Gene and Protein Profiling of the Preeclamptic Placenta

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Gene and protein profiling of the preeclamptic placenta.

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Gene and protein profiling of the preeclampic placenta

Abstract

Aims

State-of-the-art methodology was used to screen and profile the placenta, gene and protein expression, for changes related to preeclampsia (PE) and cases with increased resistance in the uterine arteries. Women with increased resistance in the uterine arteries have increased risk of developing PE. Since not all of them develop PE, this group, identified by Doppler ultrasound, was included to search for genes and/or proteins that may protect them from developing PE.

Results

The PE placenta showed increased gene expression of fetal hemoglobin (Hb). Protein expression analysis confirmed the accumulation of free Hb, particularly the gamma chain was detected in the vascular lumen. Patients with increased resistance in the uterine arteries, expressed as a notch in blood velocity tracings recorded with Doppler ultrasound. Notching without PE, showed increased expression of genes related to apoptosis and antigen presentation in their placentas. In the notch placentas that later developed PE, an increased expression of genes related to inflammatory cell movement was seen. Antibody microarray screening of maternal plasma showed that late and early onset PE as well as PE with notching and IUGR showed different inflammatory responses.

Conclusions

The changes in gene expression suggested that PE may be a three-stage disease with notch as a reversible middle stage. Accumulation of inflammatory cells in the notch placenta may cause inflammation that drives the pathophysiology into PE. Increased expression of antigen presenting genes may protect the notch placenta from pro-inflammatory damage thereby preventing progression into PE. Free fetal Hb was identified as a possible placental factor that further induces inflammation and tissue damage. Increased maternal plasma levels of free fetal Hb may be used as a prognostic and diagnostic marker for PE. The maternal immune reaction and inflammatory response may be important factors that further determine the severity and the clinical manifestations of PE.

Key words: Microarray, 2D-PAGE, antibody-array, hemoglobin, APOA1, antigen presentation, immunohistochemistry, in situ hybdridization

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To my loved ones

“The more things change, the more they stay the same.”
– Jean-Baptiste Alphonse Karr, Les Guêpes, Janauary 1849.

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Abbreviations

1D-PAGE – one-dimensional polyacrylamide gel electrophoresis
2D-PAGE – two-dimensional polyacrylamide gel electrophoresis
AP – alkaline phosphatase
APOA1 – apolipoprotein A1
AUC – area under the curve
BASE – bioarray software environment
BSA – bovine serum albumin
CASP – caspase
cDNA – complementary deoxyribonucleic acid
CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
cRNA – complementary ribonucleic acid
DAVID – database for annotation, visualization, and integrated discovery
DNA – deoxyribonucleic acid
ELISA – enzyme-linked immunosorbent assay
EPE – early onset preeclampsia
EVT – extravillous trophoblast
GO – gene ontology
Hb – hemoglobin
HbA – adult hemoglobin (2α and 2β chains)
HbF – fetal hemoglobin (2α and 2γ chains)
Hbα – hemoglobin alpha
Hbγ – hemoglobin gamma
hCG – human chorionic gonadotropin
HELLP – hemolysis, elevated liver enzymes, and low platelets
HLA – major histocompatibility complex (human leukocyte antigen)
HO – heme oxygenase
HP – haptoglobin
HPX – hemopexin
IEF – isoelectric focusing
IgG – immunoglobulin G
IL – interleukin
IUGR – intrauterine growth restriction
KEGG – Kyoto encyclopedia of genes and genomes
LPE – late onset preeclampsia
MAPK – mitogen-activated protein kinase
MALDI-TOF – matrix-assisted laser desorption/ionization - time of flight
MES – 2-(N-morpholino)ethanesulfonic acid
MOPS – 3-(N-morpholino)propanesulfonic acid
mRNA – messenger ribonucleic acid
N – notching (without preeclampsia)
PAGE – polyacrylamide gel electrophoresis
PE – preeclampsia (without notching)
PEwN – preeclampsia with notching
PIN – preeclampsia with notching and intra-uterine growth restriction
PP13 – placental protein 13
PVDF – polyvinylidene fluoride

RNA – ribonucleic acid
ROC – receiver operating characteristics curve
ROS – reactive oxygen species
rt-PCR – real-time polymerase chain reaction
SDS – sodium dodecyl sulphate
sENG – soluble endoglin
sFLT1 – soluble FMS-like tyrosine kinase 1
SVM – support vector machine
TGFB1 – transforming growth factor beta 1
TNFα – tumor necrosis factor alpha
TPM1 – tropomyosin 1
UTP – uridine 5'-triphosphate
VEGF – vascular endothelial growth factor

Introduction

Preeclampsia (PE) affects 3-7% of all pregnancies worldwide (1), which amounts to roughly 8,000,000 cases per year, making PE the major cause of maternal mortality and morbidity. There are several risk factors associated with PE. Primigravidas have almost three times the risk to develop PE in their pregnancy (2). Women who had PE in the first pregnancy have seven to eight times increased risk of developing PE in their second pregnancy (2). Multiparous women that become pregnant with a new father have the same risk as in their first pregnancy (3). The ethnicity also affects the risk of developing PE. African and Afro-American women have generally a higher risk of developing PE (4). Other risk factors for PE include chronic renal disease, chronic hypertension, obesity and diabetes. Clinically, PE manifests after 20 weeks of gestation with hypertension (above 140/90 mmHg) and proteinuria (above 0.3 g/l) (5, 6). The symptoms of PE are diverse and include diffuse signs such as swelling, headache and abdominal pain to mention a few. There are no exact means of diagnosis and more importantly, there is no cure. The sole curative treatment for PE is delivery and removal of the placenta. This suggests that the placenta is the main cause of the disease. However, in PE there are two patients to consider, the mother and the fetus. It is sometimes necessary to deliver the baby prematurely in order to cure the mother, in fact PE accounts for 15% of all premature births. Thus the art in taking care of PE patients is to optimize the situation for the mothers while extending the pregnancy as far as possible.

The Placenta

Since delivery with removal of the placenta is curative, the placenta is considered the main cause of the disease. The formation of placenta, or placentation, begins as the blastocyst is implanted into the uterine epithelium – decidua. The blastocyst is surrounded by a layer of cells that anchor the blastocyst to the uterine epithelium. After implantation cytotrophoblasts begins to invade the uterine stroma in order to locate the maternal spiral arteries. These extravillous trophoblasts (EVT) invade deep into the maternal tissue throughout the first trimester. After approximately eight weeks the EVTs have reached the maternal spiral arteries (7). The EVTs that reach the spiral arteries are termed endovascular trophoblasts. These trophoblasts clog the maternal arteries to hinder maternal blood from reaching the developing intervillous space, thus maintaining a hypoxic environment at the implantation site, this in order to drive fetoplacental vascular development. At the end of the first trimester the endovascular trophoblast plugs disappear and maternal blood enters the intervillous space, providing oxygenated blood to the placenta. Another set of EVT are the interstitial extravillous trophoblasts that invade 3-5 mm into the vessel walls of maternal spiral arteries where they remodel the artery walls by replacing the smooth muscle cells with fibrinoid matrix. The remodeling of spiral arteries prevents these vessels from constricting, creating a placental vasculature characterized by low-resistance and high blood flow (Figure 1A). The villi, where gas exchange takes place, are comprised of two layers of cytotrophoblasts/syncytiotrophoblasts which together with the vascular endothelium constitute the blood-placenta barrier, separating the maternal and fetal blood circulations. The inner layer is made up of the remnants of the original invading cytotrophoblasts.

The main function of the placenta is to ensure gas exchange and provide the nutritional needs to the fetus. Oxygen diffuses over the placental barrier whereas larger molecules such as glucose, lipids, and amino acids require active transport into the fetal circulation. The transport is facilitated by specific membrane transporters, the glucose transporters 1 and 3, the

system A and I transporters for neutral amino acids, the cationic amino acid transporters 1-4, as well as several high-affinity sodium dependent amino acid transporters (8-11). The placenta barrier functions as an immune barrier protecting the fetus from the maternal immune system, it also prevents harmful molecules from crossing over from the maternal circulation to the fetus. For example, stress hormones, such as norepinephrine and serotonin, are actively taken up and degraded by the placenta (12-14).

