected for further analysis based on their algorithm prediction scores as well as being known to be involved in placental and/or cardiovascular function as well as PE. The gene expression levels were examined using RTqPCR. All genes, except FLT1, were predicted target genes by all three prediction

databases. FLT1 was chosen due to its significance in PE research as well as being a validated target for mir-517a/b and mir-517c[59].

Real time quantitative PCR for target gene analysis. The HCAECs were, as described above, treated with normal or PE STBEVs. Total RNA was extracted, and transcribed using TaqMan® Reverse Transcription Reagents according to manufacturer's instructions (Applied Biosystems). The following pre-designed qPCR assays (Applied Biosystems) were analysed: FLT1, TGFBR2, PDGFD, ESR1, VLDLR, PDCD1LG2, HMOX1, and GAPDH. RTqPCR was performed as described above.

Transmission Electron Microscopy. *STBEV characterisation.* The STBEVs were visualized and identified using TEM, as previously described[60]. Briefly, TEM was performed using the NDOG2 antibody against PLAP (made in-house and provided by Prof Sargent), the microRNA Assay primer hsa-mir-517c (Applied Biosystems Inc., Foster City, CA, USA) and an antibody against the human foetal Hb (HbF) protein. All antibodies and primers were labelled with colloidal gold (BBI International) of different sizes; NDOG2 (anti-PLAP, 20 nm), mir-517c primer (5 nm) and anti-HbF antibody (5 nm). The samples were processed for negative staining and sections were examined with a transmission electron microscope (CM100 Twin, Philips, Eindhoven, Holland) operated at a 60 kV accelerating voltage. The images were recorded with a side-mounted Olympus Veleta camera (Olympus, Münster, Germany).

Cell incubation with foetal haemoglobin. To evaluate the effect of free HbF on STBEV uptake and miRNA transfer, HbF (0.7 mg/mL) was added to the cultured cells, with or without STBEVs. HbF was prepared from human cord blood and diluted in 15 mM Tris-HCl pH 8.0, 20 mM NaCl according to a previously published protocol[14].

Cell analysis. For cell analysis, the HCAECs were incubated for 6 hours with normal or PE STBEVs, or normal STBEVs with HbF. Cells treated with 1xPBS only were used as controls. The TEM procedure was as described above. For a quantitative evaluation, 100 cellular profiles ($n = 100$) were analysed in each case from random distributions on different locations.

Statistical analysis. Statistical analysis was performed using Origin 9 software (Microcal, Northampton, MA, USA). The non-parametric Mann-Whitney *U*-test was used and a p-value < 0.05 considered statistically significant.

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Acknowledgements

The authors would like to thank Irene Larsson, Maria Baumgarten, Eva Lindqvist and Karin Mattsson for excellent technical assistance. We also thank Lena Erlandsson for proof-reading the manuscript.

Author Contributions

T.C., M.F., D.T., M.M., M.B., I.S. and S.R.H. conceived the experiment(s). T.C., M.F., D.T., M.M. and M.B. conducted the experiment(s). T.C., M.F. D.T., M.M. M.B., I.S. and S.R.H. analysed the results. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-04468-0

Competing Interests: T.C., M.F., D.T., M.M., M.B and I.S. declares no potential conflict of interest. S.R.H. holds patents for the diagnosis and treatment of preeclampsia. S.R.H. is also a co-founder of the companies Preelumina Diagnostics AB and A1M Pharma AB (www.a1m.se).

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Paper IV

Placental syncytiotrophoblast extracellular vesicles enter primary endothelial cells through clathrin-mediated endocytosis

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Abstract

Preeclampsia is a pregnancy associated syndrome characterized by hypertension and organ dysfunction and develops in two stages. During stage one, the placenta is inadequately implanted and subjected to oxidative stress, releasing different factors into the maternal circulation. These factors cause systemic endothelial damage and maternal symptoms in stage two. Low dose aspirin can reduce the risk of developing. During preeclampsia the placenta releases increased amounts of syncytiotrophoblast extracellular vesicles (STBEVs), which have been linked to endothelial dysfunction. In the present study we analysed STBEV uptake by primary endothelial cells, how STBEVs affect endothelial cell gene expression and cell activation as well as the effect aspirin. The STBEVs were derived from normal or preeclamptic placental perfusions. The STBEV uptake was analysed with flow cytometry. Uptake was blocked using potential inhibitors; Cytochalasin D, Chlorpromazine hydrochloride, Methyl-B-cyclodextrin, Dynasore and Wortmannin. Surface expression of ICAM-1 was evaluated after STBEV exposure, with and without aspirin treatment. Endothelial cell biology related genes were analysed using a qPCR array. Chlorpromazine, Dynasore and Wortmannin almost completely blocked STBEV uptake. Methyl-B-cyclodextrin blocked uptake by 45-60%. Cytochalasin D did not block uptake. Neither normal nor preeclamptic STBEVs had significant effects on endothelial gene expression. Normal STBEVs down-regulated cell surface ICAM-1, with and without aspirin co-treatment. Aspirin had no effect on STBEV uptake or cellular gene expression, however it down-regulated ICAM-1 when co-treated with preeclamptic STBEVs. **N**ormal and preeclamptic STBEVs uptake was blocked in similar ways. Uptake occured primarily through clathrin-mediated endocytosis. Neither of the STBEVs had any significant effects on endothelial cell gene expression. The effects of STBEVs appear to be primarily on activation by regulating ICAM-1 expression, rather than on gene expression. The preventive effects of aspirin may be achieved by dampening the activation of target endothelial cells.

Introduction

Preeclampsia (PE) is a pregnancy associated syndrome, diagnosed by new-onset hypertension (>140 mmHg systolic or >90 mmHg diastolic) after 20 weeks of gestation in combination with proteinuria or systemic organ dysfunction such as thrombocytopenia, impaired liver function, renal insufficiency, pulmonary oedema or neurological disturbances. The only cure is to induce delivery, and thereby the removal of the placenta (1, 2). Today, the best way to predict women who are at high risk of developing preeclampsia is by identifying certain clinical risk factors, such as previous PE, family history of PE, multiple pregnancies, diabetes, chronic hypertension and kidney disease (3). Several predictive biomarkers have been investigated, particularly the antiangiogenic factors soluble Flt1 (s-Flt1) and placental growth factor (PlGF) (4). It is well established that low dose acetylsalicylic acid (ASA, or aspirin) is risk reducing and prevents early onset PE if given prophylactically to high risk patients (5-7). The aetiology is still not fully understood but PE develops in two stages, originating in a placenta affected by oxidative stress. In the first stage, during early placentation, the placenta is under-perfused, which causes oxidative stress and damage to the syncytiotrophoblast (STB) cells. The oxidative stress causes release of STB debris, microvesicles and anti-angiogenic factors such as sFlt-1 and soluble endoglin, into the maternal circulation. In the second stage, the released factors causes general endothelial damage in the maternal circulation, which leads to organ dysfunction and the maternal manifestations of hypertension and proteinuria (2, 4, 8-10).

