Endogenous and Exogenous Drivers of Oxidative Stress and Inflammation in Preeclampsia

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LUND
UNIVERSITY

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Abstract

Preeclampsia (PE), is a pregnancy related condition afflicting 3-8% of all pregnancies and a main cause of maternal and infant morbidity and mortality. It is thought to originate from a malfunctioning placental that eventually leads to damage to the maternal endothelium, which in turn gives rise to hypertension and organ dysfunction. Oxidative stress holds a central role in the development, progression and aggravation of the disease. This thesis explores two key contributors to the oxidative stress seen in PE; I) free foetal haemoglobin (HbF) and II) particulate matter generated by ambient and household air pollution (PM2.5).

A rabbit PE model was developed and evaluated. Cell-free HbF administered to pregnant rabbits during the second half of gestation mimics PE showing placental tissue damage and disrupted kidney function. Intravenous administration of recombinant human alpha-1-microglobulin (A1M), an endogenous scavenger protein that reversed the HbF-induced tissue damage, indicating a clear protective effect. The PE-specific renal effects were examined in a human cohort were increased plasma levels of HbF and A1M in the maternal circulation was associated with podocyte specific extracellular vesicles in the urine, pathognomic for PE. These findings were validated in the rabbit PE model showing a correlation between plasma HbF levels and kidney damage.

Air pollution is the single largest environmental threat to human health of our time. During pregnancy it is associated with an increased risk for birth- and pregnancy complications, conditions related to placental function. Particulate matter (PM) consists of, and carry, a broad range of toxic substances that can penetrate the respiratory tract and hence gain access to the blood stream and thereby the placenta.

The effects of PM of different sizes and combustion methods were explored *in-vitro* using an immortalized human first trimester cell line (HTR8). The trophoblast cells showed impaired cellular growth, morphological changes, endoplasmic reticulum stress, inflammation, disrupted mitochondrial function and altered protein secretion and expression.

This thesis supports and contributes to the body of evidence showing that oxidative stress and subsequent inflammation, play a pivotal role in the aetiology of PE. Free foetal HbF has been shown to be an endogenous oxidative stress trigger causing damage to the kidneys. The adverse impact of PM2.5 and indoor pollution on trophoblast cells offer a mechanistic explanation to previously well-established epidemiological associations. During pregnancy there is an elevated vulnerability to exposure-related alterations, potentially causing long-term effects for mother and child. Hence, this thesis expands prior work that has offered empirical and theoretical evidence that call for novel legislation that could make the air that we breathe safe for mothers, children and future generations.

Key words oxidative stress, air pollution, placenta, preeclampsia, PM2.5, HbF, α_1-microglobulin

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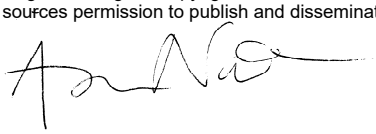
Signature  Date 2020-04-03

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Till Familjen

"A scientist in his laboratory is not a mere technician:
he is also a child confronting natural phenomena that
impress him as though they were fairy tales."

Marie Curie

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Preface

Preeclampsia (PE) is a pregnancy-related syndrome that affects 3-8% of all pregnant women worldwide and it is associated with devastating complications for both mother and the unborn child. To date there is no cure for PE, no effective treatment and no means by which it can be predicted early or prevented nor is there a specific diagnostic tool. Preeclampsia is generally managed through careful antenatal surveillance, symptomatic treatment with anti-hypertensive medication and timing of delivery. Despite several years of vigorous efforts to understand its etiology, it still remains an elusive disease. This is in large part the reason why a therapy still is lacking and delivery remains the only "cure". Uncovering the mystery behind PE is of highest priority in the continuous endeavor to reduce maternal and perinatal mortality across the globe.

While the precise mechanisms behind PE are poorly understood, there is a general consensus that a central driver of the progression is placental dysfunction. There is evidence indicating that oxidative stress is an important mechanism. Whether the placental dysfunction causes the oxidative stress or if the reverse is true remains to be revealed. Oxidative stress can be caused by the endogenous production of reactive oxygen species, such as occurs during cellular respiration or as part of the immune systems defense against microbial agents. Exogenous environmental sources can be such as particulate matter from air pollution.

The aims of the thesis are to investigate the effects of endogenous as well as exogenous stressors affecting placental function and consequently their role in the PE etiology. Building on previous studies, the role of 1) foetal haemoglobin (HbF), and 2) the effects of air pollutants will specifically be studied.

The work in this thesis is based on different *in-vitro and in-vivo* models. While studying the effects of HbF, a novel animal PE model was created in which pregnant rabbits were transfused with rabbit HbF. The effect of air pollutants, in particular fine particulate matter and wood smoke particles, were studied in cultured trophoblast cells *in-vitro*.

List of papers

The following papers are the basis of the doctoral thesis and will henceforth be referred to by their roman numerals as indicated below:

I. A1M Ameliorates Preeclampsia-Like Symptoms in Placenta and Kidney Induced by Cell-Free Fetal Hemoglobin in Rabbit. **Nääv Å**, Erlandsson L, Axelsson J, Larsson I, Johansson M, Wester-Rosenlöf L, Mörgelin M, Casslén V, Gram M, Åkerström B, Hansson SR. PLos One. 2015 May 8;10(5):e0125499. doi: 10.1371/journal.pone.0125499. eCollection 2015. PMID 25955715

II. Urinary Extracellular Vesicles of Podocyte Origin and Renal Injury in Preeclampsia. Gilani SI, Anderson UD, Jayachandran M, Weissgerber TL, Zand L, White WM, Milic N, Suarez MLG, Vallapureddy RR, **Nääv Å**, Erlandsson L, Lieske JC, Grande JP, Nath KA, Hansson SR, Garovic VD. J Am Soc Nephrol. 2017 Nov;28(11):3363-3372. doi: 10.1681/ASN.2016111202. Epub 2017 Jul 20. PMID 28729288

III. Exposure of Trophoblast Cells to Fine Particulate Matter Air Pollution Leads to Growth Inhibition, Inflammation and ER Stress. Familari M, **Nääv Å**, Erlandsson L, de longh RU, Isaxon C, Strandberg B, Lundh T, Hansson SR, Malmqvist E. PLoS One. 2019 jul 18;14(7):e0218799. doi: 10.1371/journal.pone.0218799. eCollection 2019. PMID: 31318865

IV. Urban PM2.5 Induces Cellular Toxicity, Hormone Dysregulation, Oxidative Damage, Inflammation and Mitochondrial Interference in the HRT8 Trophoblast Cell Line. **Nääv Å**, Erlandsson L, Isaxon C, Åsander Frostner E, Ehinger J, Sporre MK, Krais AM, Strandberg B, Lundh T, Elmér E, Malmqvist E, Hansson SR. Accepted in Frontiers in Endocrinology, section Translational Endocrinology 4 Feb, 2020

V. Exposure to Wood Smoke Particles Leads to Inflammation, Disrupted Proliferation and Damage to Cellular Structures in a Human First Trimester Trophoblast Cell Line. Erlandsson L, Lindgren R, **Nääv Å**, Krais AM, Strandberg B, Lundh T, Boman C, Isaxon C, Hansson SR, Malmqvist E. Submitted Feb 2020.

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Papers not included in the thesis

Inventory of Novel Anmial Models Addressing Etiology of Preeclampsia in the Development of New Therapeutic/Intervention Opportunities. Erlandsson L, **Nääv Å**, Hennessy A, Vaiman D, Gram M, Åkerström B, Hansson SR. Am J Reprod Immunol. 2016 Mar; 75(3):402-10. doi: 10.1111/aji.12460. Epub 2015 Dec 18. **Review**. PMID: 26685057

Oxidative Stress in Preeclampsia and the Role of Free Fetal Hemoglobin. Hansson SR, **Nääv Å**, Erlandsson L. Front Physiol. 2015 Jan 13;5:516. Doi: 10.3389/fphys.2014.00516. eCollection 2014. **Review**. PMID 25628568

Abbreviations

A1M	Alpha-1-microglobulin
BMI	Body mass index
BP	Basal plate
CBT	Cytotrophoblast
CNS	Central nervous system
CO	Carbon monoxide
CO_2	Carbon dioxide
CP	Chorionic plate
CTB	Cytotrophoblasts
CV	Chorionic villi
CVD	Cardiovascular disease
eNOS	Endothelial nitric oxide
ENVT	Endovascular trophoblast
ER	Endoplasmic reticulum
EV	Extracellular vesicle
EVT	Extravillous trophoblast
Fe^{2+}	Ferrous haemoglobin
FGR	Foetal growth restriction
GFR	Glomerular filtration rate
Hb	Haemoglobin
HbF	Foetal haemoglobin
hCG	Human chorionic gonadotropin
HELLP	Haemolysis, Elevated Liver enzymes and Low Platelets
HIC	High income country
HLA	Human leukocyte antigen
DBP	Diastolic blood pressure
HO	Hemoxygenase
IL-6	Interleukin-6
IS	Intervillous space

IUGR	Intrauterine growth restriction
IVF	*In-vitro* fertilization
LBW	Low birth weight
LIC	Low income country
LMIC	Low- and middle-income countries
MHC	Major histocompatibility complex
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NK cell	Natural killer cell
NO	Nitric oxide
PAH	Polycyclic aromatic hydrocarbons
PE	Preeclampsia
PFEC	placental-foetal endothelial cell
PlGF	Placental growth factor
PM	Particulate matter
PM2.5	Particulate matter <2.5 μm
PM10	Particulate matter <10 μm
ROS	Reactive oxygen species
RUPP	Reduced uteroplacental perfusion pressure
SA	Spiral arteries
SBP	Systolic blood pressure
sFlt-1	Soluble fms-like tyrosine kinase 1
SGA	Small for gestational age
SOD	Superoxide dismutase
STB	Syncytiotrophoblast
TEM	Transmission electron microscopy
Treg	Regulatory T-cells
UC	Umbilical cord
UV	Uterine veins
UPR	Unfolded protein response
VOC	Volatile organic compound

Background

Preeclampsia

Introduction

Preeclampsia (PE) is a pregnancy associated syndrome. Globally approximately 3-8% of all pregnant women develop PE, and it is the cause of more than 76 000 maternal deaths and half a million neonatal and foetal deaths every year (Brown et al., Abalos et al). The term PE stems from the Greek word "eklámpein", meaning to shine out or burst forth suddenly. Eclampsia is a late complication of PE and is characterized by the onset of grand mal seizures that are life-threatening if left untreated (Ghulmiyyah et al). Preeclampsia can be diagnosed after 20 weeks of gestation (table 1). Preeclampsia is categorized into early- and late-onset, depending on whether it manifests before or after 34 weeks of gestation, and the causative factors behind the syndrome are believed to differ, at least partly, between the two sub-types with early onset relying more heavily on a placental component in the development of the syndrome. Early onset PE is to a greater extent associated with intrauterine growth restriction (IUGR) (Staff et al 2019).

Diagnostic criteria for Preeclampsia		
i)	**Systolic 140 mmHg and diastolic 90mmHg**	New onset hypertension after 20 weeks of gestation
		And one or more of *ii*
ii)	**Proteinuria, kidney injury, liver involvement, neurological or haematological complications**	
		And or *iii*
iii)	**Uteroplacental dysfunction**	

Table 1. Diagnostic criteria for PE. Adapted from Swedish guidelines preeclampsia (SFOG 2019)

Previously, PE was divided into mild or severe depending on its clinical presentation. This categorization has largely been abandoned, since PE is a volatile

condition that can rapidly progress to a more serious manifestation from an initially quiescent presentation (Brown et al., Burton et al 2019).

To date there is no way of predicting with any accuracy who will develop PE. However, there are well-established risk factors (table 2).

A wide range of biomarkers have been suggested and tested, some with promising results such as placental growth factor (PlGF) and soluble fms-like tyrosine kinase 1 (sFlt-1) (SFOG guidelines preeclampsia). Additional studies and validation are recommended prior to full scale use in a clinical setting in Sweden (SFOG guidelines preeclampsia).