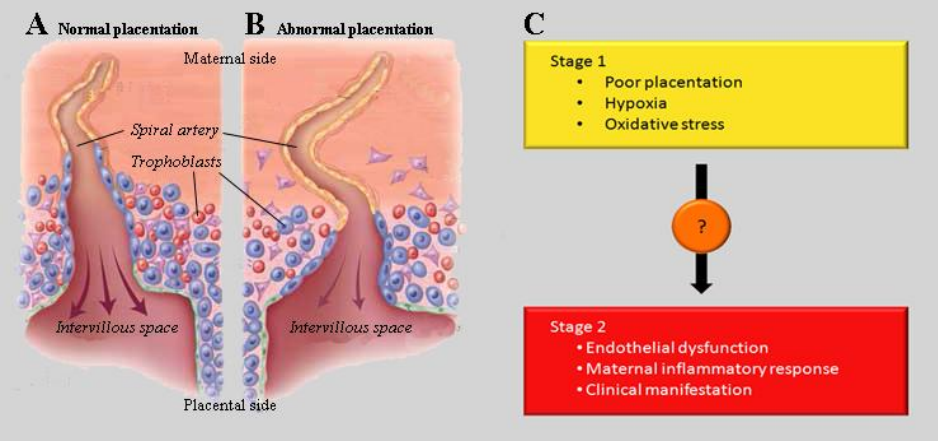

Figure 1. A schematic overview of spiral artery remodeling. A) In normal placentation the trophoblasts invade deep into the maternal endometrium. After replacement of vascular smooth muscle by a trophoblast lining, the vessels are fully dilated resulting in high blood flow. B) In PE there is abnormal placentation and the trophoblasts do not invade as deeply as in normal placentation resulting in impaired blood flow. C) The two-stage PE model suggests PE begins with poor placentation, leading to underperfusion of the placenta. The second stage is the maternal response characterized by inflammation and endothelial dysfunction. Modified from Redman et al. 2005 (15)

Furthermore, the placenta also acts as an endocrine organ. As early as four days after fertilization the trophoblasts begin to synthesize human chorionic gonadotropin (hCG) to ensure proper implantation. During pregnancy the placenta synthesizes several hormones such as placental growth hormone and epidermal growth factor, estrogen and progesterone (16-18). The endocrine functions of the placenta help to ensure that the fetal demands are met, by increasing the utero-placental blood flow and the maternal heart rate. The placenta also produces hormones for fetal growth and development, including human placental lactogen (hPL) and corticotrophin-releasing hormone (19).

The placenta is crucial for a successful pregnancy but when it is dysfunctional, it is also responsible for the most common and severe pregnancy related disease: preeclampsia (PE).

Preeclampsia

Today, PE is believed to be a two stage disease (20) where the first stage involves abnormal placentation. Normally trophoblasts invade deep into the maternal spiral arteries to create the

necessary high flow, low resistance vascular bed (Figure 1A). In PE the trophoblasts only invade half the distance of the normal placenta (Figure 1B). Hence, the PE placenta is thought to be under-perfused and thereby hypoxic (21, 22). Due to the abnormal placentation the spiral arteries retain their ability to contract which is thought to cause sporadic re-perfusion of the hypoxic and ischemic areas (23). The repeated reperfusion leads to irregular oxygen tension which in turn results in formation of reactive oxygen species (ROS) and a state of oxidative stress occurs. The oxidative stress damages the placental cells and thereby induces an inflammatory response. When the inflammation affects the vascular endothelium, the blood-placenta barrier is broken, allowing placental, and fetal, cells and cell debris to leak into the maternal circulation (24-26).

Poor placental perfusion is believed to trigger the second stage of PE: the maternal reaction and manifestation of the typical clinical symptoms: hypertension and proteinuria. The maternal symptoms are caused by general inflammation of the maternal vascular endothelium compromising organ functions. Several placental factors linking the two stages have been suggested (as summarized below), but the complete mechanisms connecting stage 1 with stage 2 are still unclear (Figure 1C). There have been numerous studies focusing on identification of the exact mechanisms responsible for the maternal inflammatory state in PE, although these studies remain inconclusive. Several inflammatory markers such as tumor necrosis factor α, interleukin (IL-) 6, 8, and 12 have been reported to be increased in PE. (27-30), However, there are also contradictory reports showing these markers to be unaltered (29, 31).

Recently, biomarkers for predicting and diagnosing PE have been discovered and three of them are of particular interest. The soluble FMS-related tyrosine kinase 1 (sFlt), is a soluble form of the vascular endothelial growth factor (VEGF) receptor. sFlt is a potent angiogenetic factor, acting as an antagonist of circulating VEGF. Studies have shown both increased gene expression in the placenta and high protein levels of sFlt in plasma (32, 33). Increased plasma sFlt appears before clinical manifestation of PE, and after delivery sFlt levels drop to normal in parallel with regression of PE symptoms (34). When injected into pregnant rats, sFlt causes the classic PE symptoms: hypertension and proteinuria. Since sFlt gene expression and synthesis are induced by low oxygen tension, it has been speculated that the hypoxic environment in the PE placenta induces the production (35).

A second factor that is increased in the PE placenta and plasma is endoglin (36, 37). Endoglin is a transmembrane protein with both an extracellular and an intracellular domain. Soluble endoglin (sEng) is detected in the maternal circulation when the extracellular endoglin domain sheds from the vascular endothelium in the placenta. The effects of sEng, shown in animal models, are hypertension and increased vascular permeability. Thus, sEng has been proposed to work in combination with sFlt to drive PE.

A third, recent, marker of PE is the placenta protein 13 (PP13). In contrast to sEng and sFlt, PP13 gene expression has been shown to be decreased in the PE placenta (38). Moreover, PP13 plasma levels do not correlate well with PE at term. However, decreased plasma levels of PP13 in early pregnancy seem to correlate with adverse pregnancy outcome in terms of PE (39).

Uterine artery notching and intrauterine growth restriction

Defective placentation can in some cases result in increased resistance in the uterine arteries. Clinically, this can be detected by Doppler ultrasound as early as 12-13 weeks in gestation

(40). The increased maternal resistance to flows shows a “notching” pattern when recording the blood flow velocity in uterine artery (Figure 2). Increased uterine artery resistance has been linked to increased risk of developing PE later in pregnancy (41, 42). Should PE manifest in combination with bilateral notching the pathology of the placenta is further compromised (43). In general, the earlier the onset of PE, the more severe is the placental pathology and commonly also manifests with IUGR.

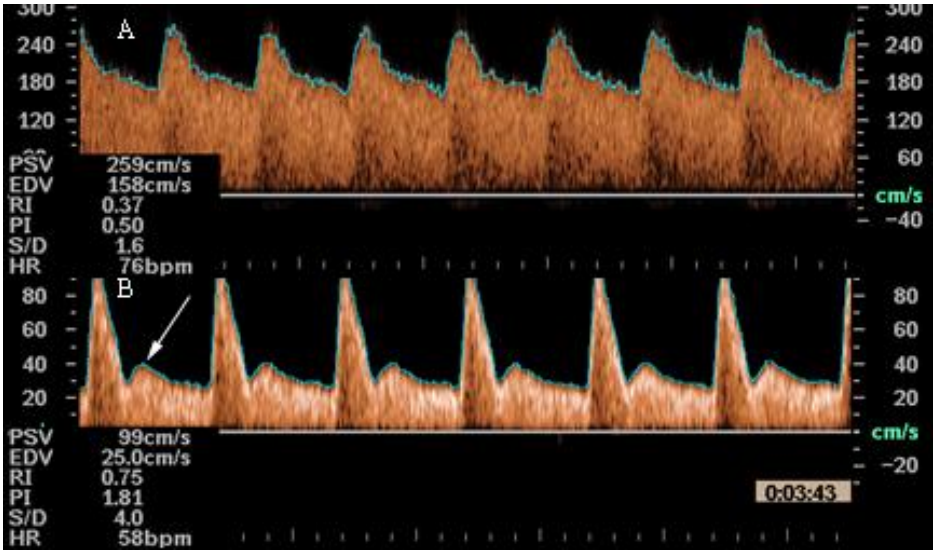

Figure 2. Blood velocity signals recorded from uterine arteries using spectral Doppler ultrasound. A) Uterine artery velocity waveform in an uncomplicated pregnancy. The high proportion of diastolic velocities indicates low resistance to flow in uteroplacental circulation. B) Doppler velocimetry of uterine arteries in a pregnancy with preeclampsia and intrauterine restriction. The proportion of diastolic velocities is decreased and in the early diastole there is a “notch” (arrow), indicating increased resistance.