Extracellular vesicles (EVs) are membrane vesicles released by all cells studied to date. The EVs have been shown to transfer functional mRNA and micro-RNA (miRNA) to recipient cells, and thereby regulate gene expression. Generally, EVs are divided into two subgroups; the larger microvesicles (100nm-1µm in diameter) that are blebbed off the cell membrane, and the smaller exosomes (30-100nm) that are derived from multivesicular bodies and released by exocytosis (11). During pregnancy, the placental STB cells, continuously release EVs named syncytiotrophoblast extracellular vesicles (STBEVs), into the maternal circulation. Normal STBEVs are believed to adapt the maternal immune system to the on-going pregnancy (12-14). Significantly higher levels of STBEVs are found in plasma from women with established PE compared to normal pregnancies (15). When comparing normal and PE STBEVs, the PE STBEVs show different characteristics, such as an increased

expression of tissue factor (16), phosphatidylserine (17), sFlt-1 (18) and sialic acid (19). Women with PE have increased arterial stiffness, which is an indicator of endothelial dysfunction, that correlates with PE severity (20). Women with PE also have an increased risk of developing cardiovascular disease later in life, possibly due to the endothelial and vascular dysfunction (21, 22).

Already in 1996, one of the first studies was published that indicated that STBEVs have effects on ECs by suppressing their growth (23). Both normal and PE vesicles, from placentas as well as from cell culture media and sera, increased cell expression of ICAM, VCAM and selectins (24-26). One study showed that the EC activation and ICAM up-regulation by PE placental vesicles was due to the vesicle bound sFlt-1 (18). In a recent study by our group, we showed internalisation of STBEVs by primary ECs (27). The STBEVs carried placenta specific miRNAs, which were transferred to different compartments within the cells, and altered the gene expression of several target genes. The PE STBEVs were, in a higher degree, transported in a to the ER, and the normal STBEVs to the mitochondria. Furthermore, the PE STBEVs caused extensive cell membrane ruffling, further supporting the idea that the STBEVs play a role in causing endothelial damage and potentially also a permanent reprogramming of the cells (27). These results lead to the hypothesis that normal and PE STBEVs might have different uptake routes.

Many different pathways for cellular uptake have been suggested for EVs, such as different types of endocytosis, phagocytosis and macropinocytosis (11). Uptake of placental vesicles have been suggested to occur by internalization by the ECs by either phagocytosis and/or endocytosis (28). In the present study we focused primarily on endocytosis, which can roughly be divided into two pathways; I) clathrin-mediated (CME), and II) clathrin-independent (CIE) endocytosis. After uptake, endosomes transfer the endocytosed material to either lysosomes for degradation or to compartments such as Golgi and ER (29). In CME, clathrin is recruited from the cytoplasm to the plasma membrane, clathrin coated pits are formed into clathrin coated vesicles, that are finally pinched off in a dynamin-dependent manner (29, 30). The CIE is largely dependent on lipid rafts for uptake. One CIE pathway is macropinocytosis, where the plasma membrane form ruffled regions to take up extracellular material. Cholesterol is needed to create these ruffles (30). Even though cholesterol is mainly

suggested in CIE, in CME the absence of cholesterol can lead to flattening of clathrin coated pits, and thereby to decreased activity of the CME (30).

In this paper the aim was to examine different mechanisms involved in the uptake of STBEVs by primary ECs, and to further analyse the effect of STBEVs on EC gene expression and activation. To elucidate whether there are differences in uptake mechanisms between normal and PE STBEVs, we added specific uptake inhibitors to the primary ECs, before adding the STBEVs. This study also investigated whether the PE preventive drug ASA had any effects on STBEV uptake, cellular activation and gene expression.

Materials and methods

Ethics statement

The perfusion studies were approved by the Oxfordshire Research Ethics Committee C at Oxford University, UK, and informed written consent was obtained from all participants. All experiments were performed in accordance with relevant guidelines and regulations.

Placenta perfusion

The STBEVs were derived from perfused placentas. Dual *ex-vivo* perfusion of isolated human placental cotyledons from normal (n=5) and PE (n=5) pregnancies was performed as previously described (31). Briefly, the perfusion experiment consisted of one equilibration phase of 30 minutes, from which the maternal perfusate was discarded. After equilibration, the maternal circuit was closed, and the placenta was perfused for 3 hours. Only perfusions with a foetal return rate of >80% were included. The perfusate was collected from the maternal side at the end of the 3 hours perfusion and used for isolation and analysis of STBEVs.

STBEV isolation

The STBEV isolation was performed using sequential centrifugation steps. After perfusion, the maternal perfusate was centrifuged at 1,500 xg to remove red blood cells, and the supernatant was frozen at -80^{o}C until further analysis, as previously described (27). The supernatant was centrifuged for 20 minutes at 3,500xg at 4^{o}C to remove cellular debris. The 3,500xg supernatant was further ultra-centrifuged for 3 hours at 110,000xg at 4^{o}C, and the pelleted material used for further study and referred to as the STBEVs. The STBEVs were re-suspended in 1xPhosphate Buffered Saline (PBS), and stored at -80^{o}C until further analysis.

Fluorescence labelling of STBEVs

The STBEVs were labelled fluorescently before being added to cell cultures and cellular uptake analysed by flow cytometry. Using the PKH67 Fluorescent Cell Linker Kit (Sigma-Aldrich, St. Louis, MO, USA), the STBEV membranes were labelled with PKH67 dye according to manufacturer's instructions. Briefly, the STBEV sample was mixed with diluent C and dye for 5 minutes, and the reaction stopped by adding foetal bovine serum (FBS). The

dyed STBEVs were washed and recovered by centrifuging for 2 hours at 100,000xg twice before resuspension in 1xPBS and stored at -20^{o}C until uptake experiments were performed. The STBEV size distributions were analysed before and after PKH-labelling, using Nanoparticle Tracking Analysis (NTA, described below). The STBEV protein concentrations were determined using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, USA), performed after PKH-labelling.

STBEV characterisation with Nanoparticle Tracking Analysis (NTA)

For determination of STBEV size distribution, NTA was performed using a NanoSight LM10 (Malvern Instruments, Malvern, UK) equipped with a 488nm laser. The samples analysed were STBEV preparations from normal (n=5) and PE (n=5) placentas, before and after PKH-labelling, and diluted in 1xPBS. Measurements were performed in 90 seconds triplicates using manual gain, camera level set to 10 or 11 and detection threshold set to 3. The obtained files were analysed using the NTA 3.2.16 software (Malvern Instruments).

Cell culture

Primary HCAECs (Lonza Walkersville, Inc., MD, USA) were cultured in endothelial cell basal medium-2 (EGM-2MV) that was supplemented with human epidermal growth factor (hEGF), hydrocortisone, human recombinant fibroblast growth factor-beta (hFGF-b), vascular endothelial growth factor (VEGF), insulin-like growth factor (R3-IGF-1), ascorbic acid, FBS and gentamicin/amphotericin-B. The cells were cultured in a humidified incubator with a gas supply of air and 5% CO_2 at 37^{o}C.