Clinical management

High risk patients (table 2), are closely monitored in antenatal care units throughout pregnancy (SFOG guidelines preeclampsia). Early pregnancy prophylactic treatment with acetylsalicylic acid has been shown to lower the risk of PE and is now widely recommended (Hendersen et al., Dutta et al 2014., Rolnik et al).

Maternal risk factors for PE	
High risk	Moderate risk
Antiphospolipid syndrome	Nullipara
Systemic Lupus Erythemtosis	BMI>30
Previous PE or Eclampsia	Age>40
Previous gestational hypertension with delivery prior to 34 weeks of gestation/IUGR/intrauterine foetal death/ablatio placentae	Systolic blood pressure>130 mmHg or diastolic blood pressure over>80 mmHg at booking at antenatal care
Diabetes Mellitus type 1 & 2	Pregnancy spacing >4 years
Duplex or Triplex pregnancy	Heredity
Chronic kidney disease	African decent
Chronic hypertension	Verified sleep apnoea
Proteinuria at booking at antenatal care	
IVF with oocyte donation	

Table 2. Risk factors for PE. Adapted from Swedish guidelines preeclampsia (SFOG 2019)

Women who develop the PE syndrome are currently running short of effective treatment, let alone a cure. In addition to close monitoring of the mother and unborn child, the mainstay of treatment consists of symptomatic treatment of the blood pressure with anti-hypertensive drugs and magnesium to prevent seizures for the mother and administration of corticosteroids for the foetus to stimulate the production of surfactant and thereby stimulate lung maturity (Brown et al., Mol et al). In order to fully alleviate PE, delivery and the subsequent removal of the dysfunctional placenta is the only option (Brown et al., SFOG guidelines preeclampsia).

The placenta in normal pregnancy

The maternal-foetal interface

The placenta is the maternal-foetal interface, facilitating the exchange of gases and transmission of nutrients and excretion of waste products, essential to promote the development of the foetus. Further, the placenta stores glycogen and iron, produces steroids and hormones essential to maintain pregnancy and has protective properties for the foetus, acting as a barrier against infectious agents and some substances harmful to the growing foetus. (Burton et al 2015). At term, the disc-shaped haemochorial human placental weighs approximately 500 g, measures 15-20 cm in diameter. The chorionic plate constitutes the surface facing the foetus with umbilical vein and arteries, branching out from the insertion of the umbilical cord (UC) (figure 1). The basal plate (BP), the maternal side of the placental, is organized in cotyledons and faces the endometrium. Maternal blood fills the intervillous space (IS), the space between the endometrium and the basal plate. Chorionic villi (CV) form terminal villi protruding into the intervillous space where the foetal syncytiotrophoblasts (STB) come into direct contact with the maternal blood (Burton et al 2015., Maltepe et al., Moffett et al., Gude et al., Huppertz et al).

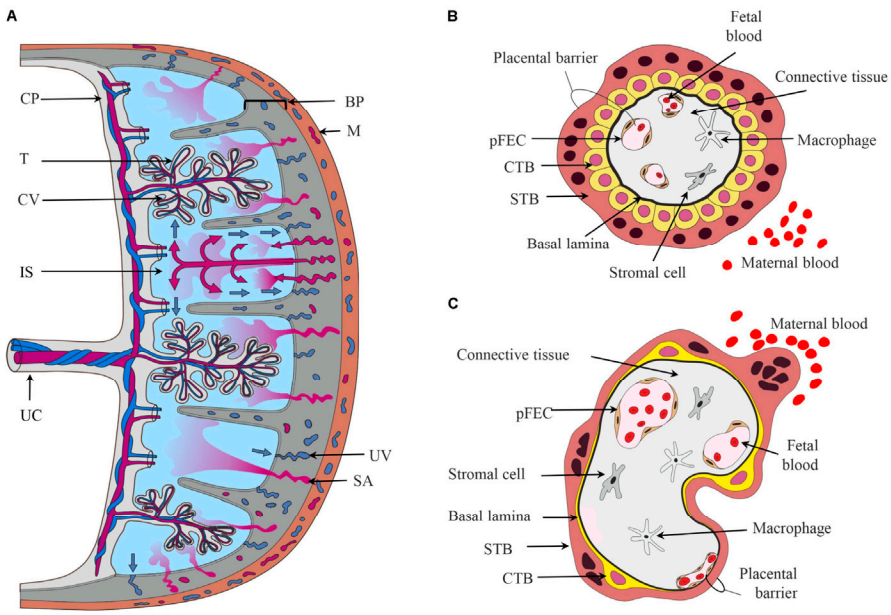

Figure 1. The placental anatomy. (A) Morphology human placenta. Chorionic plate (CP), umbilical cord (UC) and chorionic villi (CV). Intervillous space (IS) spiral arteries (SA), uterine veins (UV), trophoblasts (T). The basal plate (BP). **(B)** first trimester chorionic villi in cross section. **(C)** term trimester chorionic villi in cross section. Cytotrophoblast (CTB), Myometrium (M), pFECs, placental-foetal endothelial cells (PFECs), syncytiotrophoblast (STB). Adapted with permission from Chatuphonpraset et al.

The 1st trimester placental development

Following fertilization, the ovum transitions through a series of stages to become a blastocyst, a spherical structure composed of an inner cavity with a smaller inner cell mass and a surrounding layer of trophectoderm. The inner cell mass will eventually form the embryo and the trophectoderm will form the placenta. Once formed, the blastocyst undergoes a process known as hatching, where the outer protective layer, surrounding the trophoblast, is broken down in order to facilitate implantation (Maltepe et al). Thereafter, the connection between the blastocyst and the endometrial wall is strengthened through a succession of steps known as apposition and adhesion, followed finally by invasion of the endometrial epithelium during which trophoblastic cells rapidly proliferate and differentiate to villous or extravillous trophoblasts (EVTs) (figure 1).

A subset of trophoblast cells merges to become the STB, a multinuclear cellular mass devoid of boundaries that will line the villous trees, while other EVTs penetrate deeper into the uterine stroma towards maternal blood vessels and remodel the uterine spiral arteries (Gude et al., Roberts et al 2008). The human placental is hemochorial, which means once fully developed, the maternal and foetal blood vessels housed in the placenta are only separated by a thin layer of STB, the foetal capillary endothelium of the foetal capillary network and some mesenchyme.

The first trimester is a period of intense interaction between the maternal immune cells and the placental tissue. The conversion of the maternal vessels, the spiral arteries, in which the endothelial cells lining the blood vessels and smooth muscle cells are exchanged for trophoblasts, is a result of this fine-tuned process, driven by natural killer (NK) cells. The latter promote the migration of EVTs by releasing growth factors and cytokines (Burton et al 2015). Trophoblast development thrives under hypoxic conditions during the first trimester of pregnancy. Nature has cleverly solved this by EVTs, plugging the spiral arteries (Maltepe et al., Gude et al., Roberts et al 2008). The establishment of the spiral arteries allows for blood flow to the placenta; however, this only occurs at the 12th week of gestation when the spiral arteries are unplugged (Robbins et al).

Nutrients and gas exchange

All the nutrients needed for the growth of the foetus must pass the placenta, either by active transport or passive diffusion. Maternal blood flow is carried through spiral arteries to the placenta, where it bathes the chorionic villi and allows for exchange of nutrient and gases before being drained away through venous openings. Assuming normal placental formation, the blood flow through the placenta is determined by contractions of the uterus, intra-uterine pressure and maternal blood pressure. Oxygen passes from the maternal to the foetal circulation by passive diffusion following a concentration gradient, but this passage is facilitated by the existence HbF, which has a significantly higher affinity to oxygen than maternal haemoglobin (Hb) and can therefore extract oxygen in an efficient manner. Carbon

dioxide passes in the opposite direction, also through passive diffusion. The placental tissue utilizes a range of different mechanisms for transport; endocytosis, active transport, water-soluble substances are transported by water pores. Carrier proteins enable facilitated diffusion of glucose. Finally, the placenta acts as a storage facility for key nutrients that can be used as a backup in the case of poor maternal nutrition, storing glycogen, proteins and polypeptides. (Burton et al 2015., Maltepe et al, Moffett et al., Gude et al., Huppertz et al).

Hormonal balance and inflammatory response

In addition to the vital function of the placenta to allow passage of nutrients and oxygen to the foetal circulation and the collection of waste products and carbon dioxide, the placenta plays a pivotal role in the production of numerous hormones and neuropeptides. The most important of these is the placental hormone human chorionic gonadotropin (hCG), but the placenta also synthesizes placental variants of all the hormones released in the adult hypothalamus, as well as steroid hormones such as progesterone, estradiol, estriol and estrone.

Human chorionic gonadotropin plays a pivotal role in the initial cross-talk between the embryo and the deducida to optimize the conditions for implantation (Salomonsen et al., Sharma et al 2016., Bonduelle et al., Lopata et al). Already ten days after implantation, hCG can be measured in maternal blood, with peak concentration at 11 weeks of gestation. The production of progesterone is safeguarded by hCG by the latter upholding the corpus luteum (Shikone et al). Human chorionic gonadotropin drives the differentiation of cytotrophoblasts (CTB) to STB (Shi et al). It has been shown that hCG is involved in the immune-tolerance and immune modulation during implantation and a recent study shows regulatory T-cells (Treg) differentiation to be influenced by hCG (Schumacher et al., Diao et al).

A suggested inflammatory marker in PE is the pro-inflammatory cytokine interleukin 6 (IL-6) (Black et al). In some studies, increased levels of IL-6, together with interleukin 8, have been observed to be associated with PE (Ouyang et al., Vrachnis et al). The effects of Il-6 can be seen on the immune system as well as other cell types, for example, hepatocytes (Rose-John et al). Monocytes are the main producers of IL-6, but also trophoblasts produce IL-6 (Parker et al., Svinarich et al).

Immunological considerations

The placenta acts as the life-line for the growing foetus, and the finely tuned balance between the maternal and foetal blood flow must be constantly maintained in order for the foetus to thrive. As previously mentioned, the maternal blood occupies the cavity between the endometrium and the basal plate (BP), the intervillous space (IS). The foetal blood vessels embedded in the villous tree are in direct contact with the maternal blood. This direct contact between STB, foetally derived cells, and maternal blood is indicative of the immense importance of immune tolerance to

establish and uphold pregnancy. In consequence, the EVTs, invading the uterine deducida, display a characteristic set of human leukocyte antigen (HLA) and the STBs express no major histocompatibility complex (MHC) antigen. The complexity of immunological processes in pregnancy is to date not fully understood and remains under intense investigation (Moffett et al., Burton et al 2015).

The preeclamptic placental

Although preeclamptic women can occasionally present physical symptoms such as abdominal pain, nausea and headaches, PE is often asymptomatic, at least initially. Considering the high prevalence of PE, it is therefore of great importance that pregnant women are frequently screened for clinical signs of PE in order to enable early detection and symptomatic treatment, highlighting the need for well-functioning prenatal care.

The two-stage model

Despite decades of research, PE remains an enigma. Due to the myriad theories that have been suggested, PE has been dubbed “the disease of theories”. It would seem that there is no single causative agent or explanation, rather PE involves a complex syndrome in which multiple systems interact. However, one overarching explanation that has been offered and gained consensus is the two-stage development model, first presented in 1991 (Redman et al 1991) and recently revised (Redman et al 2015), which is centered around an early disordered placentation (figure 1). In short, a faulty placentation during the first trimester of pregnancy results in a poorly perfused placenta which in turn results in the release of factors to the maternal circulation where they cause endothelial damage and consequently give rise to the maternal symptoms (Staff et al 2019).