Every fifth case of PE also presents with intrauterine growth restriction (IUGR). IUGR is seen when the utero-placental blood flow is reduced due to placenta pathology (44). The IUGR placenta shows signs of decreased vascular branching, a decreased vascular surface area and volume, as well as a reduced number of capillaries (45, 46). The vascular alterations in IUGR are in fact believed to cause increased uterine resistance similar to that seen in notching. Defect implantation is similar in both IUGR and PE. However, not all women with PE develop IUGR and vice versa, indicating that there are other mechanisms involved in the progression of PE.

Since the placenta is believed to be the culprit in PE, a number of methods have been used to detect differences between PE and normal placentas. In recent years, functional genomic techniques have been applied to the problem. Microarrays have been used to profile the expression of numerous genes simultaneously, and 2D-PAGE has been used to detect differences on the protein level.

Gene and protein profiling in preeclampsia

Large scale screening and profiling became possible in the 1990s after the introduction of microarray technique as it allows simultaneous gene expression analysis of the whole genome in one single experiment (47). Bioinformatics tools were developed in parallel to sort and annotate the massive amount of data generated by microarrays.

To date, several array studies have been conducted on PE samples and many genes have been reported to be altered in PE. Up to date, the PE placenta proteome is less well described. The human genome contains around 25,000 genes, whereas the human proteome is estimated to contain up to 1,000,000 different proteins that in addition are post-translationally altered by glycosylation, phosphorylation etc. Proteomics is a progressive field that is continuously improving. To compare protein expression, extracted samples are separated on polyacrylamide gels in two dimensions. In the first dimension proteins are separated on based on their iso-electric charge, and in the second dimension, by their molecular size.

2D-PAGE gives greater resolution than 1-dimensional gels. Proteomics has been successfully used to identify biomarkers for diagnosis of lung and breast cancer as well as hepatocellular carcinoma (48), and thus could be a powerful tool for examining PE. Since the placenta expresses an exceptional number of genes, it also contains a very large number of proteins. Thus, the placental proteome is much harder to study than its gene expression and so far only few studies are published on the topic.

A summary on what modern molecular biology has revealed about the progression of PE is reviewed below. Based on large scale gene and protein screening, four main pathophysiological mechanisms have been shown in the PE placenta as a consequence of impaired placentation (Figure 3).

Hypoxia and oxidative stress

The molecular evidence of hypoxia in the PE placenta results from some gene array studies (21, 22). Among the genes that respond to hypoxia are vascular endothelial growth factor (VEGF) and the hypoxia inducible factors (HIFs). VEGF is a family of growth factors involved in the formation and growth of blood vessels by stimulating endothelial cell migration and assisting in the creation of the vascular lumen. The VEGF family includes VEGF-A, B; C and D as well as the placenta growth factor (PlGF). VEGF-A, the most important member in the VEGF family, has been shown to be increased in PE placentas (21). The HIFs are transcription factors that respond to changes in tissue oxygen levels by upregulating the expression of genes that help the cell survive during hypoxia, such as VEGF-A and insulin like growth factor II (IGF-II). Several HIF target genes such as insulin like growth factor II, sFlt and ceruloplasmin, have been shown to be increased in PE (21, 49).

Formation of reactive oxygen species (ROS) is thought to occur during the repeating reperfusion of the PE placenta (as described above). ROS are highly reactive and can cause significant damage to cells and tissues. Some of the harmful ROS include super oxide, O_2-, hydrogen peroxide, H_2O_2 and the hydroxyl radical, OH^-. Moreover, molecules such as heme and free iron, Fe^{2+}/Fe^{3+}, also have potent redox abilities. Oxidative stress occurs when the production of ROS overwhelms the different anti-oxidative protection systems. Molecular indications of reactive oxygen species (ROS) formation have been shown in the PE placenta. One of the enzymes responsible for ROS formation, Cytochrome P450, has in several array studies, including Paper III, been shown to be increased in PE (50-53). Worth noting is that in

each array cited, a different family and/or subfamily of Cytochrome P450 was shown to be increased in PE, underlining the importance of these genes in the pathophysiology of PE.

ROS and redox molecules are quickly rendered harmless by different catabolic enzymes. Superoxide is turned into hydrogen peroxide and oxygen by the enzyme superoxide dismutase, while the resulting hydrogen peroxide is degraded into oxygen and water by catalase. There is evidence for induction of genes involved in anti-oxidation in the PE placenta. Catalase and superoxide dismutase 1 have both been shown to be increased in the PE placenta (50). Furthermore, peroxiredoxin 2, an anti-oxidative protein that regulates peroxide levels (another ROS member), has been shown to be decreased in the PE placenta (54). The heme oxygenases (HO) are anti-oxidative catalytic enzymes that degrade the hemoglobin metabolite, heme, into biliverdin and carbon monoxide. Three known isoforms exists: HO-1-3. HO-1 is induced by hypoxia, oxidative stress and the presence of heme whereas HO-2 is the constitutively expressed form. Due to the oxidative stress in the PE placenta, one would expect HO-1 to be induced however, gene expression from different studies show various results. The two isoforms have been reported both to be under and overexpressed in PE, although our own expression data showed HO-1 to be increased (Paper III).

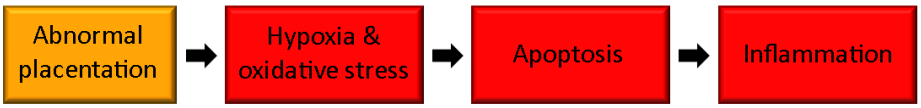

Figure 3. The progression of PE starts with an abnormal placentation. The failure to create a low resistance high blood flow in the PE placenta is thought to cause a cascade of pathological processes starting with hypoxia and oxidative stress. The oxidative stress causes damage to the placental cells resulting in apoptosis and finally inflammation. Microarray studies have shown both up- and downregulated genes related to these three functional categories summarized below.

Apoptosis

Programmed cell death is yet another important process associated with PE (55). Caspases (CASP) are a family of cysteine proteases, essential for apoptosis and inflammation and are divided into two major subgroups: initiator and effector caspases. Initiator caspases, CASP2, CASP8, CASP9, and CASP10, activate the effector caspases by proteolytic cleavage. The effector caspases, CASP3, CASP6, and CASP7, in turn cleave intra-cellular proteins eventually leading to cell death. Microarray experiments have revealed that several different apoptotic genes are differentially expressed in the PE placenta. CASP10 and CASP6 have both been shown to be increased in PE (53, 56). Other apoptosis related genes are heat shock protein 70 (HSP70) and death receptor 3, also increased in PE, suggesting greater apoptotic activity in the PE placenta. HSP70 is an anti-apoptotic protein protecting cells and proteins by inhibiting the effects of caspases. Hence the increased expression of HSP70 in the PE placenta may be a compensatory effect in an attempt to prevent further apoptosis in the placental cells.