Inhibition of STBEV uptake in primary human coronary artery endothelial cells

To analyse the cellular uptake of STBEVs by HCAECs, the STBEVs were fluorescently labelled, as described above, before being added to cell cultures. The HCAECs were plated in 24 well plates overnight, at a cell density of 0.1 x 10^6 cells per well. The next day, the cells were subjected to five different inhibitors as well as ASA for 30 minutes, before addition of fluorescently labelled STBEVs (10µg/mL) followed by an incubation for 2 hours at 37^{o}C. All samples were analysed in triplicates. After STBEV incubation, the plates were put on ice and

the wells were washed with 1xPBS. Cells were detached by trypsinization and thereafter washed and resuspended in 2% formaldehyde (FA). Cell-associated fluorescence was measured using an Accuri C6 Flow cytometer and analysed using Accuri C6 software (BD Biosciences).

Five potential pathway inhibitors were used, all purchased from Sigma. A vital part of endocytosis is actin polymerisation (11), and Cytochalasin D is a compound that blocks this event. Cytochalasin D was dissolved in dimethyl sulfoxide (DMSO). Chlorpromazine hydrochloride blocks clathrin and primarily inhibits CME (11). Chlorpromazine hydrochloride was dissolved in H_2O. To evaluate any involvement of lipid rafts in cellular uptake, Methyl-B-cyclodextrin (MβCD) can be used to extract cholesterol from the plasma membrane. Cholesterol is an important part of lipid rafts which in turn is central to the CIE pathways, including macropinocytosis (11, 30). The MβCD was dissolved in EGM-2MV. Dynasore is a compound that blocks dynamin (11), this was dissolved in DMSO. Wortmannin is a compound that blocks phosphatidylinositol 3-kinase (PI3K) and Rab5, that both are connected to early endosome fusion, and is primarily considered an inhibitor of phagocytosis (11) and macropinocytosis (30, 32). Wortmannin was dissolved in DMSO. In addition to the inhibitors, we also tested whether the clinically relevant drug Acetylsalicylic acid (ASA, Sigma) had any effect on the cellular uptake. ASA was dissolved in H_2O.

In a first set of experiments, we evaluated different concentrations of the inhibitors (specified in Fig 2) for blocking uptake of normal STBEVs. The tested concentrations were based on results from previous studies (33-38). After evaluation, we decided on the following final concentrations; Chlorpromazine 10µg/mL, Dynasore 80µM, Cytochalasin D 20µM, MβCD 5mM, Wortmannin 1µM and ASA 20µg/mL. To evaluate the diluent's effect on cells and STBEV uptake, DMSO was added as a control for Cytochalasin D, Dynasore and Wortmannin inhibition, in an equivalent concentration (0.5% of cell culture volume).

In a second setup, we compared the potential differences in uptake pathways between normal (n=5) and PE (n=5) STBEVs, using inhibitors and ASA at concentrations described above. To evaluate the energy requiring process of STBEV uptake, cells were pre-treated in either 37^{o}C or 4^{o}C for 30 minutes before addition of fluorescently labelled STBEVs (10µg/mL) and thereafter incubated for another 2 hours at 37^{o}C.

ICAM-1 expression on the cell surface after STBEV treatment

To evaluate whether the STBEVs could activate HCAECs, the level of ICAM-1 expression on the cell surface was analysed. The cells were treated with normal (n=3) or PE (n=3) STBEVs for 24 hours. To determine the effect of ASA on cell activation, the cells were co-treated with STBEVs and ASA. As a positive control for ICAM-1 activation, TNFα (1ng/mL) was used. The recombinant Human TNFα (R&D systems) was diluted in 1xPBS with 0.1% BSA, according to manufacturer's instructions. The cells were plated in 24 well plates overnight, at a cell density of 0.1×10^6 cells per well. The next day, the cells were subjected to STBEVs (10µg/mL), ASA (20µg/mL) or a combination, and incubated for 24 hours. All normal and PE STBEV samples were analysed in triplicates. After incubation, the cells were washed and detached as described above. The cells were then incubated with a specific antibody against ICAM-1 (PE-Cy™5 Mouse Anti-Human CD54 (Clone HA58), BD Pharmingen), washed and finally resuspended in 1xPBS before fluorescence was measured using an Accuri C6 Flow Cytometer and analysed using Accuri C6 software (BD Biosciences).

STBEV effect on gene expression

To determine the effect of STBEVs on gene expression, HCAECs were exposed to normal or PE STBEVs for 24 hours, with and without ASA, as described above. After 24hours incubation, the cells were trypsinized, washed, centrifuged and the cell pellet frozen at -80^{o}C until further analysis.

RNA extraction and quality assessment

RNA was extracted from cell pellets using the Qiagen RNeasy Mini Kit (Qiagen) according to manufacturer's instructions, and RNA concentration determined using a NanoDrop Spectrophotometer ND-1000. RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) to evaluate RNA integrity number (RIN) value and total RNA quality. The analysis confirmed the presence of high-quality total RNA, with RIN values above 8 in all samples.

Profiling of endothelial cell biology gene expression

To investigate the effects of STBEVs on endothelial cell specific genes, the RT² Profiler™ PCR Array Human Endothelial Cell Biology from Qiagen was used, analysing a total of 96 genes (S1 table). The samples were divided into six groups; untreated cells (control group), ASA treated cells, cells exposed to normal or PE STBEVs and cells co-treated with a combination of either normal or PE STBEVs with ASA. The RNA samples from each group (n=6 for all) were pooled and cDNA synthesis was performed using the RT2 first strand kit according to manufacturer's instructions. The cDNA was used on the real-time RT² Profiler PCR Array (QIAGEN, Cat. no. PAHS-015Z) in combination with RT² SYBR® Green qPCR Mastermix.

Each catalogued RT² Profiler PCR Array contains pathway-focused genes as well as five housekeeping (reference) genes. In addition, each array contains a panel of proprietary controls to monitor genomic DNA contamination (GDC), first strand synthesis (RTC) and real-time PCR efficiency (PPC). Generated C_T values were exported to an Excel file that was uploaded to the data analysis web portal at http://www.qiagen.com/geneglobe. Samples were assigned to controls and test groups. The C_T values were normalized, and fold change/regulation calculated using the delta delta C_T method. Fold Change is then calculated using the 2^ (-delta delta C_T) formula. The C_T cut-off was set to 35.

RTqPCR validation

From the results obtained by the PCR array, seven genes were selected for validation: Intercellular adhesion molecule 1 (ICAM1), Selectin E (SELE), Endothelin receptor type A (EDNRA), Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 (ACE), Calcitonin-related polypeptide alpha (CALCA), Natriuretic peptide B (NPPB) and Fibroblast growth factor 1 (acidic) (FGF1). Individual cDNA synthesis was performed for each sample (n=6 in each group) using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Life Technologies Corporation, Carlsbad, USA) according to manufacturer's instructions. The following pre-designed RT2 qPCR Primer Assays (Qiagen) were used; ICAM-1, SELE, EDNRA, ACE, CALCA, NPPB, FGF1 and RPLP0.