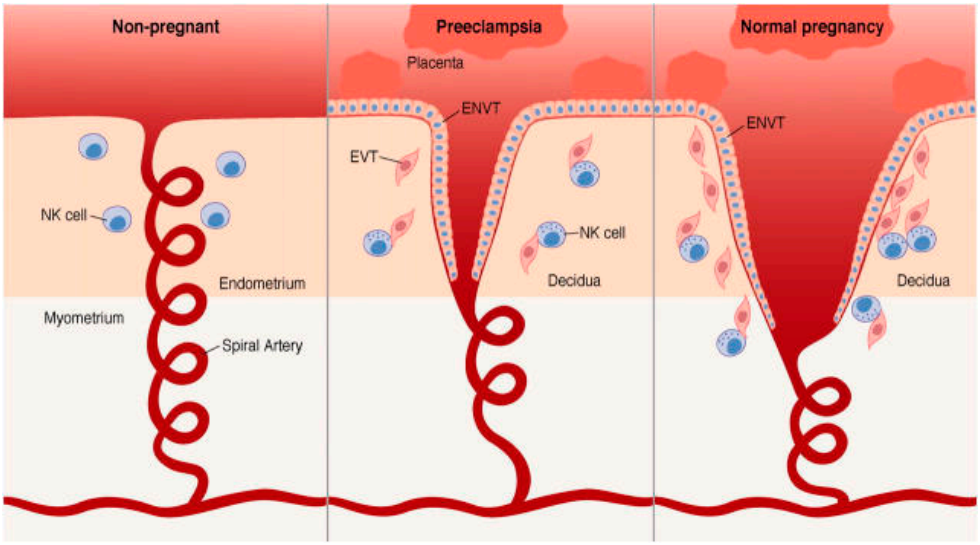

Figure 1. Spiral arteries in non-pregnant, preeclampsia and normal pregnancy
Shallow invasion of spiral arteries in the preeclampsia placenta (middle section) compared to deep spiral arteries in normal pregnancy (right side) and spiral arteries in the non-pregnant myo- and endometrium. Extra villous trophoblasts (EVT), endovascular trophoblasts (ENVT), natural killer (NK) cells. Adapted with permission from Parham et al 2004

Stage one

The primary disruption takes place in the first trimester during the implantation and invasion of the uterine wall. A defective or incomplete remodeling of the uterine spiral arteries during this period leads to an altered placental perfusion with a high-pressure pulsatile blood-flow, instead of the constant blood flow and pressure that is the result of normal placental development. This change to the placental blood flow eventually leads to OS and damage of the CV, which in response secrete various agents that contribute to the maternal syndrome. Factors such as inflammatory cytokines, anti-angiogenetic molecules and cell-free foetal DNA have all been suggested as possible links between the defect placentation and the systemic maternal response in the two-stage model.

Stage two

In the second stage of PE, the factors released by the damaged CV induce a systemic activation of maternal endothelium that results in perturbed organ damage. This might involve damaged kidneys and vasculature, as well as disrupted coagulation pathways, insulin balance and lipid metabolism.

To complicate the image delineated above, there seems to be etiological differences between early- and late onset PE. While early-onset PE is clearly related to defective

remodeling of maternal spiral arteries and uteroplacental mal-perfusion, late-onset PE seems to be more intimately related to a maternal pre-gestational morbidity, that is, risk-factors (table 2) such as high arterial blood pressure, predisposition to inflammation and a high body mass index (BMI), coupled with a mismatch between foetal metabolic demands and maternal perfusion.

Recent research has also indicated foetal sex as a potential, but important determinant of the outcome of PE, although this association seems to be complex and remains to be fully investigated. The mechanics behind the development and progression of the disease on a molecular level remain poorly understood. It is therefore important to gain a deeper understanding of this elusive disease so that global maternal and perinatal mortality and morbidity can be further reduced.

Effects on other organ systems

The kidneys

Pregnancy requires the female body to through substantial physiological changes. The increase in plasma volumes puts an extra strain on the kidneys which increase in both size, roughly 30%, and glomerular filtration rate (GFR) by up to 50% (Rasmussen et al). Glomerular endotheliosis, characteristic damage to the glomeruli giving rise to proteinuria, is pathognomic to PE (Stillman et al). The endothelial cells (podocytes) are caused to swell and lose their endothelial fenestration, in severe cases even the capillary lumens are occluded (Lafeyette et al., Stillman et al). The glomerular filtration barrier, controlling the outflux of proteins from blood to urine, is upheld by the slit diaphragms formed by the podocytes (Mundel et al). Several studies have shown an association between disruption to podocytes and proteinuria in PE (figure 2)(Garovic et al., Henao et al).

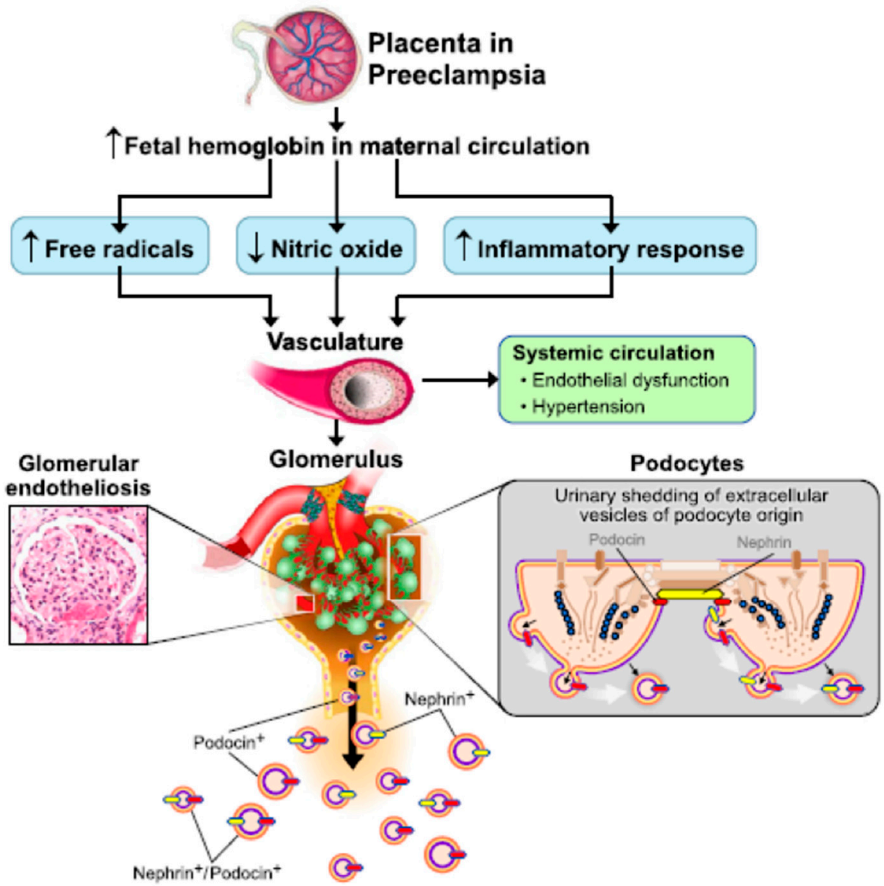

Figure 2. HbF and renal injury in preeclampsia
Urinary extracellular vesicles originating from podocytes linked to increased levels of HbF in the maternal circulation. (Adapted from Paper II)

In addition to proteinuria, podocyturia has been observed in PE patients (Garovic et al., Phipps et al). During the lifecycle of the podocyte, upon reaching maturation, it loses its ability to undergo miotic transformation. Renewal of podocyte can only occur through transformation of parietal cells (Penning et al., Appel et al). Once a podocyte goes under, either due to apoptosis or breach of junctions holding it in place, the integrity of glomerular basal membrane is affected and results in proteinuria (Shiffer et al., Kihara et al). The shedding of podocyte has been shown to be detectable prior to proteinuria in PE during the second trimester of pregnancy, thereby showing to be a promising marker for predicting PE (Craici et al). Patients with PE have a combination of reduced renal blood flow and decreased area of

filtration, resulting in a reduced GFR. The kidney suffers great insult with tubular necrosis and massive glomerular endotheliosis in 3-15 % of cases of the Haemolysis, Elevated Liver enzymes and Low Platelets syndrome (HELLP), a severe complication to PE (Fakhouri et al).

Complications to PE and risk of future adverse health

Central nervous system

PE can in 1-2% of cases lead to several complications in the central nervous system (CNS) (Sibai et al 2006). Eclampsia, the most severe manifestation of PE and potentially lethal stage in PE, all efforts in health care system aim to prevent PE developing into eclampsia. To date, eclampsia is still one of the reasons why mothers die during pregnancy or during the post-partum period (Ghulmiyyah et al). The aetiology behind eclampsia is not fully mapped out but the insult asserted by the dysfunctional placental and other factors on the endothelium resulting in hypertension is thought to be the starting point. The hypertension in turn induces vasospasms which reduce the circulation to CNS resulting in brain oedema. At this stage it is a snowball effect where the oedema exerts pressure on the CNS tissue with ischemia and tissue infarctions as a result (Zeeman et al). Clinically, it is hard to predict eclampsia. However, it has been reported that women with PE experience severe headache and blurry vision, prodromal signs, days before the onset of eclampsia (Knight et al). Clinically eclampsia is defined as tonic-clonic seizures presenting in a pregnant woman or during the post-partum period with all other explanations for seizures ruled out (for example, brain haemorrhage, epilepsy, CNS infection) (Knight et al). Prophylactic treatment for convulsions with magnesium sulphate is administered to women at high risk of eclampsia (Brown et al 2018).

HELLP

PE can in some cases (8-10%) progress into the life-threatening condition HELLP which entails Haemolysis, Elevated Liver enzymes and Low Platelets and has a rapid development involving maternal intravascular haemolysis (Brown et al 2018., Hammound et al).

The unborn child

The placental is essential for the foetus, which relies on the placenta for nutrients and oxygen, however in the case of PE, the placenta is harmful for the mother. Due to the nature of the dysfunctional placenta in PE, the foetus is at risk of foetal growth restriction (FGR) (Staff et al 2019). Once or if the effects of PE call for delivery of the placenta and subsequently the unborn child, it is a considered a pre-term birth if delivery occurs before 34 weeks of gestation (Brown et al 2018., Mol et al 2016). Children born with FGR are at immediate risk for infant respiratory distress

syndrome, sensitivity to infections and jaundice in the neonatal period as well as at long term risk for neurocognitive disorders and asthma to mention a few (Colella et al 2018).

Risk of cardiovascular disease

Central to PE is the maternal systemic enhanced inflammatory state and the endothelial dysfunction (Tomimatsu et al). The activation of the vascular endothelium during PE is thought to cause long-term damage and increase the risk for future cardiovascular disease (CVD). Several studies found the risk of CVD, including, stroke, venous thromboembolism and ischaemic heart disease later in life to increase by two-fold in women with previous PE (Brown et al 2014., Bellamy et al., Roberts et al 2010). In addition, women who had their pregnancy complicated by PE suffer an elevated risk of hypertension, requiring pharmaceutical intervention later in life. The risk of developing hypertension peaks during the post-partum period, but according to recent studies, it remains two-fold even beyond two decades after delivery (Egeland et al., Behrens et al., Ying et al).

Not only previous PE increases the risk of CVD, other obstetric conditions of placental origin, such as IUGR and placental abruption, have shown a more than two-fold risk of developing CVD later in life (Ray et al). Like their mothers, children born from a preeclamptic pregnancy run an increased life time risk of CVD (Kajantie et al., Lu et al., Nahum et al).

However, on a theoretical level, it is not entirely clear if CVD and PE share the same risk factors or if PE itself constitutes a risk factor for CVD. Given that approximately 3-8% of all pregnant women develop PE, and subsequently are at higher risk of suffering from CVD it offers an opportunity for preventive strategies in life style changes and early detection of CVD. A recent meta-analysis concluded that all women with previous PE should be recommended life-long monitoring for CVD risk factors (Wu et al 2017). The current praxis in Sweden is for all mothers with hypertension during the gestational period to be informed about the risks and recommended to organize yearly follow-up. Mothers with repeated PE, early onset PE and severe PE are to be referred to check-ups once per year with regards to CVD and hypertension (SFOG guidelines preeclampsia).

Oxidative stress

Oxidative stress represents a shift in balance between antioxidant defenses and reactive oxygen species (ROS) in favor of the latter, resulting in cellular damage. The production of ROS is an inevitable by-product of a wide range of biochemical processes, under physiological condition only produced in limited amounts (Buonocore et al). Such processes include the immune systems production of ROS in the defense against pathogens, following ischemia/reperfusion injury to bodily

tissues, and the respiratory chain, which occur in all cells in the body. Reactive oxygen species are highly reactive and can result in damage on proteins, membranes and DNA when produced in excess and when the balance between oxidative and antioxidant substances is offset (Tjoa et al., Valko et al).