Inflammation and immunoregulation

There is an increased inflammation and maternal immune response in PE (28, 57, 58). There are two main types of immune systems: the innate and the adaptive. Innate immunity is

characterized by a rapid inflammatory response, induced by foreign cells and/or cell debris, resulting in attraction of phagocytic blood cells which invade the inflamed tissue. Cytokines such as tumor necrosis factor α (TNFα), interleukin (IL) 6 and interferon gamma, can amplify the innate response. Since the PE placenta is assumed to be in a state of inflammation, one would expect pro-inflammatory markers to stand out in gene expression experiments. Inflammatory genes that consistently have been reported to be elevated in PE include TNFα and the protein C receptor (55, 59). Hormones related to inflammation such as the adipose derived leptin, have also been shown to have increased gene expression in the PE placenta (59, 60). Leptin expression is induced by inflammatory agents such interferon γ, and leptin in turn regulates the release of pro-inflammatory cytokines.

In contrast, the adaptive immune response is slower but far more specific, only targeting identified agents. After phagocytosis, a foreign cell is catabolized into smaller fragments and attached to the major histocompatibility complex (HLA) molecules, which are transported to the surface of the antigen presenting cells, enabling the adaptive immune response to recognize and target the foreign cell. Antigen presentation further activates the adaptive immune cells leading to phagocytosis or cytolysis of the foreign cells. While microarray experiments were expected to reveal changes in adaptive immune related mediators in the PE placenta, the data generated so far is inconclusive. HLA-G expression has been reported to be either increased or decreased (60, 61). The discrepant findings may be due to differences in the HLA-G isoforms present on the arrays. HLA-D expression has been shown to be increased in the PE placenta (62).

Angiogenetic factors

Angiogenetic factors are the most recent focus of attention. A major player is sFlt shown to be increased both on placental gene level and increased as a circulating protein (32). Members of the VEGF family have been shown to be increased in the PE placenta (63). The tenascin system, responsible for endothelial cell movements during vascular growth, is also induced in PE (64). Hence there appear to be increased angiogenesis in the PE placenta, which may be an attempt to compensate for the reduced perfusion (Figure 1).

In summary, the pathophysiological mechanisms in stage one begin with inadequate placentation leading to decreased blood perfusion that induces oxidative stress, inflammation and apoptosis. As a result of the tissue damage the blood placenta barrier is broken, allowing leakage of cells and debris in to the maternal blood system. What further gives rise to the maternal endothelial dysfunction and the clinical symptoms are yet unknown.

The screening techniques for both genomics and proteomics become more and more efficient. In the present study we have used state-of-the art technology to study gene and protein expression in the PE placenta. To further bring clarity to the complex disease, or rather syndrome, well defined clinical subgroups of PE have been studied.

Aims

The general aim of this study was to develop tools and optimized methods to screen the PE placenta and by using large scale screening methods for both gene and protein detection to generate new hypotheses about the pathophysiological mechanisms behind PE.

The specific aims were:

- To create a PE associated cDNA subtraction library in order to create a PE associated microarray chip
- To use the PE associated microarray chip and a whole genome microarray chip to gain new insight in the PE pathology
- To characterize and define inclusion criteria for PE, including risk group patients with bilateral uterine artery notching
- To optimize and develop methods for protein extraction and 2D-PAGE separation
- To correlate gene and protein expression data to identify new biomarkers for PE
- To screen for inflammatory markers in plasma from PE women using antibody microarrays

Material and Methods

Patients

Defining PE and the clinical subgroups is important to be able to relate the molecular findings with clinical aspects of the disease. Patient groups are presented in Table 1. PE was defined as hypertension (> 140/90 mmHg) and proteinuria (> 0.3 g/l). PE was further divided into early and late onset PE. Early onset PE was defined as manifestation and delivery before 35 gestational weeks and late onset thereafter. The HELLP syndrome, *h*emolysis, *e*levated *l*iver enzymes and *l*ow *p*latelets, is thought to be a severe form of PE. HELLP diagnosis was based on presence of hemolysis (serum lactate dehydrogenase > 600 U/l), elevated liver enzymes and low platelets (> 100,000 / µl). Uterine artery notching is a risk factor for PE although not all women with bilateral notching develop PE (41). To detect genes and proteins that may protect against PE, notching that later developed PE was included. Uterine artery Doppler velocimetry was used to determine presence of bilateral uterine artery notching at 18 gestational weeks. The uterine artery was visualized with color Doppler and presence or absence of diastolic notch in the waveform was visually assessed. A subgroup of PE with notching and IUGR was also included. IUGR was defined as an estimated fetal weight deviation of at least 2 standard deviations below the gestational age-related mean weight (65, 66). In the most severe PE cases, pregnancy was terminated as early as 24-25 gestational weeks. Thus matching these with full term normotensive pregnancies may introduce errors in the analysis, since gestational age might per se affect the gene profile. To account for differences in gestational age between PE and normotensive controls, pre-term placentas from uncomplicated pregnancies could have been used as controls. However, as the genetic profile of the preterm placenta so far is unknown it is hard to say whether it actually constitutes a healthy control, and therefore not used in our studies.

Table 1. Study groups and number of included samples.

Paper	Controls	LPE	EPE	N	PEwN	PIN	HELLP
I[a, c]	15	10	–	5	5	–	–
II[b, c]	30	30	–	–	–	–	–
III[a, c]	15	10	–	5	5	–	–
IV[b,c]	27	29	–	–	–	–	5
V[c]	11	10	11	–	–	8	–

LPE = late onset PE, EPE = early onset PE, PEwN = PE with bilateral notching, PIN = PE with bilateral notching and IUGR, HELLP = hemolysis, elevated liver enzymes and low platelets.

[a] The same samples were used in the studies.

[b] The same patients were used in the studies. Placenta samples were used in Paper II and plasma samples in Paper IV.

[c] Some overlapping in samples was present in all studies to be able to correlate gene expression, protein expression and plasma protein expression.

Tissue sampling

Tissue and blood samples were collected at the Department of Obstetrics and Gynecology, Lund University Hospital after written consent was obtained. The studies were approved by the Research Ethics Committee Board at Lund University for studies on human subjects. A 10 mm cubic placenta tissue sample was collected after delivery from a central portion of the placenta. Areas with necrosis and/or thrombosis were avoided. Samples were snap frozen on dry ice and kept at -80°C until use. Blood was collected before and after delivery in EDTA vacuette tubes (6 ml) and in PaxGene blood RNA tubes (10 ml). Following sampling the tubes were spun at 2,000g for 20 minutes after which the plasma was recovered and stored at -80°C until use. PaxGene tubes were stored according to manufacturer's instructions (PreAnalytiX Gmbh).

Methods to study gene expression

RNA extraction (Papers I and III)

Total RNA was extracted by Trizol™. Approximately 125 mg placenta tissue was used and homogenized in 2.5 ml Trizol. RNA was extracted according to the instructions provided by the manufacturer. An additional precipitation with sodium citrate was added to remove contaminating proteoglycans and polysaccharides Extracted RNA was quantitated using a Nanodrop spectrophotometer. In order for extracted samples to be included in the microarray experiments, the extracted RNA had to pass the following quality criteria: 260/280 ratio above 2, 260/230 ratio above 1.8, as well as the presence of clear 18s and 28s bands on a 2% agarose gel.

DNA Subtraction library (Paper I)

Tissue from five women with severe PE (two of which also had notching) and eight controls was used to prepare two subtracted libraries (67). These samples were not included in the groups used for gene expression analysis in order to avoid bias. First full-length, cap-trapped cDNA libraries were made from both normal and preeclamptic placental RNAs. Subsequently, the two libraries were subtracted. Drivers were made from each of the amplified libraries using the vector sequences. Then the driver from the preeclamptic placental library used to subtract the normal placentas library and vice versa. At the end, chromatography allowed removal of double strand DNA. The subtracted cDNA was subsequently sequenced and identified.