The qPCR was performed using the standard protocol supplied by the manufacturer Qiagen's RT2 qPCR Primer Assays on CFX Connect Real-Time System (Bio-Rad). Each reaction was run in duplicate. Negative controls as well as RTC were included for each assay. Data were normalized to RPLP0. Using the $\Delta\Delta C_T$ method described by Livak et al (39), fold changes were calculated relative to untreated samples. A fold change of >1.5 was defined as an increased mRNA expression, while a fold change <1.5 was defined as a decrease.

Statistical analysis

Statistical analysis was performed using Origin 9 software (Microcal, Northampton, MA, USA). The non-parametric Mann-Whitney *U*-test was used and a p-value <0.05 was considered statistically significant.

Results

STBEV characterization with Nanoparticle Tracking Analysis

To determine the size distributions of normal and PE STBEVs, before and after PKH-labelling, we used the NTA methodology. Mean vesicle sizes for normal and PE STBEVs were 186 ± 22 nm and 185 ± 6 nm respectively, before PKH-labelling, and 176 ± 15 nm and 180 ± 17 nm after PKH-labelling. No significant differences were observed between normal and PE STBEVs or when comparing STBEVs before and after PKH-labelling (data not shown).

STBEV uptake and inhibition quantified by flow cytometry

The uptake of PKH-labelled STBEVs by primary HCAECs was quantified using flow cytometry. Initially, concentrations of normal STBEVs ranging from 2.5µg/mL to 20µg/mL were tested, and the uptake increased with increased vesicle concentration (Fig 1). The concentration 10µg/mL was chosen for further analysis.

Figure 1. Flow cytometry analysis showing increased STBEV uptake with increased exposure concentration. The horizontal axis shows mean fluorescent values; a.u., arbitrary units.

In order to find the optimal concentration of inhibitor to use for the experiments, to assure as strong inhibition as possible but with minimal cell death, different concentrations of inhibitors were evaluated (Fig 2).

Figure 2. Flow cytometry analysis showing uptake of normal STBEVs after exposure to different concentrations of inhibitors. MβCD (a), Cytochalasin D (b), Chlorpromazine (c), Dynasore (d) and Wortmannin (e) all showed increased inhibition with increased concentration. ASA (f) showed no effect on STBEV uptake. DMSO (g) which Cytochalasin D, Dynasore and Wortmannin were diluted in, showed a surprisingly strong inhibition of

STBEV uptake and was used as a control in all following experiments. The horizontal axis shows % of internalized STBEVs, with the value for normal or PE STBEVs set as 100%.

The inhibitor MβCD (Fig 2a) showed an increased inhibition of normal STBEV uptake with increased MβCD concentration, although a complete inhibition was not achieved due to signs of cell death already at 10mM. At 20mM there was extensive cell death. An optimal concentration of 5mM MβCD was chosen. Cytochalasin D (Fig 2b) also showed increased inhibition with increased concentration. A complete inhibition was not achieved due to cell death occurring at 50µM therefore 20µM Cytochalasin D was chosen for further analysis. Cytochalasin D was diluted in DMSO and a 20µM solution corresponded to 0.5% DMSO in cell cultures, which was tolerated by the cells. For Chlorpromazine (Fig 2c) 10µg/mL was chosen, which resulted in an almost complete (91%) inhibition of STBEV uptake. Dynasore (Fig 2d) was diluted in DMSO which limited the highest concentration to 160µM to avoid DMSO levels >1% in the cell cultures. A concentration of 80µM was chosen for Dynasore since the inhibition was comparable to the inhibition at 160µM. Wortmannin (Fig 2e), diluted in DMSO, showed an increased inhibition with increased concentration. A strong inhibition was seen already at 0.2µM. At 1µM the inhibition was nearly complete and stable, and the DMSO concentration 0.5% in cell cultures. There was no effect by ASA (Fig 2f) on STBEV uptake, and for further analysis 20µg/mL was chosen due to its clinical significance, corresponding to the recommended 75mg ASA for prophylactic treatment in high-risk pregnancies. As three of the inhibitors were diluted in DMSO, the effect of DMSO (Fig 2g) on STBEV uptake was also evaluated. Interestingly, DMSO blocked up to 50% of STBEV uptake but did not lead to increased cell death at 0.5% concentration. Therefore, all analysis with Cytochalasin D, Dynasore or Wortmannin treatments were performed in parallel with DMSO-only treated cells.

Next, any potential differences in inhibition of normal and PE STBEVs uptake were investigated (Fig 3). Both normal and PE STBEV uptake were completely blocked by keeping the cells at 4^{o}C (Fig 3a). As mentioned before, as a control to Cytochalasin D, Dynasore and Wortmannin, cells were pre-treated with DMSO-only at equivalent volumes (0.5% of final well volume) used for the uptake inhibitors. DMSO inhibited normal STBEV uptake by 32%

(p=0.01208) and PE STBEVs by 26% (p=0.01208), with no difference between the groups (shown in Figs 3c, e and f).

Figure 3. The difference in inhibitory effect on normal and PE STBEV uptake, was evaluated using flow cytometry. Pre-treating the cells at 4^{o}C (a) inhibited all STBEV uptake. The inhibitor MβCD (b) blocked uptake of both normal and PE STBEVs. Cytochalasin D (c) initially appeared to inhibit uptake, but when comparing to pre-treating with DMSO before addition of STBEVs, all inhibition was due to the effect of DMSO. Chlorpromazine (d) lead to an almost complete block of STBEV uptake. Dynasore (e) and Wortmannin (f) showed strong inhibitory potential, also when comparing with DMSO. Finally, the clinically important drug ASA (g) showed no effect on STBEV uptake. The horizontal axis shows % of internalized STBEVs, with the value for normal or PE STBEVs set as 100%. * representing significantly inhibited STBEV uptake, after accounting for DMSO effect.

The inhibitor MβCD (5mM) blocked normal STBEVs by 60% (p=0.01208) and PE STBEVs by 45% (p=0.01208), although the difference between normal and PE was not statistically significant (Fig 3b). Initially, Cytochalasin D appeared to inhibit STBEV uptake. However, there was no significant difference between the inhibition caused by Cytochalasin D and by DMSO-only (Fig 3c), and therefore the inhibition could completely be ascribed to the effect of DMSO. Chlorpromazine (10µg/mL), caused an almost complete inhibition of both normal (98%, p=0.01208) and PE (95%, p=0.01208) STBEVs (Fig 3d). When evaluating Dynasore (80µM), DMSO controls were run in parallel. Normal STBEVs were blocked by 86% (p=0.01208) and PE STBEVs by 76%, (p=0.01208) with no statistically significant difference between groups (Fig 3e). There was a significant difference between Dynasore and DMSO-only for both normal (p=0.01208) and PE STBEVs (p=0.02144), demonstrating that Dynasore has an effect in itself on inhibiting STBEV uptake. Wortmannin (1µM) inhibited normal STBEVs by 94% (p=0.01208) and PE STBEVs by 89% (p=0.01208), with no significant difference between the groups (Fig 3f). The difference in inhibitory effect between Wortmannin and its diluent DMSO, was significant for both normal (p=0.01208) and PE (p=0.01208) STBEVs. The effect of ASA (20µg/mL) had no significant effect on uptake for neither normal nor PE STBEVs (Fig 3g).