Antioxidants are similarly produced ubiquitously in bodily tissues and act as a defense and counterbalance to the harmful effects of oxidative molecules (Hansson et al 2013). Categorized into two sub-groups, enzymatic and non-enzymatic, antioxidants serve various functions and neutralize or inhibit the production of free radicals and ROS through their interaction with substances such as superoxide, hydrogen peroxide, heme groups and vitamin C and E (McCord et al). Examples of non-enzymatic antioxidants are nicotinamide adenine dinucleotide (NADH), Nicotinamide adenine dinucleotide phosphate (NADPH) (Pompella et al) and glutathione while enzymatic antioxidants include catalase, superoxide dismutase (SOD) and hemoxygenase (HO) (Scholz et al). The HO which exists in different isoforms, is responsible for the degradation of heme groups into iron, biliverdin and carbon monoxide (Kikuchi et al). The endogenous antioxidant defenses, if not impaired or overwhelmed, can manage the ROS and uphold the redox-balance. However, if the production of ROS exceeds the capacity of the antioxidant defense, the result is OS with consequent damage to tissue.

Research has demonstrated several links between PE and OS with lowered antioxidant capacity of PE placentas (Wang et al., Walsh et al). As previously mentioned, a central tenant of the two-stage PE model is the oxidative stress to the chorionic villi that occurs as a consequence of disrupted uteroplacental perfusion. Damage to the chorionic villi in turn results in the leakage of substances into the maternal circulation that can cause systemic oxidative stress. During stage two of PE, placental factors gain access to the maternal circulation resulting in endothelial damage and dysfunction and oxidative stress (Smarason et al., Tjoa et al). In support of this, studies have found reduced antioxidant capacity in the placentas, as well as in the blood of women with PE (Hubel et al., Raijmakers et al). Microparticles, containing microRNA and HbF, shedded from the placenta can add to the oxidative stress (Redman et al 2008., Tanetta et al., Cronqvist et al 2014., Rudov et al). While many endogenous substances can cause oxidative stress, the work in this thesis has placed a particular focus on free HbF, which has strong oxidative reactivity and has been implicated as an important contributing factor in the development of PE.

Endothelial dysfunction and damage

The endothelium is a core concept in the development of PE. The clinical symptoms (stage two) are a manifestation of the events that occur in the maternal endothelium (Roberts et al 1998). It has been debated whether the insult to the endothelium is an activation, rather than a damage (Roberts et al 1998).

The endothelium is the cell layer lining the vessels in the vascular system. The endothelium enables the vascular system to regulate and uphold a physiological tone in the vascular bed to ensure an optimal flow of blood. To achieve this, the endothelium relies on vasodilatory and anti-thrombotic mediators (Hadi et al). Once the endothelium no longer can uphold these tasks due to endogen or exogen disturbances, it will transform into a pro-inflammatory state (Endemann et al., Bonetti et al).

The endothelial cell has several protection mechanisms by which it attempts to diminish the insult caused by ROS. For example, nitrite oxide formations (NO) prevents platelet-aggregation, has anti-inflammatory and vasodilatory properties (Endemann et al). Consumption of NO leads to depletion of NO, partly due to the increased activity of endothelial nitric oxide synthase (eNOS) and in part due to ROS binding to NO. The vasodilatory effect of NO is subsequently reduced, in both activity and bioavailability (Bonetti et al). Reactive oxygen species interact with the endothelium and cause leukocytes to adhere to the endothelium by upregulation adhesion molecules, in addition ROS increases the permeability of the endothelium (Lum et al).

Foetal haemoglobin

Properties and effects

Haemoglobin normally resides in red blood cells and is vital in the transport of oxygen to bodily tissues. Composed of four globin chains surrounding a central iron-containing heme group, Hb is categorized into an adult and a foetal form depending on the variant of globin chains it contains. HbF has significantly higher affinity to oxygen than adult Hb, enabling it to capture oxygen efficiently from the maternal circulation and deliver it to the growing foetus (Hansson et al 2014). Due to the central iron atom and its redox activity, Hb has potent oxidative reactivity and can therefore disrupt the oxidative balance and cause tissue damage when released from its normal confinement to red blood cells (Nääv et al 2015., Schaer et al 2009., Schaer et al 2013., Hansson et al 2014).

Three known mechanisms have been described through which HbF can carry out oxidative damage: 1) generation of free oxygen radicals, 2) binding of ferrous haemoglobin (Fe^{2+}) to nitric oxide (NO), resulting in vasoconstriction (Cindrova-Davis et al), and 3) induction of cytokine production and neutrophil activation, eliciting an inflammatory response (Kumar et al, 2005).

HbF in Preeclampsia

As for the role of HbF in PE, analysis of placental gene expression has revealed an elevated expression of HbF both in protein and mRNA form in the placentas of preeclamptic women and cord blood (figure 4) (Centlow et al., 2009, Mazoumi et al).

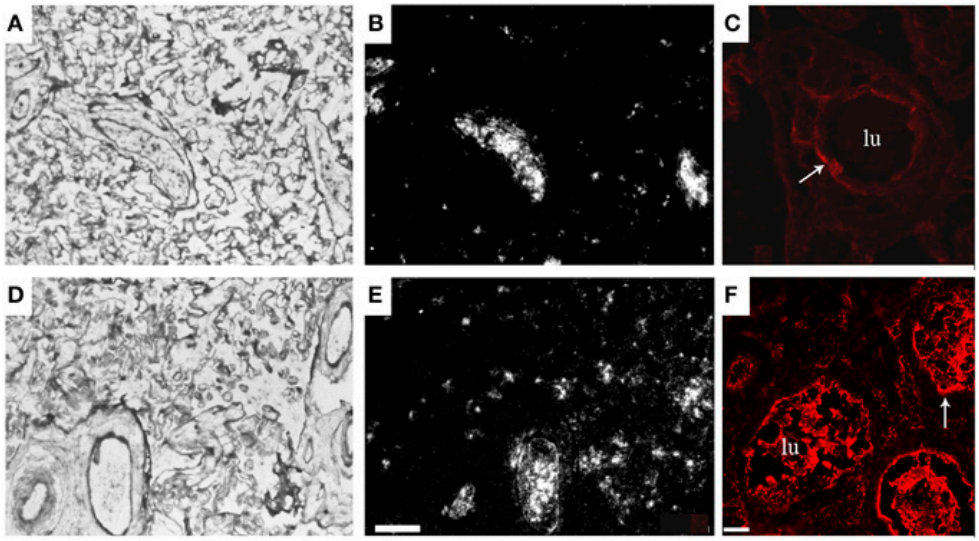

Figure 4. Placental villi display increased expression of HbF in preeclamptic placentals compared to controls. Increased placental expression of HbF in preeclampsia. Representative images from in situ hybridizations of human placenta, displaying the villous section of the placenta. In control sample, HbF mRNA expression (A) and (B), in preeclampsia samples HbF detected within and in close proximity blood vessels (D) and (E). In control sample HbF protein expressed tagged with red fluorescent marker (C) and in preeclamptic sample high levels of HbF expressed in the lumen of blood vessels (lu) and vascular endothelium (arrow) (F). Scale bars for (A, B, D, E) = 100 µm and for (C, F) = 25 µm. Adapted with permission from Hansson et al

In addition, our group has –through the use of the placenta perfusion model – shown how the exposure to free Hb creates a preeclampsia-like condition, at least in an *ex vivo* system (May et al., Centlow et al., 2009). Finally, using animal models with rabbits and ewes, free Hb has also been revealed to cause placental and glomerular kidney damage similar to that seen in PE (Nääv et al 2015., Wester-Rosenlof et al., 2014). Syncytiotrophoblast extracellular vesicles have been shown to have the ability to carry HbF and also to deposit HbF in endothelial cells (Cronqvist et al). It has been hypothesised that free HbF enters the maternal circulation and contributes to the oxidative stress and damage to the maternal endothelium, thus qualifying as a relevant factor in the development of PE. Levels of free HbF in maternal blood are significantly increased in PE cases compared to controls, with a dose-response association between increasing levels of free HbF and blood pressure elevation, elevated levels of free HbF in cord blood has also been associated with FGR (Olsson et al 2009., Gram et al 2015., Brook et al 2018). As a tool for prediction and diagnosis, the HbF/Hb ratio together with alpha-1-microglobulin (A1M) and Hb/heme have shown promising results (Anderson et al. 2011, Anderson et al. 2016, Kalapotharakos et al 2019, Murtoniemi et al 2019).

Alpha-1-Microglobulin

The endogen protein A1M has an innate capacity to bind heme and free radicals hence providing cellular protective properties (Allhorn et al., Olsson et al 2012., Åkerström et al 2007). Alpha-1-microglobulin protective capacity against ROS has been evaluated in multiple studies, showing effect *in-vitro* as well as *in-vivo* models (Olsson et al 2010., Olsson et al 2007). In the *ex-vivo* placental perfusion model, A1M reversed the tissue damage caused by free HbF (May et al). In cohort studies, A1M has been tested as a predictive tool, elevated levels of A1M during the 1st trimester could indicate who later in pregnancy would develop PE. A similar association, but as a diagnostic tool, has been shown between elevated levels of A1M in term pregnancies with PE compared to controls (Olsson et al 2009., Anderson et al 2011, Kalapotharakos et al 2019, Murtoniemi et al 2019).).

Air pollution

Introduction

Ambient air pollution consists of gases and a range of particles in different fractions based on size: coarse, fine and ultrafine. In 2015, long-term exposure to particulate matter <2.5 PM2.5 μm (PM2.5) was determined to be amongst the top five factors for leading to premature death on a global level (Cohen et al). Approximately 90% of the world population live in areas where air pollution exceeds the World Health Organization's (WHO) limits, and the WHO estimates that approximately 7 million people annually die prematurely due to air pollution (WHO).

The detrimental effects of rising carbon dioxide levels on the global climate has been established for decades, but its ubiquitous and far-reaching consequences have recently become widely and more publicly recognized. Few citizens remain unaware of the messages propagated by the global climate research community, including recent mass-demonstration movements such as Fridays for Future, warning us that the anthropogenic global warming is to blame for the rapidly escalating climate crisis. Arctic ice depletion, droughts, wild-fires, floods, hurricanes, ocean acidification, coral bleaching and wildlife extinction have all been blamed on the rise in global temperatures, driven by increased carbon dioxide levels (Mora et al).

Properties

Complete combustion of fuel would result in minimal release of air pollutants, but with existing technology and fuel types this is not possible. Instead, incomplete combustion results in the creation of numerous air pollutants. These include carbon dioxide (CO_2), particulate matter (PM), carbon monoxide (CO), polyaromatic hydrocarbons (PAH), methane, volatile organic compounds (VOC), black carbon and metals. Studies have shown a reduction in immune cells in human lungs exposed to wood smoke generated by incomplete combustion (Muala et al). The particulate matter is further divided into the sub-categories PM10, PM2.5 and PM0.1 depending on whether the specific diameter is less than 10, 2.5 or 0.1 µm respectively (figure 5). The composition and extent of pollution created depends on the combustion method and type of fuel.