PCR amplification (Paper I)

cDNA templates were generated from the 800 unique plasmids by means of polymerase chain reaction using a mix of T3/T7 primers. Acquired cDNA templates were purified on filter plates and quality was controlled by means of agarose gels.

Microarrays (Papers I and III)

Microarray chips were manufactured at the Swegene microarray center, Lund University. The 800 cDNA clones were printed in triplicate on each microarray slide. In addition, one copy of a whole genome oligo set, containing approximately 27,000 genes, was included on each array chip. Sample RNA was labeled with Cy3 fluorescent dye and reference RNA with Cy5, mixed and hybridized on the chips. One patient sample per chips was used. After hybridization, slides were scanned with a laser scanner to obtain intensity values for the color ratios. Quality controls were carried out in Genepix Pro (MDS Analytical Technologies), where spots contaminated by for instance grains of dust were removed. Following image analysis, ratio values were uploaded into BASE for statistical analysis.

Microarray statistics (Papers I and III)
Firstly, all data were filtered for intensity. The spots were required to have an intensity of 250 optical density units or higher in one of the two channels to be included. Spots were then filtered for signal to noise ratio, by comparing the actual spot- with the background signal. A ratio above 2 was required for further analysis. The resulting data were normalized by Lowess normalization adjusting for differences in the number of inserted fluorescent labels. Data were then normalized for reference interference bias by means of median centering. In Paper I, the three replicates for each subtracted cDNA, were analyzed by comparing their variation with a global variation coefficient. Variance was required to be within 2 standard deviations (SD) for a replicate to be further analysis. The study groups were analyzed for differences in gene expression using a false discovery rate (FDR) based analysis method (see Methodological consideration). In Paper I, a q-value below 0.05 and FDR of 0.5% was considered significant and in Paper III a cutoff at $p \leq 0.005$ was used. The lower cutoff value for Paper III was chosen to limit the result to the most significant genes, thereby reducing FDR.

Bioinformatics analysis (Papers I and III)
The use of bioinformatics analysis allows one to transform gene expression data into biological information. These tools permit one to catalog genes according to similarity in function, participation in signaling pathways, protein domains, etc, clarifying the biological relevance of the microarray data. Gene ontology (GO) is the most commonly used database for biological functions, http://www.geneontolgy.org. GO annotates genes according to their molecular function, cellular location or biological process and categorizes them into a tree like hierarchical database as exemplified in Figure 4. Another bioinformatics database is the Kyoto encyclopedia of genes and genomes (KEGG), http://www.genome.jp/kegg/. KEGG annotates genes based on their involvement in known signaling pathways. The pathways included range from different metabolic pathways to translational and signal transduction pathways. Unlike GO, KEGG is not hierarchically organized; instead KEGG only correlates genes with their signaling pathways. Thus, KEGG makes it possible to ascertain whether any specific signaling pathways are transcriptionally altered.

Two database programs were used to annotate differentially expressed genes and analyze differences in GO between groups: 1) GoMiner (68) and 2) the Database for Annotation, Visualization and Integrated Discovery (DAVID) (69). A whole-genome gene-set, the same as printed on the array chips, containing approximately 27,000 genes, was used as background. DAVID was employed to compare changes in pathways between groups. Genes were classified according to presence of protein domains by means of the InterPro database. DAVID was used to compare differences in the presence of protein domains between groups.

Real-time PCR (Papers I and III)
Real-time primers were ordered premade from Applied Biosystems or constructed using Assays-by-Design™. The primers were designed to target exon borders in order to exclude amplification of genomic DNA. Quantification was achieved by adding a 4-fold dilution series as calibration for each of the DNA primers. Each sample was assayed in duplicate. A negative control was added on each assay to ensure unspecific amplification. B-actin was used as endogenous reference, so called house-keeping gene. The results are presented as scatter plots (Paper I) and as box plots in Paper III in order to include percentile (75^{th} and 95^{th}) values. Statistical differences were calculated using a Kruskal-Wallis test with a *post hoc* Dunn's multiple comparison test. A p-value below 0.05 was considered statistically significant.

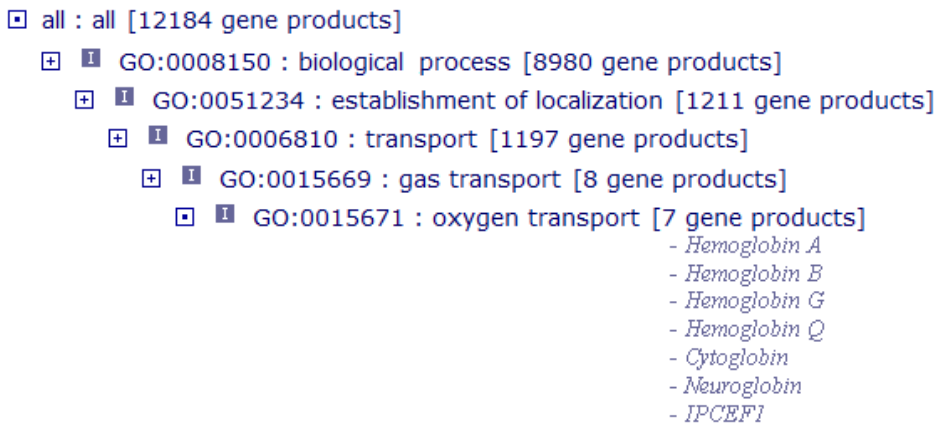

Figure 4. Gene ontology is a hierarchical database most often depicted as a tree. The uppermost categories (biological processes, molecular mechanisms, and cellular components) are very broad, covering all physiological processes and components. Branching out from the three main categories are more defined annotations, and down the tree, the branches (or biological functions) become more specific. The outermost branches are specific categories containing few genes, here exemplified by hemoglobin, one of the most significantly altered genes in PE (Paper I).

In-situ hybridization (Paper I)
In situ hybridization was performed as previously described (70). DNA templates were generated by polymerase chain reaction PCR from cDNA using modified primer pairs consisting of either a T7 RNA promoter and a downstream gene-specific sequence (anti-sense) or a T3 RNA promoter and an upstream gene-specific primer (sense). Complementary RNA (cRNA) probes were transcribed from gel-purified DNA template using ^{35}S-UTP and either T3 or T7 RNA polymerase according to the manufacturer's instructions (Ambion MAXIscript) to generate sense and antisense probes respectively.

Frozen cryo sections (12 µm), were cut on a cryostat and thaw mounted on silanized slides. The sections were stored at -80°C until use. Tissue sections were fixed with formaldehyde and dehydrated before hybridization as previously described (71), after which the sections were hybridized 20-24 h with 2×10^6 cpm of denatured ^{35}S labeled cRNA probe. Excess probe was washed away and the sections were apposed to Kodak hyperfilm for three days and then coated with film emulsion. After 3-4 weeks exposure at 4°C sections were developed, fixed and counterstained with a Giemsa stain. Microphotographs were prepared using an Olympus BX-60 micro-scope equipped for darkfield and brightfield microscopy with a Olympus DP50CU digital camera. Captured images were assembled using Adobe Photoshop 7.0.

Methods to study protein expression

Protein extraction (Paper II)
Approximately 30 mg of frozen placental tissue was pulverized and lysed in either urea/CHAPS or Hepes buffer. Excess glycogen was removed by centrifugation at 43,000g for

2 h. Protein concentrations were determined using the bicinchoninic acid (BCA) method with BSA as a standard according to manufacturer's instructions (Pierce).

Protein precipitation (Paper II)
Six precipitation methods were used to remove sample contaminants: 1) ice cold acetone 2) ice cold acidified acetone 3) ice cold ethanol 4) dichloromethanol/methanol as previously described (72) 5) trichloroacetic (TCA) acid and 6) TCA followed by ethanol wash.