ICAM-1 expression after STBEV exposure

To evaluate whether the HCAECs could be activated by normal (n=3) or PE (n=3) STBEVs, cells were exposed to vesicles for 24 hours, with or without ASA. The ICAM-1 surface expression was measured using flow cytometry (Fig 4). As a reference for cell activation, TNFα was tested at different concentrations (Fig 4a), showing that the cells could indeed be activated and up-regulate the protein expression of surface bound ICAM-1. Combining TNFα with ASA decreased the ICAM-1 expression by 6%. After co-treating the cells with TNFα and normal or PE STBEVs there was 34% and 19%, respectively, less ICAM-1 expression on the cell surface. Next, the cells were co-treated with normal or PE STBEVs in combination with ASA (Fig 4b). Normal STBEVs +/- ASA down-regulated ICAM-1 surface expression by 25% and 26% respectively, compared to untreated cells. The PE STBEVs increased ICAM-1 surface expression by 6%, but when combined with ASA, the surface expression declined by 10% compared to untreated cells.

Figure 4. The effect on cell activation was evaluated by analysing ICAM-1 surface expression using flow cytometry. TNFα (a) increased ICAM-1 surface expression with little additional effect by ASA. Normal and PE STBEVs decreased the activation caused by TNFα. In another set of experiments (b), treatment with ASA again showed no effect on ICAM-1 expression. Normal STBEVs decreased the ICAM-1 expression by almost 25%, with no additional effect when co-treated with ASA. PE STBEVs slightly increased ICAM-1 levels, which appeared to be counteracted by ASA. The ICAM-1 expression of untreated cells was set as 1. The horizontal axis shows mean fluorescent values; a.u., arbitrary units.

RT^2 array analysing endothelial cell biology gene expression

The STBEV effect on genes related to endothelial cell biology, was evaluated using the RT^2 Profiler PCR Array from Qiagen (96 genes, see S1 table). The cells were incubated with normal or PE STBEVs, with or without ASA, for 24 hours. In total 26 genes expressed a fold change >1,5 in at least one group compared to untreated control cells (S1 table and Fig 5). Treatment with only ASA had no effect on any genes analysed. Both normal and PE STBEVs caused both up- or down-regulation of various genes. Groups of genes that were affected by

the STBEVs, were those related to inflammation (IL11, IL3, CCL2, IL1B, FASLG, PTGS2, CCL5), cell adhesion and activation (SELE, CDH5, ICAM-1), vascular tone (CALCA, ACE, NPPB, EDNRA, NOS3) as well as coagulation (THBD, PTGIS) and cancer (KLK3, KIT). We chose to focus on genes involved in blood pressure regulation, vasoconstriction/dilation, vascular resistance and activation. The following seven genes, marked with an asterisk * in Figure 5, were chosen for RTqPCR validation; ICAM1, SELE, EDNRA, ACE, CALCA, NPPB and FGF1.

Figure 5. Heat map of all up- or downregulated genes (green and red, respectively) analysed with the RT² Profiler PCR Array focusing on genes related to endothelial cell biology. For a complete list of all analysed genes, see S1 table. Genes marked with an asterisk * were chosen for validation with RTqPCR. Underlined values represent fold changes >1,5.

RTqPCR validation of selected genes

Seven genes (ICAM1, SELE, EDNRA, ACE, CALCA, NPPB and FGF1) were analysed with RTqPCR for validation of the RT²array results (Fig 6). Treatment with ASA had no effect on the selected genes, as was also seen in the RT² array. No genes were significantly affected and displayed great variability between samples, although ICAM1 and SELE showed fold changes >1.5 (S1 fig). The ICAM1 gene had a 1.5-fold change compared to untreated cells when treated with PE STBEVs as well as a 2-fold change, when co-treated with PE STBEVs and ASA. For the SELE gene, PE STBEV +/- ASA treatment lead to a 3.22- and 3.85-fold up-regulation, respectively, while normal STBEVs +/- ASA lead to a 1.55- and 1.79-fold up-regulation, respectively. The remaining genes, EDNRA, ACE, CALCA, NPPB and FGF1, all had fold changes <1.5 after both ASA and/or STBEV treatment.

Figure 6. Heat map of all genes analysed with RTqPCR. Only two genes, ICAM1 and SELE, were up-regulated due to STBEV and ASA treatment. All other genes were unaffected by treatments. Underlined values represent fold changes >1,5.

Discussion

The aim of this study was to investigate the routes for cellular uptake of normal and PE STBEVs by primary ECs, as well as their effect on EC activation and gene expression. Furthermore, we aimed to evaluate the effects of ASA on STBEV uptake, EC activation and gene expression. By blocking different parts of the endocytic pathways, we found that primary ECs internalize the STBEVs primarily through CME. The interaction between normal STBEVs and the ECs leads to a decreased activation of the cells as shown by the ICAM-1 surface expression. Uptake of normal and PE STBEVs did not lead to an alteration of the selected endothelial cell biology genes. The effect of ASA was primarily seen as decreased ICAM-1 expression, but no effects of gene expression were seen. In a previous study, we saw effects on gene expression when analysing specific target genes for the miRNAs found inside STBEVs (27). In the present study the genes were chosen due to their connection to endothelial cell biology.

We have previously shown how placental miRNAs in STBEVs were taken up by HCAECs and transferred to endosomes, the ER and to mitochondria. Since normal STBEVs were transferred to a higher degree to the mitochondria, and PE STBEVs to the ER (27), we speculated that this might be due to differences in uptake mechanisms. Both normal and PE STBEV uptake was completely blocked by keeping the cells at 4^{o}C prior to exposure experiments, suggesting that the uptake is not passive but energy requiring. This confirms what has been shown previously for many different vesicles and cell types (11). We tested the uptake inhibitor MβCD, a cholesterol-depleting agent which affects lipid rafts. It is most commonly described to affect CIE, but has also been shown to inhibit CME to some extent (40, 41). In this study we could not reach more than 50% uptake inhibition before the cells were clearly disrupted, which prohibits us to draw any conclusion as to whether lipid rafts or cholesterol are always involved in STBEV uptake or not. The fact that MβCD affects cells negatively is hardly surprising considering that cholesterol is an important part of the cell membrane. However, the presence of cholesterol is clearly important for uptake. Cytochalasin D, a cell permeable compound known to inhibit actin polymerisation (11), has previously been used with good inhibitory effect on uptake, but our results showed that Cytochalasin D could not inhibit STBEV uptake any more than its diluent DMSO. Inhibition of uptake with Chlorpromazine resulted in an almost complete block of both normal and PE STBEV uptake.