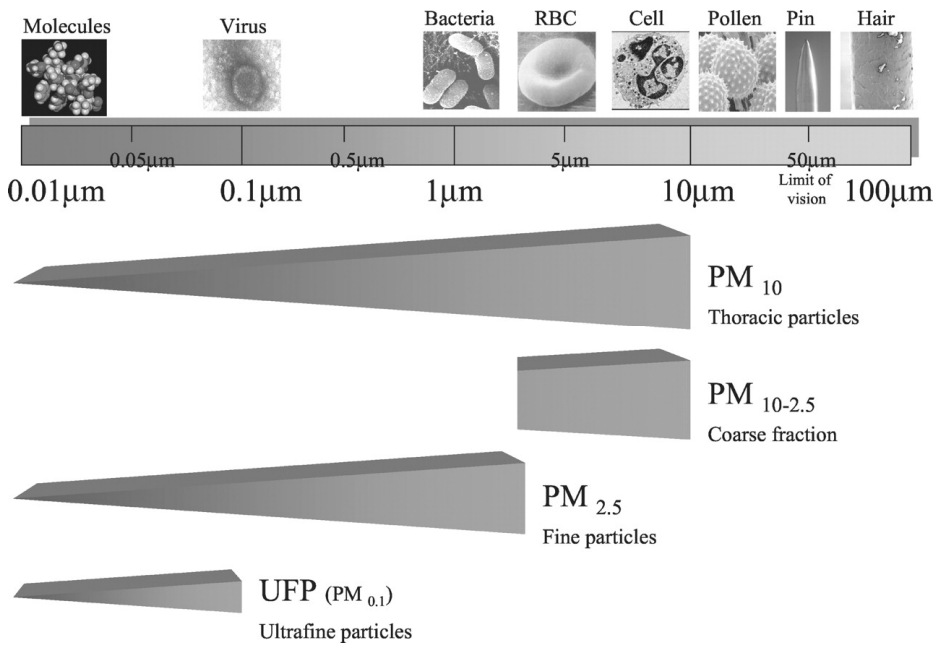

Figure 5. Particle size, definitions and nomenclature. Adapted with permission from Brook et al

Sources and geographical distribution

Air pollution is often discussed in the context of anthropogenic sources – most notably the combustion of fossil fuels in traffic, industry, heating systems and power generation – but air pollution can also be produced by naturally occurring events,

such as volcanic eruptions and bushfires. As a result of climate change, wildfires are predicted to continue to increase on both intensity and frequency (Flannagan et al). In the most hard-hit regions the mortality attributed to pollution is ranging from 151-316 deaths in 100 000 people (figure 6). In low- and middle-income countries (LMIC), another major source of air pollution exposure is smoke from fires used for cooking, heating and trash burning. Indoor air pollution is a health hazard, 4,3 million fatalities each year are due to indoor air pollution (G.B.D.F). Wood, coal and dung are commonly used, often with inadequate ventilation in open hearths or inefficient stoves that result in high level of air pollution indoors in low income countries (LIC). Exposure to emissions from cook stoves using firewood as fuel has been shown to induce pathological changes to placental tissue as a result of hypoxic conditions (Dutta et al).

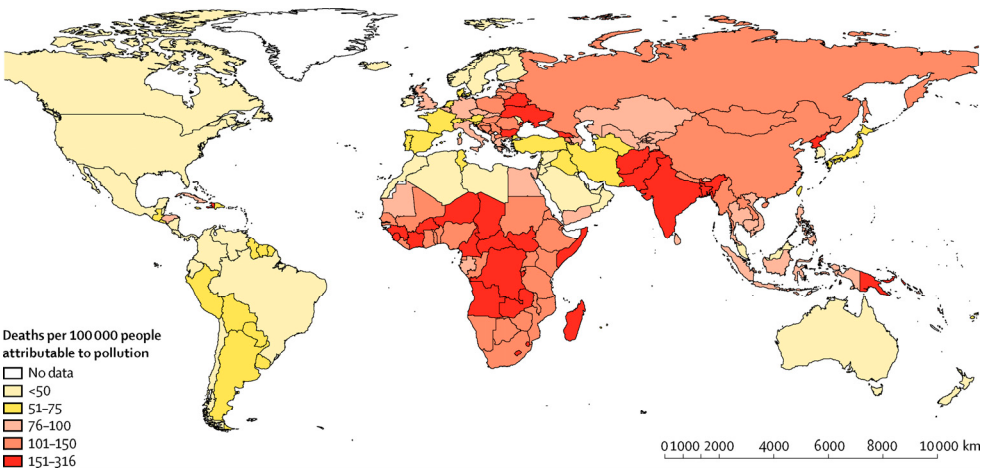

Figure 6. Mortality attributed to pollution. Number of deaths per 100 000 people that are attributable to pollution, 2015, adapted with permission from Landrigan et al.

Effects on human health

The main drivers behind the increased mortality coupled to air pollution are cardiovascular and pulmonary disorders, such as heart disease, stroke, lung cancer and chronic obstructive pulmonary disease (figure 7). Premature deaths in the pediatric population attributed to PM2.5 are associated with lower respiratory tract infections (Cohen et al). Pregnant women and the child they carry are particularly vulnerable to the adverse health effects exerted by exposure to air pollution (Koman et al., Westergaard et al).

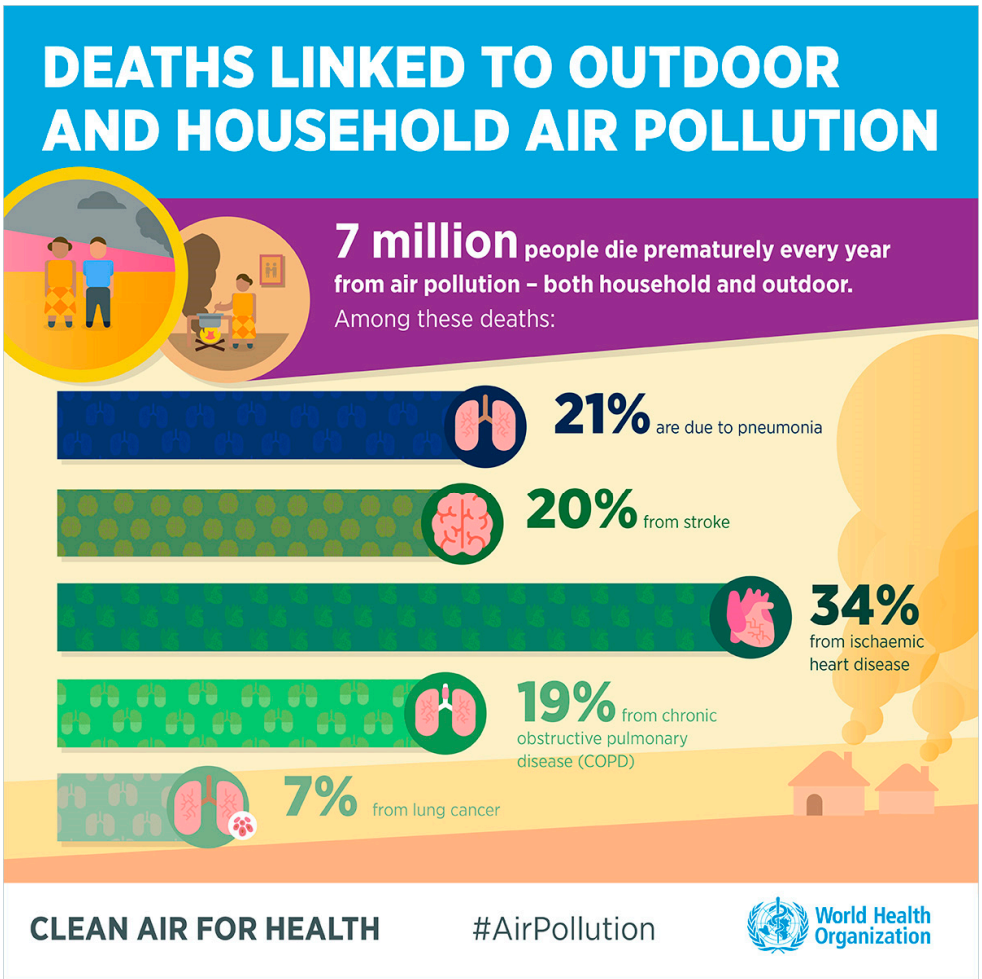

Figure 7. Deaths linked to outdoor and indoor air pollution. Adapted from WHO ambient air pollution.

While many air pollution particles are deposited in the lungs, or captured by pulmonary macrophages, some particles by-pass the respiratory defenses and enter directly into the bloodstream (Kastury et al., Tschernig et al). This is particularly true for fine particulate matter particles, PM2.5, due to their small size (Yixing et al). Once they have entered the bloodstream, particles can enter other bodily tissue, and the effects of this dispersion are still under intense investigation. In this thesis, the focus has been placed on the association between air pollution and negative effects on maternal and foetal health. As will be discussed below, epidemiological studies have already demonstrated a connection between air pollution and pregnancy complications (Malley et al., Pedersen et al 2013), but there is limited

knowledge regarding the mechanisms responsible for this association. Our aim thus is to investigate the molecular foundations linking air pollution and adverse pregnancy outcomes.

Pregnancy and air pollution

Pregnancy is a vulnerable period for both mother and child. Extensive physiological changes take place in the mother to accommodate for the metabolic demands of the growing fetus, with alterations in respiration, hormonal balance and the cardiovascular system (Soma-Pillay et al). The fetus, meanwhile, is undergoing intense development with rapid cellular proliferation. During this period, the placenta holds an essential role to safeguard the fetus and ensure continued growth and development (Burton et al). Although the placental barrier offers protection against harmful substances entering into the maternal blood stream, this defense is by no means complete. Pathogens, drugs and other agents may cross over the blood-placenta barrier and cause adverse outcomes to the developing child (Koren et al., Tetro et al). Adverse pregnancy outcomes such as PE and IUGR share an etiology of a faulty placentation, suspected to be caused by extravillous trophoblasts and an impaired capacity to invade the myometrium during the first trimester of pregnancy. Studies investigating cellular response to PM2.5 and PAH exposure show cell-cycle arrest and reduction in invasive capacity as well as inhibited migration in exposed trophoblast cells (Li et al 2007., Liu et al 2016., Qin et al)

Epidemiology

Numerous epidemiological studies have indicated air pollution as a disruptive force during this finely tuned and sensitive developmental period. Associations have been found between air pollution and negative birth outcomes including prematurity and low birth weight (LBW) (Malley et al., Pedersen et al 2013., Wang YY et al). Gestational complications such as gestational hypertension and diabetes have similarly been associated to air pollution (Malmqvist et al 2013., Pedersen et al 2014., Malmqvist et al 2016), and a multitude of studies have found that exposure to PM2.5 and PM10 during pregnancy is associated with an increased risk of developing PE (Wang Q et al 2018., Lee et al., Dadvand et al 2013., Dadvand et al 2014). The source of air pollution has relevance, with traffic-derived air pollution shown to have the strongest negative impact on pregnancy outcome (Dadvand et al 2014., Pereira et al). However, results regarding air pollution and preeclampsia are not entirely consistent, with some studies reporting no association (Madsen et al., Savitz et al., Wesselink et al). Epidemiological studies are particularly sensitive to confounding factors, and can never demonstrate direct causality. In this setting, possible confounding sources include factors relating to maternal health such as low

or high BMI, low socioeconomic status, gestational diabetes, maternal age under 20 or over 35 and maternal country of origin (Westergaard et al., Malmqvist et al 2013., Khalil et al, Magee et al., Neal et al, Valensise et al., Wright et al). It has also been suggested that these discrepancies could be due to different study settings, as well as the use of various models and methods to assess PM exposure (Mandakh et al). However, it is an established assumption that exposure levels are generally underestimated in the models currently used, hence driving the results towards the null. In other words, the effect of air pollution on adverse health outcome is underestimated.