1D-PAGE (Paper II and IV)
In order to separate extracted proteins based on size (one dimension), samples were loaded onto either NuPage Bis-tris gels or SDS gels and run for 1 h at 200 V. MES or MOPS was used as running buffer and proteins were visualized with Coomassie brilliant blue.

2D-PAGE (Paper II)
To further separate the proteins, 2D-PAGE was used. In the first dimension, to separate proteins based on charge (iso-electric focusing), the samples were loaded onto immobilized pH gradient (IPG) blue native strips. The strips were later equilibrated and soaked in electrophoresis buffer. The second dimension was run on SDS PAGE over night. Gels were then stained with either SYPRO Ruby or silver stain (73) and scanned. Gels stained with SYPRO Ruby were sent to Ludesi 2D analysis (http://www.ludesi.com) for spot detection, matching and analysis. The two groups (PE and controls) were run on three replicate gels which were then compared for spots with a fold change above 1.5. For inclusion and evaluation each spot had to be present on all three gels.

Mass spectrometry identification (Paper II)
The differentially expressed spots and bands were cut out from the gels and digested into smaller peptide fragments using trypsin. The peptide fragments were analyzed using MALDI-TOF MS to obtain a peptide mass fingerprint (PMF). For protein identification, human protein sequences in the SwissProt database (http://www.expasy.ch/sprot) were searched using the Mascot Software (Matrix Science Ltd).

Western Blot (Paper II)
Samples were extracted using the optimized extraction used in 2D-PAGE. Samples were loaded and separated in 1D on NuPage gels as above. Gels were blotted onto polyvinylidene fluoride (PVDF) membranes for 8 minutes using the iBlot dry blotting system (Invitrogen), blocked with milk for 1 h and incubated with primary antibody (mouse anti-human APOA1) for 1 h at room temperature (RT). After washing, incubation with the secondary antibody (goat anti-mouse IgG) was done for 1 h at RT. The membranes were then washed and exposed to enhanced chemilumeniscence and developed on autoradiographic film. The films were scanned with ultraviolet light and the optical densities obtained with SynGene GeneTools. A Mann-Whitney rank sum test was used to determine the statistical difference between the groups. A p-value below 0.05 was considered statistically significant.

Immuno-histochemistry (Paper I)
Fresh frozen sections were fixed with formalin and blocked in blocking solution (Powerblock). Sections were incubated with a primary sheep anti-Hbγ antibody for 1 h in RT. Following rinses, the sections were incubated with a secondary donkey anti-sheep IgG antibody for 1 h in RT. The sections were rinsed, dried and cover-slipped. A Leica inverted fluorescent microscope (Leica Microsystems) was used and images captured using Volocity software (Improvision).

Enzyme-linked immunosorbent assay (ELISA) (Paper IV)
A competitive ELISA was used in order to measure the free levels of adult Hb levels (HbA). A 96-well plate was coated with a commercial HbA antibody (Sigma) and then incubated with either standard rabbit anti-HbA or directly with patient plasma samples. After rinsing, the wells were incubated with AP-conjugated swine anti-rabbit IgG antibody. Following rinses, a substrate solution was added and absorbance was measured at 415 nm at the onset of reaction and then every 10 minutes.

A sandwich ELISA was used for measurements of free levels of fetal Hb (HbF). A 96-well plate was coated with an affinity-purified rabbit anti-HbF. A standard series of HbF solution or plasma samples were then added and incubated. After incubation, biotin labeled anti-Hb antibodies were added followed by incubation with extravidin-alkaline phosphatase (AP). A substrate solution was added and absorbance at 415 was measured as above. Student's t-test was used to determine the statistical difference between the groups. A p-value below 0.05 was considered statistically significant.

Antibody microarrays (Paper V)
Samples were labeled and biotinylated using previously optimized protocols (74). Unbound biotin was removed by dialysis. Antibodies were produced in bacterial cultures and purified. Purity and integrity were evaluated by means of 1D-PAGE. Antibody microarrays were created by spotting two drops of antibodies onto plastic microarray slides. Eight replicates of each antibody were spotted. 144 human recombinant antibodies were selected for the antibody microarrays, some of which had shared targets. The biotinylated samples were incubated on the slides for 1 h in RT. Following washes, arrays were dried and scanned for spot intensity quantification. The background was automatically deducted. Spots with the highest and lowest intensity were also excluded before the mean intensity was calculated. The microarray data were chip-to-chip normalized by identifying the 15% of analytes with the lowest coefficient variation across all slides. These values were used to calculate a chip-to-chip normalization factor that each data set was normalized to. The support vector machine (SVM) was used to classify samples as healthy or non-healthy and differentially expressed protein analytes were identified using a non-parametric test (Wilcoxon)

Results

Gene expression results

cDNA subtraction library microarrays – Paper I

Subtracting PE with controls revealed approximately 800 unique cDNAs that differed between the groups (543 increased in the PE and 251 increased in the control subtraction library). To elucidate the biological functions of these cDNAs GO analysis was performed which showed several significant GO families such as: *carbohydrate binding* (GO:0030246), *apoptosis inhibitor activity* (GO:0008189) and *peroxidase activity* (GO:0004601).

Microarray experiments based on the subtracted cDNAs showed altered expression for 30 genes in PE, notch and controls (Table 2). The most significant genes, hemoglobin α and γ (**Hbα** and **Hbγ**) chains were overexpressed in PE (Figure 5). Bilateral notching exhibited increased expression of the gene encoding for major histocompatibility complex **HLA-DPA1** compared to the control group. Other differentially expressed genes are presented in Table 2. Bioinformatics analysis showed functional categories related to hemoglobin, *oxygen transport* and *hemoglobin complex*, but also *actin polymerization* (Figure 4).

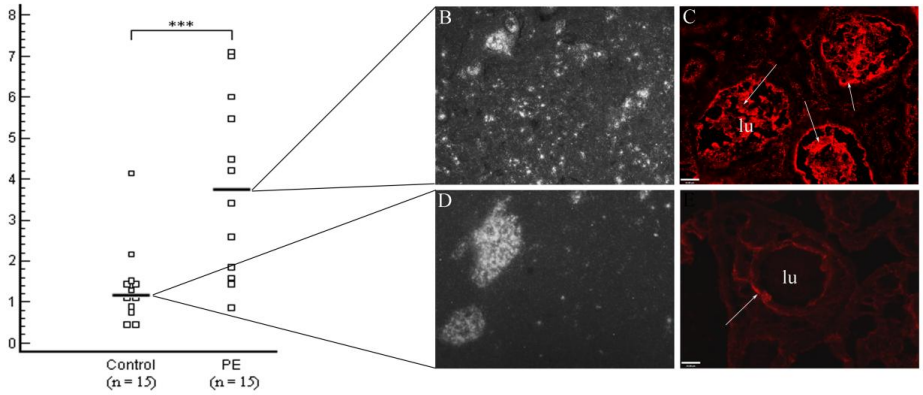

Figure 5. Both Hbγ mRNA expression (A) and presence of Hbγ protein were increased in the PE placenta. B and D show the Hbγ RNA expression in the placenta, PE and controls respectively. Presence of Hbγ mRNA was found in vessel lumen and in PE also in scattered cells throughout the intervillous space. Immunohistochemistry showed accumulation of free hemoglobin γ in the vascular lumen in PE (C). In controls (E), there was no free Hb filling the vascular lumen.

The increased gene expression for the **Hbα**, **Hbγ**, and **HLA-DPA1** was verified by means of real-time PCR. Maternal RNA from blood was analyzed in order to exclude that maternal blood contamination accounted for the results obtained. Genes significantly altered in the placenta were unchanged in maternal blood RNA. Microarray analysis of maternal blood RNA showed significantly increased gene expression for **prostate differentiating factor** and

nuclear receptor coactivator 4. To determine histological Hb mRNA expression, *in situ* hybridization was performed on placental tissue sections. The Hb mRNA expression pattern was similar in both study groups for all four hemoglobin chains (Hb α, γ, β and δ). *In situ* hybridization revealed that nucleated Hbγ expressing cells were mainly located in the lumen of the vessels in both PE and control samples (Figure 5 B and D). In contrast, the PE placenta showed Hb mRNA expressing cells scattered throughout the intervillous space, and the signals per cell appeared to be more intense in PE than in controls. Interestingly, no signal was seen in trophoblasts. Based on their location and distribution, these cells are likely to be blood derived and of fetal origin.