This chemical has been suggested to block several receptor families, such as monoamines (dopamine, serotonin, histamine) and the cholinergic muscarinic receptors. It is commonly used in experimental settings to block CME, through clathrin translocation (37). The results from Chlorpromazine, strongly suggests that STBEV uptake mainly occurs by CME. Dynasore, a dynamin inhibitor (36, 42), caused an almost complete block of uptake. Dynamin is mainly a part of CME but have been described to also participate in macropinocytosis (30). Wortmannin was the third inhibitor leading to almost complete block of uptake. As a PI3K inhibitor, it also affects Rab5-dependent endosome fusion (34) and has its primary effects on endosome recycling. It is also known to participate in the membrane ruffling seen in macropinocytosis (30). Since we previously showed increased EC cell membrane ruffling due to PE STBEVs, we speculate that the vesicles could have a long-term effect on which uptake pathways are activated. In previous studies, DMSO has been shown to induce ion leakage from cells as well as diverse effects on processes such as inflammation, metabolism and apoptosis (43). We show that DMSO affects the uptake of vesicles but have not investigated which mechanisms is involved or if it interferes with the endocytic pathways directly.

In conclusion, our results show support for uptake through CME with indication of CIE being involved. We draw the conclusion that clathrin, lipid rafts and dynamin are all essential components in the STBEV uptake pathway by primary endothelial cells. Even though we found no significant differences in inhibitor effects between normal and PE STBEVs, all the inhibitors and DMSO, had a consistently lower uptake inhibition on the PE STBEV uptake. Since PE STBEVs have different properties and surface molecules (16, 17, 19) compared to normal STBEVs, as well as causes membrane ruffling (27), alternate uptake mechanisms for PE STBEVs are still possible. Different surface molecules could allow interactions with other cell surface receptors, and thereby activation of different pathways. It is also described that internalized material can affect the integrity of clathrin coated pits and vesicles, thereby redirecting the endosome to different compartments (30).

The differences in surface molecules could also explain the differences seen in cell activation between normal and PE STBEVs. Activation of immune and endothelial cells is an important part of the second stage of PE (10).The flow cytometry data showed that normal STBEVs down-regulated ICAM-1 expression while the expression was unaffected by exposure to PE

STBEVs. This suggests that normal STBEVs may have a protective role for the endothelium, that is lacking in PE. In contrast to our results, previous studies have shown an up-regulation of ICAM-1 surface protein after exposure to PE placental vesicles or sera (24-26). This might simply be due to differences in isolation techniques, vesicle sources and experimental setup in general. Previously, it has been shown that different preparations of placental vesicles have different properties (44). A study by O'Brien et al (45) suggested that soluble factors, such as sFlt1, rather than vesicles are the cause of endothelial dysfunction in PE, and another study suggested that PE vesicles need monocytes to be present to activate the ECs (26). When isolating vesicles with different techniques, the contamination of other factors might vary and therefore also affect the end results. It is likely that vesicles need interaction with soluble factors and other cells to function physiologically. One further explanation for the decrease in surface ICAM-1 following treatment with normal STBEVs, could be the fact that activation of ECs can lead to release of soluble ICAM-1 (46, 47).

We analysed endothelial cell gene expression and found that no genes were altered significantly when analysed by RTqPCR, although several genes were indicated as altered by the RT^2 array. The gene SELE (E-selectin) showed the greatest fold change >2 by PE STBEVs. This supports previous studies demonstrating increased levels of E-selectin in PE (48, 49) and increased E-selectin expression on ECs that were co-cultured with PE trophoblasts (25). Previous studies have also shown that placental vesicles do not affect angiogenic genes (45).

The current results on the effects of ASA, showed no effect regarding STBEV uptake or endothelial gene expression. However, ASA did interestingly down-regulate the surface protein ICAM-1 after co-treatment with PE STBEVs, suggesting an interaction between ASA and PE STBEVs that does not occur between ASA and normal STBEVs. Future studies should reveal whether ASA could affect the communication between STBEVs and endothelial cells as well as cell activation. Further, it would be interesting to study whether ASA also affects the release of STBEVs from the placenta, since it has been shown that the PE placenta releases significantly higher levels of STBEVs than in normal pregnancy (15). We speculate that ASA could affect the levels of STBEVs in PE.

In summary, the present study shows that both normal and PE STBEVs are primarily taken up by endothelial cells via CME. The small differences in uptake between normal and PE STBEVs could be accounted for by differences in surface molecules but require further studies. Instead, there was a difference in how the normal and PE STBEVs activate endothelial cells, where the surface molecule ICAM-1 was decreased by normal STBEVs suggesting a protective role of normal STBEVs on the maternal endothelium, as part of their physiological adaptation to normal pregnancy. The effects of ASA did not interfere with STBEV uptake or endothelial gene expression. Yet, ASA did decrease ICAM-1 protein expression when co-treated with PE STBEVs suggesting this as one protective mechanism of ASA in PE. The beneficial effects of ASA in preventing PE, could be an effect on the surface markers of the excessively shedded PE STBEVs' and their ability to activate ECs. For future perspectives, it would be interesting to look further into the mechanisms of STBEV cell interaction and activation, and how ASA interplays with these. There is also a need for more functional studies, looking at the effect and interaction of STBEVs and ASA on vessels.

Acknowledgments

The authors would like to thank Eva Hansson, Oonagh Shannon and Mikael Evander for excellent technical assistance and scientific discussions.

Author contribution statement

T.C., L.E., D.T. and S.R.H. conceived the experiment(s). T.C., L.E. and D.T. conducted the experiment(s). T.C., L.E. and S.R.H. analysed the results. All authors reviewed the manuscript.

Competing financial interests

T.C., L.E. and D.T. declares no potential conflict of interest. S.R.H. holds patents for the diagnosis and treatment of preeclampsia. S.R.H. is also a co-founder of the companies Preelumina Diagnostics AB and A1M Pharma AB (www.a1m.se).