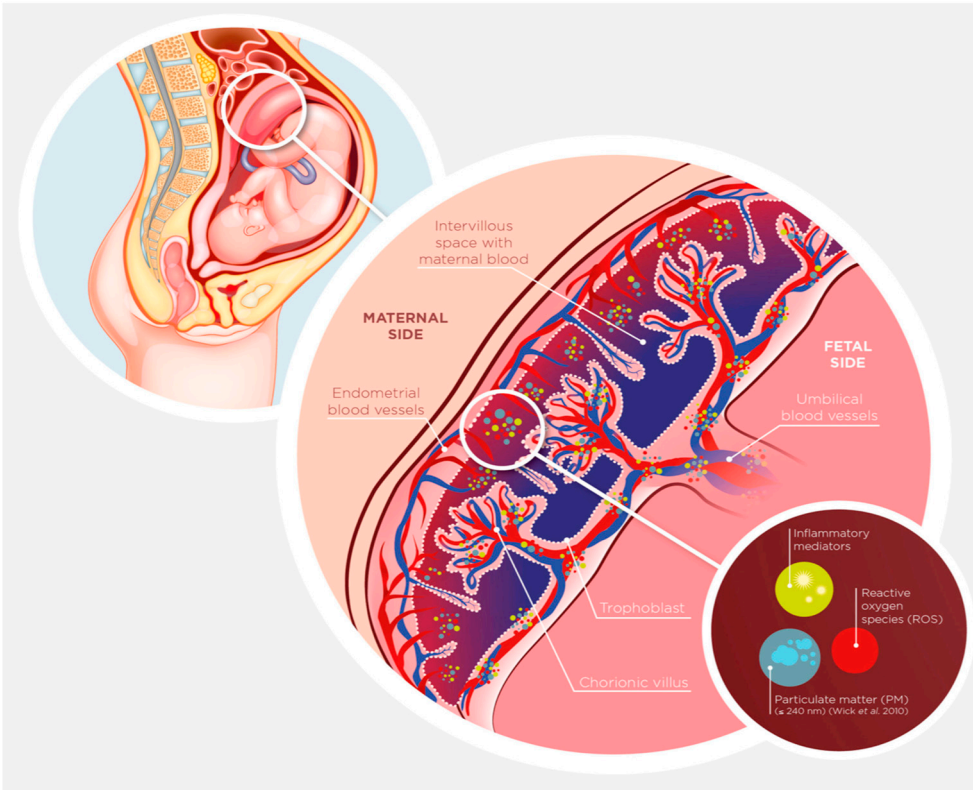

Figure 8. Illustration of PM2.5 effect on the placenta. PM2.5, ROS and inflammatory mediators in the placental circulation. Adapted from Luyten et al.

Biological mechanisms

Supporting the findings from the aforementioned epidemiological studies with experimental and clinical research is vital in order to validate the reported correlations, and in order to elucidate the mechanisms through which this effect is carried out. In animal inhalation studies using rodents, it has been demonstrated that PM reaches the placenta, inducing local effects in the placenta and effecting the fetus (Liu et al., Blum et al). Particulate matter has been seen to induce inflammation in placental tissue and to result in elevated levels of cytokines in the circulatory system (Gurgueira et al., Hougaard et al., Campagnolo et al). In humans, particles have been detected in the circulatory system after less than 1 minute after exposure through breathing (Nemmar et al). On a molecular level, the effects of PM exposure include elevated levels of interleukin 6 (IL-6) and interleukin 1b, both proinflammatory mediators (van Eeden et al). Recently, researchers were able to identify black carbon particles on the foetal side, of the placenta through a histological study, and found the density of said particles to be associated with the maternal level of exposure (figure 8) (Bouvé et al). Numerous studies have reported on air pollution exposure resulting in alterations in placental gene expression, mitochondrial dysfunction and changes to placental mitochondrial DNA content, effects on levels of global DNA methylation, oxidative stress and morphological changes to the endothelium and villi (Saenen et al., Dutta et al., Janssen et al 2012, Clemente et al., Maghbooli et al). More research is needed, and the work of this thesis is an attempt to further scientific knowledge regarding the effects of air pollution on placental tissue.

The present investigation

Aims of the thesis

The overall aims of this thesis were to study the effects of endogenous as well as exogenous sources of oxidative stress on placental health and their potential roles in the development and exacerbation of PE.

Specific aims:

Paper I: 1) To establish a preeclampsia *in-vivo* rabbit model to investigate to role of cell-free HbF in the pathogenesis of preeclampsia and 2) to evaluate the therapeutic potential of A1M to relieve preeclampsia symptoms.

Paper II: To investigate the presence of podocyte-specific proteins and extracellular vesicles in the urine of women with preeclampsia and in the urine from the rabbit model.

Paper III: To determine the effects of fine particulate matter air pollution on trophoblast health in an *in-vitro* exposure trophoblast culture model.

Paper IV: To further investigate the effects of fine particulate matter (PM2.5) on trophoblast survival, mitochondrial function, membrane integrity, hormonal regulation and particle uptake.

Paper V: To determine the effects of wood smoke particles on cellular survival and proliferation using the *in-vitro* exposure trophoblast culture model.

Materials and methods

The planning of any experimental or clinical study requires careful consideration regarding the choice of method and study population in relation to the purpose of the inquiry. There are advantages and disadvantages of every method, and factors such as ethical justification, efficacy, reproducibility and time- and resource-consumption are but a handful of the factors that need to be considered. In this thesis, two main research methods were employed – the preeclampsia rabbit model and the trophoblast cell culture model. Below is a description of each model as well as a summary of the advantages and disadvantages of both. Details regarding the

assays, methods and statistical methods used for data gathering and analysis can be found in the “materials and methods” section of each paper.

The preeclampsia rabbit model

Drawing on previous studies that have indicated HbF as a factor of etiological significance in the development of preeclampsia (see background), we here sought to induce preeclampsia in pregnant rabbits by transfusing them with free rabbit HbF. The aim was partly to validate the theory of HbF being a pivotal etiological factor, but also to create a model through which further research on preeclampsia could be conducted.

Description

Rabbit foetal blood was harvested from rabbit fetuses, following 29 days of gestation. The blood was subsequently centrifuged and red blood cells collected and washed. In order to isolate foetal haemoglobin molecules, red blood cells were lysed and membranes separated through centrifugation, followed by dialysis of the supernatant and additional centrifugation and filtration. The HbF fractions were thereafter collected and endotoxins removed by EndoTrap and endotoxin levels determined by Limulus Amebocyte Lysate.

As a second step, pregnant rabbits were administered the acquired HbF intravenously at a dose of 20 mg/kg at gestational day 20, following three days of acclimatization to the test facility. Thereafter, rabbits were injected with HbF every second day until termination of the study (figure 9). The dose of 20 mg/kg was determined following a pilot study in which a dose of 10 mg/kg did not induce any measurable symptoms and a dose of 40 mg/kg was not well tolerated. Urine was collected and blood-pressure was measured every day, and blood samples were collected every second day, until day 29 when the trial was terminated. Upon termination, the animals were anesthetized, kidney functioned evaluated and organs and fetuses collected.

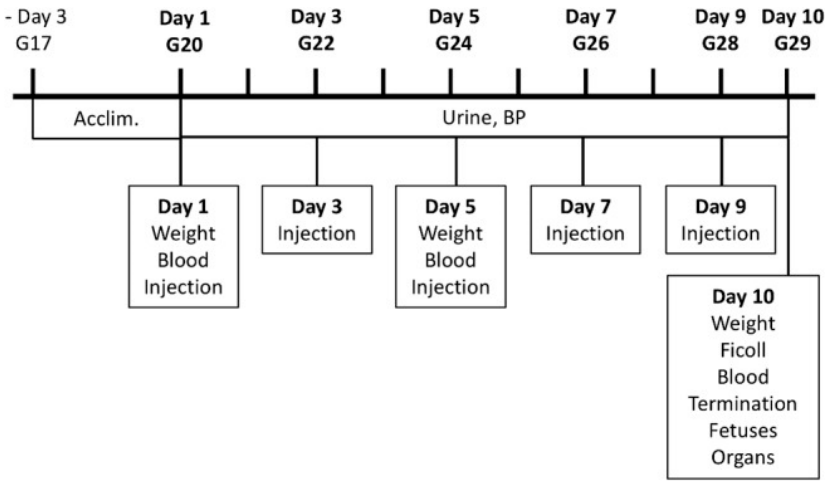

Figure 9. Rabbit PE *in-vivo* model timeline. Dam were treated every second day starting day 20 of gestation. Adapted from Nääv et al, paper I.

Disadvantages

- The study design mimics stage two of PE.
- No hypertension was observed.
- The dams were infused with HbF. In human PE, HbF is suggested to enter the maternal circulation by leakage from the placenta. The administration of HbF intravenously could possibly result in that the model does not fully display the placental tissue damage.

Advantages

- The PE-rabbit model has a gestational period, suitable for administration of HbF.
- The placental structure of rabbits is more similar to human placental structure compared to other rodents.
- The rabbit is a suitable animal model given its size, and it is possible to house multiple dams during the same period and thereby diminishing confounding factors.

The trophoblast cell culture and particle *in-vitro* exposure model

Trophoblasts are cells that compose the outer layer of the blastocyst during embryonal development and that hold a vital role in embryonal implantation and formation of the placenta. The trophoblasts used here, HTR8, are a commercially available human first trimester trophoblast cell line that have been employed extensively in placental research. Although it is possible to harvest trophoblast cells from term placentae, there is a greater amount of uncertainty in using such samples due to variability in yield and genetic heterogeneity. The novel aspect of the usage of HTR-8 in this thesis is the exposure to well characterized pollution particles.

Brief description

Using a high-volume cascade impactor, PM2.5 particles were collected in an urban traffic environment on a polypropylene filter. Particles were subsequently extracted using pure methanol and dried in a vacuum evaporator. Dried PM2.5 were analysed for the presence of PAH using gas chromatography-mass spectrometry and for metal compositions by inductively coupled plasma-mass spectrometry. For the trophoblast culture experiments, dried PM2.5 were dissolved in cell medium, subjected to indirect and direct sonication and diluted to desired concentration. Thereafter, HTR-8 cell cultures incubated with a mixture of cell medium, foetal bovine serum and antibiotics were exposed to the PM2.5 particles and cultured for a pre-determined duration and finally extracted and analyzed.

In paper V, wood logs from four different species were combusted in a wood stove in carefully controlled laboratory settings. Wood smoke particles were gathered on polytetrafluoroethylene filters employing a Dekati Gravimetric Impactor. The particles were analyzed for PAH and metal content using methods as described above.

Disadvantages

- This study used the HTR8 cell line with limited capacity to form a syncytium.
- The HTR8 is an immortalized cell line, it is therefore difficult to draw any extensive conclusion on cytotoxicity.

Advantages

- This study used HTR8 cell line, a cell line that is highly stable in culture and that offers excellent conditions for producing reproducible results.
- The HTR8 is a first trimester cell line, hence displaying properties relevant to investigate the effects of PM during the first trimester.
- A strength of the study is the characterization of PM and PAHs in exposed cells.

Physiological dose

Whilst it is indisputably proven that PM2.5 particles from air pollution can pass over from alveoli in the lungs to the circulation (Kastury et al), the dose that the placenta is exposed to during pregnancy remains unclear. However, histological analysis has in previous studies documented air pollution particles in placental tissue (Bouvé et al, Liu et al 2018), demonstrating that some exposure does occur. Furthermore, as epidemiological studies have demonstrated associations between air pollution and pregnancy complications, it is reasonable to believe that this placental accumulation of pollution particles carries some etiological importance. In an effort to calculate a realistic dose of exposure, the mean ambient PM2.5 rate of Malmö (where particles were collected) was combined with lung PM clearance capacity and a daily exposure dose was estimated to 50-500 ng of PM2.5 per day. In high exposure settings, such as mega cities, the daily exposure is estimated to be the equivalent of 10 000 ng/ml. During pregnancy, plasma volume is increased and there is a 20% increase in consumption of oxygen, resulting in a 40-50% increase in minute ventilation, and while respiratory rate remains unchanged, there is also an increase in tidal volume (Soma-Pillay et al). These pregnancy related alterations in cardio-pulmonary physiology were considered when calculating daily exposure.

Results

Paper I

Renal damage and vascular dysfunction, two of the hallmarks of PE, can be caused by oxidative stress. Numerous particles can contribute to oxidative stress, including extracellular free Hb and its metabolites. As it has been demonstrated that an increased accumulation and synthesis of free HbF occurs in the placenta during PE, it is of interest to elucidate the etiological importance of this molecule. The aim of this study was 1) to create a novel animal preeclampsia-model in which PE is induced through the intravenous administration of HbF and 2) to reverse the oxidative damages by administrating A1M, a known scavenger of cell-free heme groups.

Results in brief

- Pregnant rabbits infused with HbF, starting mid-gestation until term, developed proteinuria and a significantly increased glomerular sieving coefficient, indicating renal damage.