To correlate mRNA expression with protein expression, immunohistochemistry was performed for both Hbα and Hbγ chains. In the control placentas, a weak staining for Hb was detected on the vessel walls whereas the vascular lumen in the PE placentas was congested with free Hb protein (Figure 5 C and E).

Table 2. Summary of the most significant genes (Papers I and III). A total of 138 genes showed altered gene expression in the two array studies presented. The differentially expressed genes were associated with several biological functions and signaling pathways.
⇧ – upregulated **genes** and *functions*, ⇩ – downregulated, ⇨ – unchanged.

Individual genes	N	PE	PEwN
Hemoglobin γ [a]		⇧	⇧
Hemoglobin α [a]		⇧	⇧
Major histocompatibility complex DPA1 [a]	⇧	⇨	⇨
Major histocompatibility complex B [b]	⇧	⇨	⇨
Transforming growth factor β1 [a, b]		⇧	⇧
Haptoglobin [b]	⇩		
Glutamine synthase [b]		⇩	⇩
Inhibin A [b]		⇧	⇧
Actin, alpha 2 [a]	⇨		⇩
Gene ontology			
Hemoglobin complex [a]		⇧	
Hemoglobin metabolism [b]		⇧	⇧
Actin polymerization [a]		⇧	
Transcription factor binding [b]		⇧	
Chemotaxis [b]	⇨		⇧
Cytokine binding [b]	⇨		⇧
Regulation of apoptosis [b]	⇧		
Signaling pathways			
Neurodegenerative disorders [b]		⇧	
Antigen processing and presentation [b]	⇧	⇨	⇨
MAPK signaling pathway [b]	⇨	⇧	
Leukocyte transendothelial migration [b]	⇧		

[a] Gene or function was altered in the cDNA subtraction library microarray experiment (Paper I)
[b] Gene or function was altered in the whole genome microarray experiment (Paper III)

Whole genome screening – Paper III

Whole genome microarray analysis using microarray chips containing 27,000 genes was performed in order to extend the gene expression differences seen in Paper I. Moreover, an additional group with bilateral notching with PE was included to more specifically reveal protective or harmful genes (N – notching without PE, PEwN – notching with PE). A total of 138 genes were significantly altered (94 upregulated and 44 downregulated) in at least one inter-group comparison. The most significant genes included **transforming growth factor β1** (**TGFB1**), **inhibin α**, and **neural cell adhesion molecule 1** (Table 2). Six of the most significantly altered genes were selected and verified with rt-PCR.

One advantage of whole genome screening is the ability to produce more accurate and informative bioinformatics results. Bioinformatics analysis revealed several differences between the four study groups. There were increased expression of genes related to *leukocyte migration*, *regulation of apoptosis* and *phosphorylation* between N and controls. When comparing N with PE, genes related to *antigen presentation and processing* were increased in N. Genes associated with *chemotaxis* and *cellular movement* were increased in notch with PE. Genes associated with *hemoglobin metabolism*, *transcriptor factor binding* and the *neurodegenerative disorder pathway* were altered in PE compared to controls.

Protein expression results

Differential 2D-PAGE analysis – Paper II

Two protein extraction methods were combined with six precipitation methods in order to find the best method to achieve reproducible as well as high-quality 2D-PAGE expression. The best separation and largest amount of spots were obtained with urea/CHAPS as lysis buffer in combination with dichloromethanol protein precipitation. During pregnancy there is a gradual accumulation of glycogen in the placenta (75). Removal of the glycogen by centrifugation was crucial since the glycogen clogged the IPG-strip. Hence, following lysis, samples were spun at 43000 g to remove the contaminating glycogen and tissue debris. Without removal of glycogen no proteins entered the IEF-strip which resulted in a totally blank 2D-PAGE. Freezing of samples did not affect the separation or numbers of spots on 2D-PAGE.

To examine the differential protein expression between PE and controls, 30 samples from each group were pooled (Table 1). Three replicates of 2D-PAGE were run for each pool. In total, 51 proteins were altered in the comparison, 28 increased and 23 decreased in PE. Due to low amounts of protein in the spots, identification was only possible for two of the spots. Apolipoprotein A1 (APOA1) that was shown to be increased 1.63 times in PE. Tropomyosin 1 (TPM1) was only detectable in the controls. The APOA1 increase was verified by means of western blot which revealed a similar increase (1.66 times, $p = 0.01$).

Maternal levels of free fetal Hb – Paper IV

To evaluate the clinical relevance of the accumulation of free Hb in the placenta with a potential of leaking into the maternal circulation, free fetal Hb (HbF) was analyzed in plasma and urine in three different study groups: PE, HELLP and controls (Table 1). HbF levels were eight times higher in both PE and HELLP plasma compared to controls. No difference in HbF levels were detected between PE and HELLP. In contrast, HbF was only elevated in urine samples from the HELLP patients. The plasma level of free adult Hb (HbA) was 1.5 times higher in PE and 4 times higher in HELLP compared to the control group.

The performance of Hb-F and Hb-A as diagnostic markers of PE was evaluated by receiver operating characteristics curve (ROC) analysis. The area under the curve (AUC) was 0.95 and 0.98 for HbF and HbA respectively. The sensitivity and specificity for Hb-F was 93.1 % and 96.3 %, respectively, using the 95-percentile value as cut-off between healthy and diseased as predicted by the ROC curve. The values only relate to term pregnancy and could at this point not be applied in the first trimester.

Screening for inflammatory markers in maternal plasma– Paper V

Results from our whole genome microarrays (Paper III) suggest that a pro-inflammatory response may drive the progression from notch to fully developed PE. To elucidate the maternal inflammatory response, plasma samples from women with late onset PE (LPE), early onset PE (EPE) and PE with notching and IUGR (PIN) were analyzed using antibody microarrays. Groups were compared by constructing a ROC curve, from which the AUC was calculated.

In the initial analysis only the control group (C) separated from the other groups (Figure 6A). Early onset PE (EPE) did not differ from late onset (LPE) which in turn did not differ from the PE group with notching and IUGR (PIN). However, after clustering, the EPE and LPE groups further, they divided into two subgroups (Figure 6B). The EPE subgroups reached a ROC value of 1. Both EPE subgroups appeared to separate equally from the other groups (LPE, C and PIN). The LPE also divided into two subgroups. LPE1 shared profile with the control group but differed from LPE2 and PIN. The LPE2 subgroup shared similar profile with the PIN group but separated from the control and LPE1 group (Figure 6B).

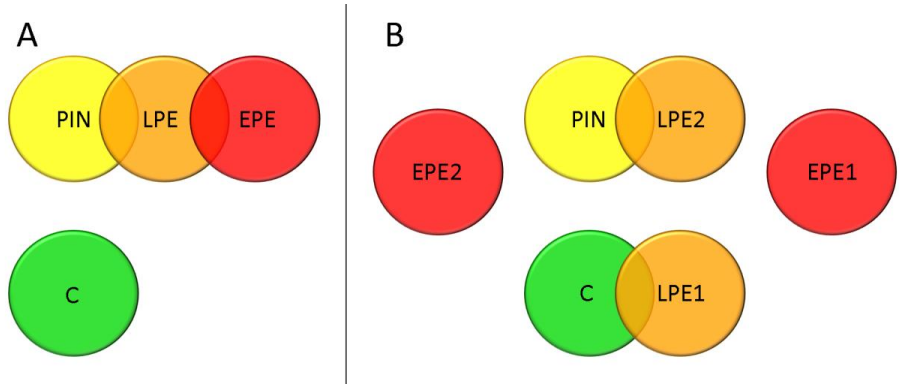

Figure 6. A schematic image of AUC values shown as distance between circles. A) In the first analysis the three PE groups shared similarities hence only separated significantly from the control group (C). B) When examining profiles from late and early onset PE (LPE and EPE respectively) subgroups, a different pattern was detected. The EPE groups did not share any similarities between the others. The LPE separated into two subgroups. The LPE2 subgroup was analogous to the severe PE group with notching and IUGR (PIN) and the LPE1 subgroup similar to the control group.