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Figures

Figure 1

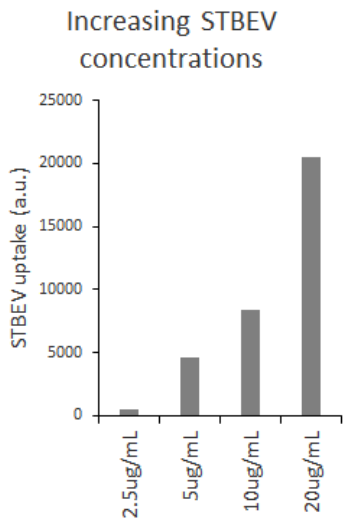


Figure 2

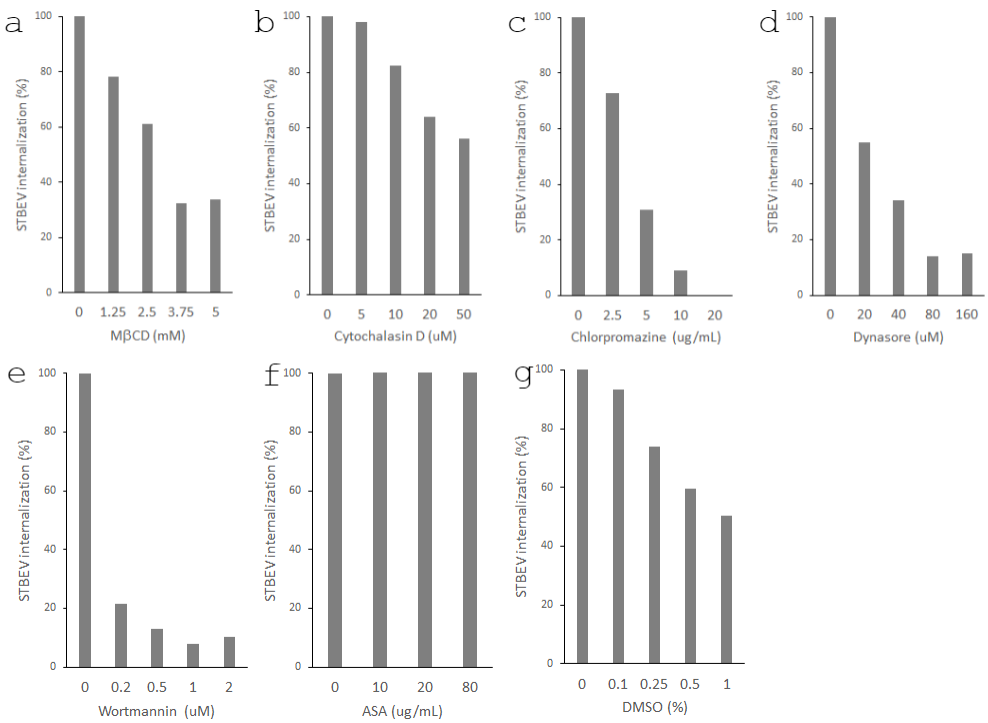


Figure 3

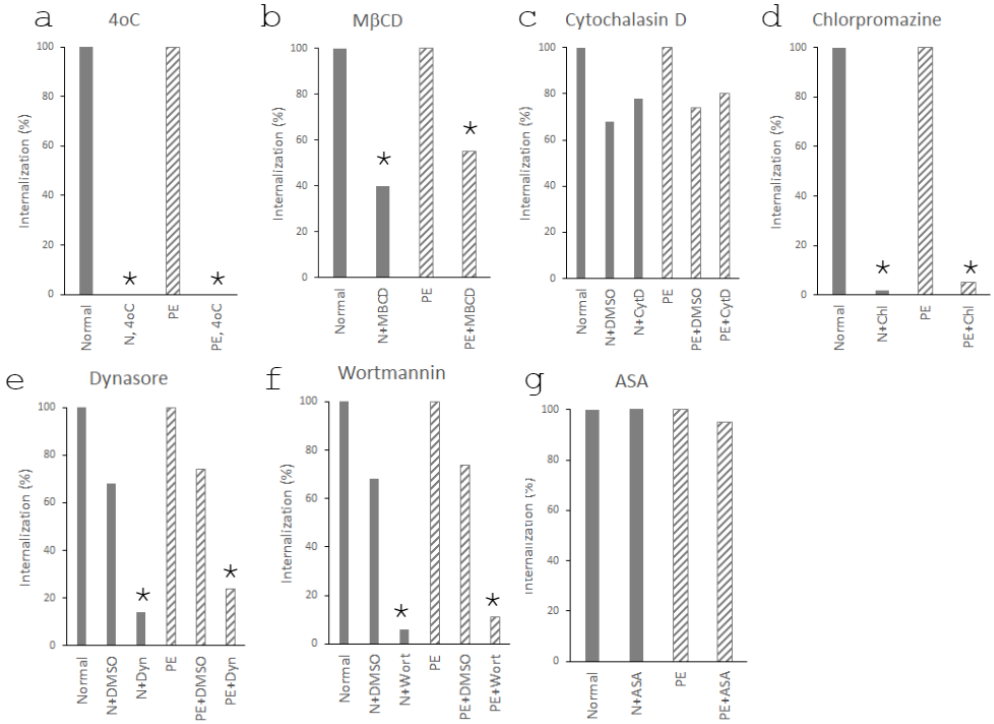


Figure 4

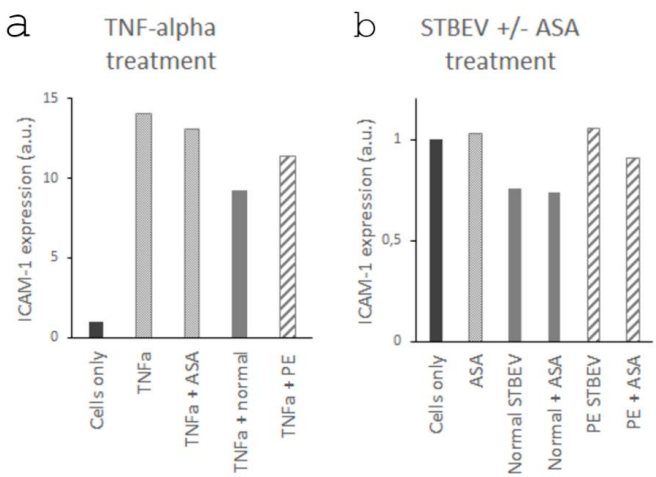
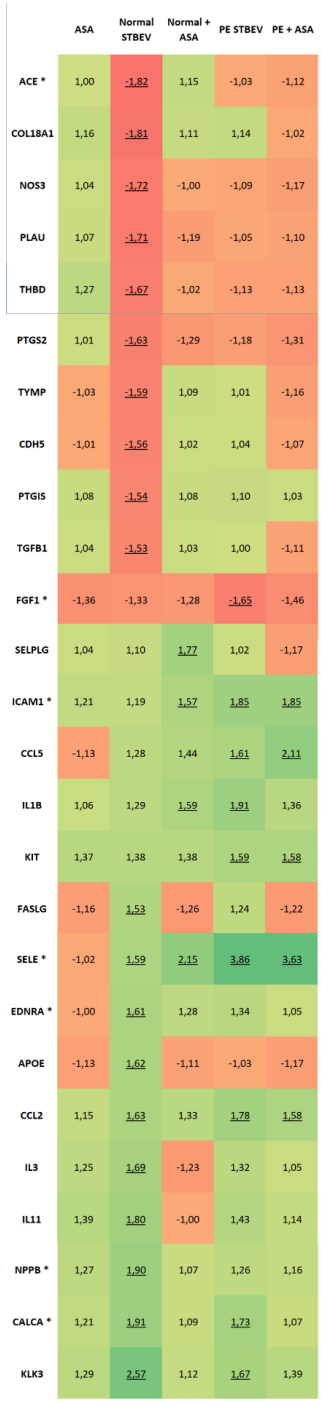