- The above-mentioned changes were significantly ameliorated through the co-administration of recombinant A1M.
- Transmission electron microscopy revealed, intracellular as well as extracellular, tissue damage to both the kidneys and the placenta after administration of HbF.
- Tissue damage to kidneys and the placenta was significantly ameliorated in the group receiving A1M in addition to HbF.
- No significant changes were noted in blood-pressure in the two study groups.

Paper II

Renal podocytes are a group of cells located at the glomerular basement membrane in the kidneys, and due to their position and ability to form a glomerular filtration barrier, they hold a pivotal role in upholding normal kidney function. Previous research has indicated selective damage to podocytes as an important factor in the development of proteinuria seen in preeclampsia. In particular, a reduced expression of the podocyte-specific protein nephrin has been seen in the glomeruli of preeclamptic women. Using urinary samples gathered from pregnant women with and without preeclampsia, this study aimed at exploring this potential link by determining levels of podocyte-specific extracellular vesicles (EV) and proteins. In an effort to find a causative agent for the podocyte damage in preeclamptic proteinuria and renal failure, the preeclampsia rabbit model was employed to determine the possible effect of HbF on podocyte function and protein expression.

Results in brief

- When compared to controls, urine from preeclamptic women displayed a significantly higher levels of nephrin (nephrinuria) as well as a high ratio of podocin-positive to nephrin-positive EVs.
- Plasma concentrations of HbF and A1M were elevated in preeclamptic women.
- Pregnant rabbits exposed to free HbF exhibited increased proteinuria, elevated levels of podocyte-specific EVs as well as endothelial damage to the glomeruli as shown thrown transmission electron microscopy.

Paper III

Ambient air pollution in the form of gases and fine particles is increasingly being recognized as an important risk factor, both for the development and aggravation of many diseases and medical conditions. Pulmonary and cardiovascular disorders are well-recognized in this setting, but pregnancy-related conditions and complications are not exempt, and an association has been proven to exist between levels of air pollution, preeclampsia and preterm birth. Using first trimester human placental trophoblast cells (HTR-8) and exposing them *in-vitro* to pollution particles gathered from an urban environment, this study aimed at discerning any causative links between air pollution and preeclampsia.

Results in brief

- Trophoblast cells exposed to high doses of PM exhibited significantly increased levels of IL-6 and decreased levels of hCG.
- Exposure to PM resulted in trophoblast cells displaying increased endocytosis, as well as reduced cell growth compared to untreated controls.
- Proteomic analysis of cultured cells revealed an altered expression in proteins involved in processes such as inflammation, cellular survival, endoplasmic reticulum stress and molecular transport pathways.

Paper IV

As previously discussed, associative links have been revealed between air pollution and pregnancy-related complications such as preeclampsia and preterm birth. However, this connection has primarily been demonstrated through epidemiological studies, and the understanding of how these detrimental effects are carried out on a molecular basis has remained somewhat undeveloped. The aim of this study was to validate the hypothesized link and deepen our scientific understanding regarding air pollution and preeclampsia. The study was carried out using the previously established model employing trophoblast cultures exposed to various concentrations of air pollution particles for various durations, with a battery of assays and examinations, carried out post-exposure to study hormone regulation, mitochondrial function, membrane integrity and an in-depth histological analysis using TEM.

Results in brief

- TEM analysis revealed cellular uptake of PM2.5 particles and a selective localization of particles to the mitochondria. In addition, exposed cells revealed structural damage to mitochondria, DNA, cytoskeleton and the endoplasmic reticulum.
- Cultured cells exposed to PM2.5 exhibited an increase in production of IL-6 and progesterone and a decrease in production of hCG.
- Increased cytotoxicity was shown in the 48h cohort treated with PM2.5 when compared to controls.

Paper V

Although increasing levels of air pollution is a global problem, the sources and composition of such pollution can vary between different areas. While air pollution in high-income countries is largely due to combustion from traffic and factories, the populations of low-income countries are exposed to a higher degree to wood smoke, often from indoor wood burning. Additionally, different countries' access to and use of forested land as well as prevalence of wildfires can contribute to this variation. While wood smoke is known to exacerbate certain medical conditions, particularly pulmonary ones, little is known regarding its effects on pregnancy and maternal health. Employing the previously established model (III, IV) of exposing cultured trophoblast cells to pollution particles, this study aimed at evaluating the effects of wood smoke particles on trophoblast health.

Results in brief

- Wood smoke particles were visualized inside trophoblast mitochondria following *in-vitro* exposure, and structural changes were noted in mitochondria as well as ER and membranes.
- Cells exposed to wood smoke particles exhibited an increased production of IL-6 and a decreased production of hCG.
- Cytotoxicity was increased in cells treated with wood smoke particles compared to controls.

Discussion

Oxidative stress is a key element in the development and maintenance of PE. This thesis explores two sources of oxidative stress: HbF and PM2.5. Given the complexity of PE, a number of factors are thought to interact, eventually giving rise to maternal symptoms and subsequently to the diagnosis of PE (figure 10). In chronology the events are thought to occur through predisposing factors, initial placental events, inadequate placentation, reduced uteroplacental perfusion pressure (RUPP), circulating factors, endotheliosis, systemic and local tissue damage and clinical manifestations (along blue arrow figure 10).

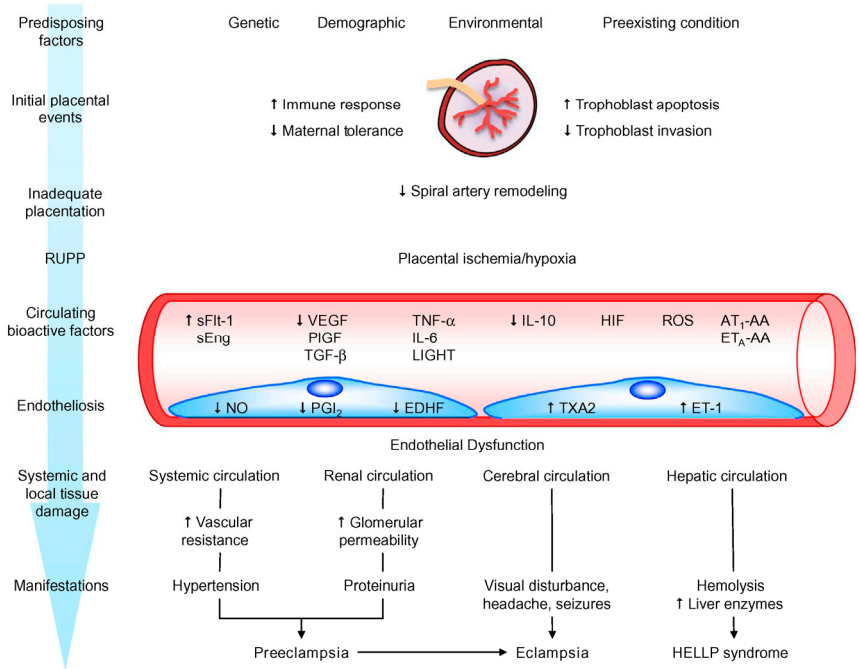

Figure 10. Multifactorial aetiology of preeclampsia. Predisposing factors, genetic, dempgraphic, envirommental and preexisting conditions, affecting initial placental events entaling immune respone, maternlal immunotolerance, trophoblast apoptosis and trophoblast invasion with subsequent inadequate placentation and immpaired spiral artery remodeling. Reduced uteroplacenal perfusion pressure results in placenal ischemia and or hypoxia. This is followed by the realse of circulationg bioactive factors causing endotheliosis gving rise to endothelial dysfunction. The systemic and local tissue damage caused by the ednothelial dysfunction effects systemic-, cerebral-, hepatic- and renal citcualtiond eventually giving rise to the clinical conditions; preeclampsia, eclampsia and HELLP syndrome. Adapted with permission from Possomato-Vieira et al.

Air pollution is a global threat to human health and the climate. It respects no national borders and can disperse over large areas. When there is a western wind, pollutants originating from China have been detected in the USA (Peuschel et al). Ambient air pollution and indoor air pollution are often categorized as two different entities due to their different origins. However, the two types of air pollution are similar in composition and often co-exist in LMIC (Hogg et al., Hollingsworth et al).

The graded response paradigm has long been central in scientific understanding of how oxidative stress exerts cellular toxicity. This entails the idea that a physiological response would be the result of low levels of oxidative stress, while high levels of oxidative stress would induce cellular toxicity (Xia et al). The current understanding of oxidative stress and its counterpart antioxidants make up the redox system. Reactive oxygen species and reactive nitrite species to mention a few, have complex roles as regulators of inflammation and mediators of cell signalling (Daiber et al., Lambeth et al., Nathan et al). This also holds true for the oxidative stress effects of air pollution and their subsequent effects on health (Li et al, Newby et al). Hence, the response to air pollution exposure does not only include the effects of the actual particles but also the downstream impact and/or alterations of endogenous redox signalling. These downstream effects have been stipulated to result in aggravated oxidative stress due to potentiated endogenous ROS. Amongst endogenous sources of ROS such as NOS, cytochrome P45O and the respiratory chain (Lambeth et al., Nathan et al., Wende et al), including HbF in paper I and II HbF, are identified as an additional important source of endogenous ROS. Building on previous findings, where a rat HbF-perfusion model reported oxidative stress -induced renal injury with increased glomerular permeability (Sverrisson et al) the rabbit PE model was established (paper I). The dams infused with HbF developed proteinuria and excreted podocyte EVs, consequently providing a substantial connection between the presence of HbF and podocyturia typical to PE. In addition, the dams displayed morphological changes in podocyte structure with mitochondrial swelling and ER disruption. In paper II, a nested case control study, a cohort of normotensive and preeclamptic pregnancies showed a significant difference in the quantity of podocyte EVs in urine in cases compared to controls. In plasma, cellfree HbF and A1M were significantly higher in the target cases, when compared to controls. These findings are in line with the A1M properties as a scavenger binding free heme, hence being upregulated as a response to the increased levels of HbF. Additional endogenous defense systems – haptoglobin and hemopexin – were significantly decreased in plasma in cases compared to controls. In the rabbit PE-model (paper I), the structural damage to placental and renal tissue was alleviated when treated with A1M. The opportunities for therapeutic avenues using A1M, given its properties as a scavenger, reach beyond the scope of finding a therapy for PE.

An essential question to ask when conducting exposure studies *in-vitro* is in what manner the results are to be interpreted. The ideal use of cell culture is to identify isolated mechanisms and pinpoint pathways. The leap to drawing overarching

conclusion on a systemic level can be bold. In fact, the actual exposure level of PM2.5 for the placenta is unknown. The study design in the trophoblast exposure model (paper III-V) attempts to mimic the exposure levels relevant to those of pregnant women. 5,000 ng/ml is assumed to correspond to PM2.5 concentrations of 25 $\mu g/m^3$, (WHO target for short term exposure), and 10,000 ng/ml is assumed to correspond to 50 $\mu g/m^3$, estimated to mirror the levels in a more polluted city. In paper III we show that within a short time period (30 minutes) after exposure, the PM is being internalized through endocytosis. This observation is further supported by the upregulated expression of proteins for transport and endocytosis. Long-term exposure to PM resulted in inhibited cell growth. Paper III and paper IV examined the effects of ambient air pollution on protein secretion, whereby hCG and IL-6 were altered in both studies. However, progesterone showed diverging results with no difference between exposed cells and controls in paper III. The same study protocol was followed in paper IV, although with a higher n-number and exposure doses in a range closer to urban environment with high levels of ambient air pollution. Progesterone secretion was significantly altered in paper IV.

In paper III-V the trophoblasts exposed to PM evinced altered hormonal response and inflammatory profile. This signifies that the sole effects of the PM, in an *in-vivo* setting, is probably sum of effects of the exogenous PM exerted on other tissues such as lung, vascular endothelium and renal endothelium potentiates the impact of oxidative stress. Other methods, beyond single cell culture, could be of interest with organoid culture closest at hand. *In-vivo* models of exposure using rodents have obstacles in extrapolating results to the human physiology, mainly due to differences in filtering systems and anatomy (Rao et al).

As discussed in this thesis and prior work, air pollution is composed of a heterogenous mixture of substances. The composition is affected by source, time and atmospheric conditions (Brook et al). These in turn, eventuate in different degrees of toxicity based on size, solubility, structure and surface reactivity to name a few (Nel et al., Oberdorster et al 1994, Oberdorster et al 2005, Rivera et al). Amongst the soluble components, PAH are generated by combustion of organic substances and has been shown to be associated with a number of adverse health outcomes. An interesting finding in this thesis, in paper IV and V, is the presence of PAHs inside trophoblasts, indicating that PAH exerts their effect on an intracellular level.

Several health conditions with endothelial dysfunction and/or activation, such as atherosclerosis and diabetes type II, share the triangulation between ER stress, oxidative stress and inflammation. (Hotamisligil et al., Lee et al). The cell has a protection system to manage misfolded proteins – the unfolded protein response (UPR) (Walter et al). In line with previous studies (Hettiarachchi et al), in paper III we show that PM induces ER stress and disturbances in ER phagosome interactions through the expression of TMX4, REEP1 and TNFAIP8 proteins.

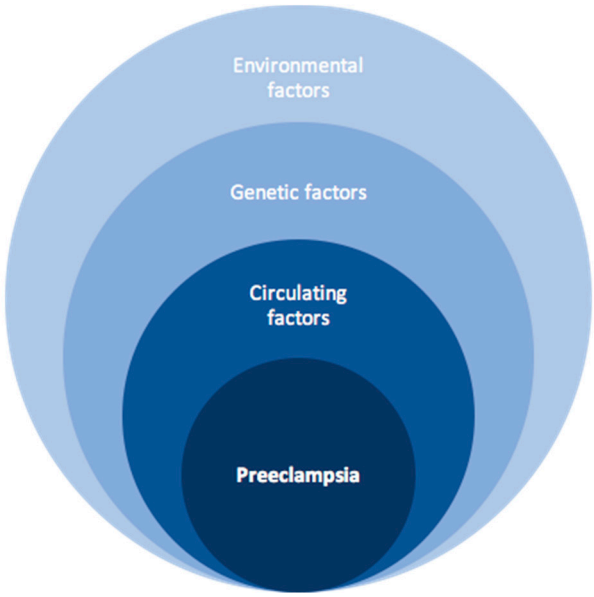

Figure 11. Conceptual image of PM effect on PE. Environmental factors ambient and indoor air pollution, genetic predisposition, circulating factors HbF.

Air pollution, poverty, and environmental injustice

On a global level, deaths due to air pollution exposure have an uneven distribution, strongly linking air pollution exposure to poverty. In 2015, 99% and 89% of deaths attributed to indoor air pollution and ambient air pollution respectively occur in LMIC (The world bank). Although densely populated areas, such as mega-cities are sites with staggering levels of ambient air pollution, the issue of ambient air pollution is ample and urgent in LMIC. Close to all, 98%, of all urban areas with more than 100 000 inhabitants in LMIC do not succeed in meeting the WHO standards, <10 μg/m^3 of PM2.5 per year (The World Bank).

Roughly 3 billion people in LMIC rely on wood, charcoal and other types of biomass, for instance, dung, to provide heat, light and means of cooking. In addition to indoor pollution being strongly linked to poverty, it is also associated with gender inequality. Compared to an urban population within the same socioeconomic bracket, rural women and children suffer from a disproportionate exposure to indoor air pollution. Traditional stoves, lack of ventilation and the use of biomass as fuel are contributing factors. The pungent association between poverty and indoor air pollution results in morbidity and premature deaths for women and children in the poorest countries of the world (Yadama et al).

It cannot be stressed enough; the world stands to confront an enormous challenge to human and planetary health (Withmee et al). Notwithstanding, alarming effects on human health and the environment, scanty measures have been taken by the global, national and local leadership to counteract this worrying development. In particular in LMIC, the outlook is vexing, with an impending large-scale industrial expansion, accompanied with scarce legislation, strategies and funding for emission and chemical pollution control (Greenberg et al, Nugent et al).

Conclusions

Preeclampsia is one of the main causes of maternal-and neonatal morbidity and mortality, and this thesis attempts to elucidate the impact of HbF, an endogenous driver of oxidative stress and air pollution, an exogenous driver of oxidative stress. In the rabbit PE-model, foetal haemoglobin has been shown to cause OS induced PE-typical lesions to kidney and placental tissue and functional disturbances to the glomeruli. In a human PE nested cohort study, similar urinary findings were established in addition to elevated levels of HbF and A1M in plasma. The impact of PM, originating from four different sources, on first trimester extravillous trophoblasts were investigated. The results showed dysregulation in hormonal secretion, cellular toxicity, ER stress, mitochondrial interference and enhanced inflammatory response.

The effects of air pollution on adverse health outcomes for mother and child cannot be overlooked. Part of the exposure studies were conducted with particles collected in real-life conditions in Malmö, Sweden. This thesis could thus be seen to mimic actual exposure conditions and PM properties. The findings are suggestive of mechanisms contributing to increased levels of oxidative stress and inflammation in placental cells, offering a foundation for further investigations regarding negative effects of air pollution during pregnancy.

Future perspectives

There is currently a lack of animal models that fully depict PE (Erlandsson et al). To some extent, this seriously limits our understanding of the syndrome. However, in recent years omics technologies have offered new avenues for furthering our understanding of PE and the complex relationship between air-pollution and PE. In addition, organoid cultures with human cells would be highly interesting in this context. In brief, this offers several new challenging questions for the research community to address.

Biological mechanisms

Deepen the understanding of the biological mechanisms behind the epidemiological correlation between air pollution and PE:

- Use different type of trophoblast cell lines in exposure studies. There are BeWo cell lines that resemble 3rd trimester trophoblasts which could be made to form a syncytium by cyclic-adenosine monophosphate treatment. Future studies that aim to investigate the effect of PM during the late stage of pregnancy and possible mechanisms in late onset PE could use the BeWo cell line.
- Alternatively, empirical work could draw on the dual-placental perfusion model. However, since the dual-placental perfusion model uses human term placenta, it must be considered that these placental will have undergone exposure to PM *in-vivo.*
- Use organoid culture when investigating placental health over time in exposure experiments. The accuracy of extrapolated to humans is likely higher in organoid culture than in exposure studies in animal models, as they often entail very high levels of PM2.5 and the placental structure of rodents differs from that of humans. To identify causal pathways and investigate those as potential targets for therapeutic or preventive strategies.

Air pollution toxicity

Identify the toxicity of air pollution pertaining to content and source:

- Differentiate and investigate the effect of components of air pollution (PAH, carbon, metals) on trophoblast cells.
- Engage in expanded particle analysis for bioaerosol in order to detect endotoxins. This could deepen our understanding of the inflammatory and innate immune response.
- Analyze the effect of different fractions of PM, including ultrafine particles.

Populärvetenskaplig sammanfattning

Havandeskapsförgiftning drabbar 3–8% av alla graviditeter och skördar varje år uppskattningsvis 76 000 kvinnors liv globalt. Havandeskapsförgiftning är en av de främsta orsakerna till mödra- och spädbarnsdödlighet i världen. Tillståndet kännetecknas av högt blodtryck och organpåverkan, och antas bero på en nedsatt funktion i moderkakan i kombination med faktorer i moderns blodomloppet. I svårare fall kan även andra organsystem påverkas, vilket ibland kan resultera i allvarliga krampanfall. Fostret kan också drabbas, och vid havandeskapsförgiftning är risken för tillväxthämning och förtidsbörd påtagligt ökad jämfört med okomplicerade graviditeter.

Havandeskapsförgiftnings uppkomst och utveckling utifrån ett cellulärt perspektiv, samt vilka mekanismer som är involverade, är dock höljt i ett visst dunkel, trots intensiva forskningsinsatser.

Mycket tyder på att havandeskapsförgiftning är intimt kopplat till det samspel som råder tidigt i graviditeten mellan livmodervägg och moderkakan; centrala mekanismer är oxidativ stress och inflammation. Oxidativ stress uppstår när reaktiva syreföreningar, sk syraradikaler, bildas och orsakar skada på celler och vävnader. Sådana syreföreningar bildas naturligt i kroppen vid olika processer, bland annat som del av immunförsvarets skydd mot infektioner, men dessa processer kan också komma i obalans och orsaka skada. Bland annat kan oxidativ stress skada kärlväggar vilket leder till bristande kärlfunktion vilket leder till generella organskador. En konsekvens av ovissheten kring uppkomsten av tillståndet är att man fortfarande inte har någon bot för havandeskapsförgiftning och inte heller någon effektiv metod för tidig upptäckt. Idag finns endast behandling för förhöjt blodtryck och vissa symptom allteftersom de tillstöter. Någon egentlig bot finns inte, att avbryta graviditeten ät enda behandlingen som ofta innebär att man kan behöva förlösa i förtid för att förhindra komplikationer.

Denna avhandling syftar till att öka kunskapen kring de grundläggande mekanismerna som utlöser och förvärrar havandeskapsförgiftning i hopp om att i framtiden kunna förebygga, tidigt upptäcka, behandla och i bästa fall bota detta vanliga och farliga tillstånd. Utgångspunkten är att undersöka inifrån kommande, endogena, och utifrån kommande, exogena, faktorer som bidrar till oxidativ stress. Del A i avhandlingen innefattar artikel I och II och belyser den endogena faktorn fetalt hemoglobin och dess effekt på moderkakan och njurarna. Studierna har utförts

i en kanin-modell och som en kohort-studie av blod och urin från gravida kvinnor. Det har i tidigare studier visats att produktionen av fetalt hemoglobin ökar i moderkakor hos kvinnor med havandeskapsförgiftning. Denna ökning leder till att moderkakan tar skada och barriären mellan den maternella och fetala blodcirkulationen störs, vilket får till följd att moderkakan frigör ämnen som orsakar oxidativ stress. Det fetala hemoglobinet leder även till skada på insidan av blodkärlens väggar vilket i sin tur leder till ökat blodtryck och skador i njuren med proteinläckage. I kaniner ses samma typ av vävnadsskador i moderkaka och njure, studien har även visat hur dessa skador kan reverseras vid tillförsel av alfa-1-mikroglobulin, ett kroppseget protein som har känd förmåga att stoppa oxidativa processer. Kvinnor med havandeskapsförgiftning uppvisar förhöjda nivåer av fetalt hemoglobin och alfa-1-mikroglobulin i sin blodcirkulation samt läckage av podocytvesiklar i urinen, små avknoppade blåsor från njurens celler.

Del B av avhandlingen innefattar artikel III-V och syftar till att undersöka de biologiska mekanismer som tros ligga bakom sambandet mellan exponering för luftförorening och den ökade risken att utveckla havandeskapsförgiftning under graviditeten. Tidigare epidemiologiska studier har fastställt ett statistiskt signifikant samband mellan exponering för luftförorening under graviditet och ökad risk för en rad graviditetsrelaterade tillstånd, däribland graviditetsdiabetes, prematurbörd och havandeskapsförgiftning. Hur detta samband uppstår på cellulär nivå är relativt outforskat men antas, i likhet med fetalt hemoglobin, drivas av oxidativ stress. I cellstudier med placentaceller från en cellinje har fyra olika typer av luftföroreningspartiklar undersökts med hänsyn till celltillväxt, hormonsekretion, inflammation och strukturella aspekter av organeller. Partiklarna härrör dels från urbana miljöer, dels från vedeldning med olika förbränningsgrad. Resultaten visar att moderkaksceller som har exponerats för partiklar uppvisar ett ökat inflammatoriskt svar, rubbad hormonsekretion och hämmad celltillväxt i jämförelse med kontrollceller som har odlats under samma förutsättningar men utan tillsatta partiklar. Vidare visar studierna att luftföroreningspartiklarna tar sig in i cellerna och uteslutande återfinns i mitokondrier - de organeller i cellen som producerar energi i form av ATP. Dessa rön underbygger den epidemiologiska forskning som har visat att låg luftkvalitet har negativa hälsokonsekvenser för både mamman och det ofödda barnet.

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