Figure 5



	ASA	Normal STBEV	Normal + ASA	PE STBEV	PE + ASA
ACE *	1,00	-1,82	1,15	-1,03	-1,12
COL18A1	1,16	-1,81	1,11	1,14	-1,02
NOS3	1,04	-1,72	-1,00	-1,09	-1,17
PLAU	1,07	-1,71	-1,19	-1,05	-1,10
THBD	1,27	-1,67	-1,02	-1,13	-1,13
PTGS2	1,01	-1,63	-1,29	-1,18	-1,31
TYMP	-1,03	-1,59	1,09	1,01	-1,16
CDH5	-1,01	-1,56	1,02	1,04	-1,07
PTGIS	1,08	-1,54	1,08	1,10	1,03
TGFB1	1,04	-1,53	1,03	1,00	-1,11
FGF1 *	-1,36	-1,33	-1,28	-1,65	-1,46
SELPLG	1,04	1,10	1,77	1,02	-1,17
ICAM1 *	1,21	1,19	1,57	1,85	1,85
CCL5	-1,13	1,28	1,44	1,61	2,11
IL1B	1,06	1,29	1,59	1,91	1,36
KIT	1,37	1,38	1,38	1,59	1,58
FASLG	-1,16	1,53	-1,26	1,24	-1,22
SELE *	-1,02	1,59	2,15	3,86	3,63
EDNRA *	-1,00	1,61	1,28	1,34	1,05
APOE	-1,13	1,62	-1,11	-1,03	-1,17
CCL2	1,15	1,63	1,33	1,78	1,58
IL3	1,25	1,69	-1,23	1,32	1,05
IL11	1,39	1,80	-1,00	1,43	1,14
NPPB *	1,27	1,90	1,07	1,26	1,16
CALCA *	1,21	1,91	1,09	1,73	1,07
KLK3	1,29	2,57	1,12	1,67	1,39

Figure 6

	ASA	Normal STBEV	Normal + ASA	PE STBEV	PE + ASA
NPPB	0,96	0,80	1,10	1,02	1,04
CALCA	0,79	0,91	1,00	0,88	1,15
FGF1	0,86	0,91	0,99	0,92	1,03
ICAM1	1,03	1,02	1,07	1,35	1,81
EDNRA	0,84	1,04	1,29	1,07	1,21
ACE	0,90	1,09	1,06	0,89	1,03
SELE	0,94	1,41	1,55	2,08	2,82

Supporting information

S1 table. The 96 genes analysed using the Rt2 array from Qiagen

Position on array plate	Symbol	Description
A01	ACE	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
A02	ADAM17	ADAM metallopeptidase domain 17
A03	AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
A04	AGTR1	Angiotensin II receptor, type 1
A05	ALOX5	Arachidonate 5-lipoxygenase
A06	ANGPT1	Angiopoietin 1
A07	ANXA5	Annexin A5
A08	APOE	Apolipoprotein E
A09	BAX	BCL2-associated X protein
A10	BCL2	B-cell CLL/lymphoma 2
A11	BCL2L1	BCL2-like 1
A12	CALCA	Calcitonin-related polypeptide alpha
B01	CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
B02	CASP3	Caspase 3, apoptosis-related cysteine peptidase
B03	CAV1	Caveolin 1, caveolae protein, 22kDa
B04	CCL2	Chemokine (C-C motif) ligand 2
B05	CCL5	Chemokine (C-C motif) ligand 5
B06	CDH5	Cadherin 5, type 2 (vascular endothelium)
B07	CFLAR	CASP8 and FADD-like apoptosis regulator
B08	COL18A1	Collagen, type XVIII, alpha 1
B09	CX3CL1	Chemokine (C-X3-C motif) ligand 1
B10	EDN1	Endothelin 1
B11	EDN2	Endothelin 2
B12	EDNRA	Endothelin receptor type A
C01	ENG	Endoglin
C02	F2R	Coagulation factor II (thrombin) receptor
C03	F3	Coagulation factor III (thromboplastin, tissue factor)
C04	FAS	Fas (TNF receptor superfamily, member 6)
C05	FASLG	Fas ligand (TNF superfamily, member 6)
C06	FGF1	Fibroblast growth factor 1 (acidic)
C07	FGF2	Fibroblast growth factor 2 (basic)
C08	FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
C09	FN1	Fibronectin 1
C10	HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
C11	HMOX1	Heme oxygenase (decycling) 1

C12	ICAM1	Intercellular adhesion molecule 1
D01	IL11	Interleukin 11
D02	IL1B	Interleukin 1, beta
D03	IL3	Interleukin 3 (colony-stimulating factor, multiple)
D04	IL6	Interleukin 6 (interferon, beta 2)
D05	IL7	Interleukin 7
D06	ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
D07	ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
D08	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
D09	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
D10	KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)
D11	KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
D12	KLK3	Kallikrein-related peptidase 3
E01	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)
E02	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
E03	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
E04	NOS3	Nitric oxide synthase 3 (endothelial cell)
E05	NPPB	Natriuretic peptide B
E06	NPR1	Natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)
E07	OCLN	Occludin
E08	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide
E09	PECAM1	Platelet/endothelial cell adhesion molecule
E10	PF4	Platelet factor 4
E11	PGF	Placental growth factor
E12	PLAT	Plasminogen activator, tissue
F01	PLAU	Plasminogen activator, urokinase
F02	PLG	Plasminogen
F03	PROCR	Protein C receptor, endothelial
F04	PTGIS	Prostaglandin I2 (prostacyclin) synthase
F05	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
F06	PTK2	PTK2 protein tyrosine kinase 2
F07	SELE	Selectin E
F08	SELL	Selectin L
F09	SELPLG	Selectin P ligand
F10	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1

F11	SOD1	Superoxide dismutase 1, soluble
F12	SPHK1	Sphingosine kinase 1
G01	TEK	TEK tyrosine kinase, endothelial
G02	TFPI	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)
G03	TGFB1	Transforming growth factor, beta 1
G04	THBD	Thrombomodulin
G05	THBS1	Thrombospondin 1
G06	TIMP1	TIMP metallopeptidase inhibitor 1
G07	TNF	Tumor necrosis factor
G08	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
G09	TYMP	Thymidine phosphorylase
G10	VCAM1	Vascular cell adhesion molecule 1
G11	VEGFA	Vascular endothelial growth factor A
G12	VWF	Von Willebrand factor
H01	ACTB	Actin, beta
H02	B2M	Beta-2-microglobulin
H03	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H04	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H05	RPLP0	Ribosomal protein, large, P0
H06	HGDC	Human Genomic DNA Contamination
H07	RTC	Reverse Transcription Control
H08	RTC	Reverse Transcription Control
H09	RTC	Reverse Transcription Control
H10	PPC	Positive PCR Control
H11	PPC	Positive PCR Control
H12	PPC	Positive PCR Control

S1 figure. Graphs depicting the RTqPCR validation results for the seven genes; ICAM1, SELE, EDNRA, ACE, CALCA, NPPB and FGF1, n=6 in each group. No gene showed significant differences for any group compared to the control group with cells only. The genes ICAM1 and SELE had several groups with a fold change >1.5, although with great variability between samples.