Discussion

Hemoglobin and preeclampsia

To generate new hypotheses for the underlying onset of PE, large scale gene expression analyses were performed, using two different array chips, to screen the PE placenta. Analysis of the custom made PE associated gene chips showed increased gene expression of two hemoglobin (Hb) chains, α and γ. Only fetal Hb contains γ chains. In adults, hemoglobin is synthesized in the bone marrow whereas during fetal development, hemoglobin synthesis occurs in the liver, spleen and lymph nodes. The Hb gene expression was observed in both the healthy and diseased placenta, suggesting that the placenta is a source of fetal hematopoietic stem cells and may act as an extramedullary hematopoietic organ during pregnancy in a similar manner as the murine placenta (76-79).

Hemoglobin in its free form can cause severe tissue damage. Free hemoglobin chains can disrupt and damage cell membranes (80). However, it is mostly the Hb metabolites, heme and iron, that are harmful. Heme can damage cells indirectly by sensitizing cell membranes as well as membrane proteins to oxidation or by direct oxidation (81). Due to heme's hydrophobic nature, it can cross cell membranes and cause damage to cytosolic proteins, organelles and DNA (82, 83). Heme and iron also oxidize proteins and lipids into cytotoxic forms that further cause oxidative damage (82). In addition, from being a strong oxidizer, heme also promotes the formation of reactive oxygen species (ROS) (84). Apart from the oxidative properties, heme also induces inflammation, either by the recruitment and activation of neutrophils or by direct action via the pro-inflammatory toll-like receptor 4 (84-86). Thus, the increased production and accumulation of free Hb may be a pathophysiological mechanism that is responsible for the oxidative stress and endothelial damage seen in the PE placenta.

In adults, Hb synthesis is induced by low oxygen tension, as seen in humans living on high altitudes (87). In the PE placenta, there is a decreased perfusion, which due to hypoxia, may promote the increased Hb gene expression described. Previously, plasma levels of two Hb stimulatory hormones, erythropoietin and activin A, have been shown to be increased in PE possibly contributing to the increased Hb synthesis described (88, 89). There are several scavenging mechanisms to degrade hemoglobin and its metabolites. The two most important scavengers are **hemopexin** (**HPX**) and **haptoglobin** (**HP**) both of which are synthesized in the liver. HPX is responsible for scavenging of free heme, whereas HP binds free hemoglobin. After binding their respective target, HPX is degraded in the liver while HP is degraded by the spleen. Interestingly, both HPX and HP genes were expressed in the placenta. HP was under-expressed in the notch (without PE) placentas compared to the control group whereas HPX gene expression was unaltered between the groups. **Heme oxygenases (HO-) 1** and **2** are two enzymes responsible for degrading heme. The HO-1 gene expression was increased in PE vs. controls indicating a demand for anti-oxidative protection. HO-2 expression was reduced in PE vs. controls which rather may suggest that the HO system may be impaired in the PE placenta contributing to the accumulation of Hb seen in the PE placenta. Another hemoglobin related gene with increased expression in PE was transforming growth factor B1 (TFGB1). TGFB1 is generally considered a pro-inflammatory agent; however, TGFB1 can also reactivate the expression of Hbγ in adult hematopoiesis, as well as induce proliferation of hematopoietic progenitor cells (90, 91). Thus, increased levels of TGFB1 may stimulate hematopoiesis in the placenta and thereby contribute to the increased expression of Hb seen in the PE placenta.

Free Hb and the metabolites can also mediate systemic effects. For instance, free Hb is able to increase vascular contractility and thereby increase blood pressure. Hemoglobin is a potent nitric oxide (NO) scavenger, reducing levels of free NO, which in turn leads to endothelial dysfunction and increased vascular tone (92-94). Hb also scavenges the endothelium-derived relaxing factor (EDRF) further increasing the vascular tone (95). Thus, free Hb may, if the accumulated free Hb leaks into the maternal circulation, by these mechanisms play a role in the hemodynamic changes that are typical in PE. Indeed, results presented in Paper IV showed significantly higher plasma levels of free fetal Hb in women with PE and HELLP further supporting this theory. Thus, increase in free Hb may be a new important etiological factor in the progression of PE and a potentially important diagnostic biomarker, possibly also reflecting the severity, of PE.

Preeclampsia – a three-stage disease

It has previously been suggested that notching may be an early form of PE, expressing a gene profile that is similar to that of PE (55). This hypothesis was strengthened by our findings in Paper III, where notch groups, with and without PE, were compared. Notch without PE did indeed differ from the control and the PE group. Bioinformatics analysis showed alterations in genes related to *regulation of apoptosis*, *response to stress* and *leukocyte transendothelial migration*. The early PE placenta is characterized by the same features, apoptosis, infiltration of leukocytes and oxidative stress (96-99). Hence, the gene expression of the notch placenta is similar to the early onset PE placenta. In fact, notch may not only be a risk factor for PE, but an intermediate stage in the PE progression. Based on the results presented in this thesis, we hypothesize that PE may be a three stage disease with notch as a reversible, middle step (Figure 7). It is interesting to note that free hemoglobin and heme mediate leukocyte migration, apoptosis and oxidative stress, categories characterizing the notch placenta. Hence, an increase in free Hb early in pregnancy may contribute to the changes described in the notch placenta. The major hemoglobin scavenger **HP** was under-expressed in the notch placenta compared to controls contributing to the accumulation.

Inflammation is an important mechanism in the PE placenta (51, 100, 101). In notch without PE, no inflammatory GO categories were altered compared to the controls. However, PE with notch expressed several pro-inflammatory genes including **TGFB1**, **inhibin A**, and **chemokine ligand 8**. Genes related to *inflammatory cell movement* and *chemotaxis* were also increased in this group. Thus, the PE placenta appears to be associated with an increased recruitment of pro-inflammatory cells, eventually leading to inflammation. The placental cytokine gradient and accumulation of free Hb may drive the chemotaxis of inflammatory cells into the placenta. T-cell proliferation was yet another immune related category that was elevated in PE compared to notch. Consequently, the progression from notch into PE may begin with recruitment of inflammatory cells to the placenta which later induces inflammation.

Not all women with uterine artery notching progress to PE. Thus, these placentas were analyzed in search for genes that protect them against developing PE. When comparing the two notch groups, with and without PE, there were increased expression of genes related to *antigen presentation and processing* such as **HLA-B**, **CD74**, and **PSME1** and **2** in the notch without PE group. Paper I revealed increased gene expression of **HLA-DPA1** in the same group. PE has previously been shown to be associated with an increased leakage of fetal cells and cell debris into the maternal circulation, which was suggested to contribute to the maternal inflammation (24, 26, 102). Increase in antigen presenting genes may help the

adaptive immune response to identify, bind and neutralize the foreign cells and debris, preventing leakage and activation of inflammation. In fact, no inflammatory genes were altered these notch placentas, further supporting this hypothesis.

Accumulation of inflammatory cells in the notch placenta may cause inflammation that drives the placental pathophysiology in PE. Possibly, the general maternal inflammatory response determines the clinical manifestations of PE, i.e. time of onset, severity and/or fetal involvement. In order to more specifically profile the maternal immune response, plasma samples from early and late onset PE (EPE and LPE respectively), PE with notching and IUGR (PIN), and healthy controls were collected before delivery and analyzed using antibody microarrays.

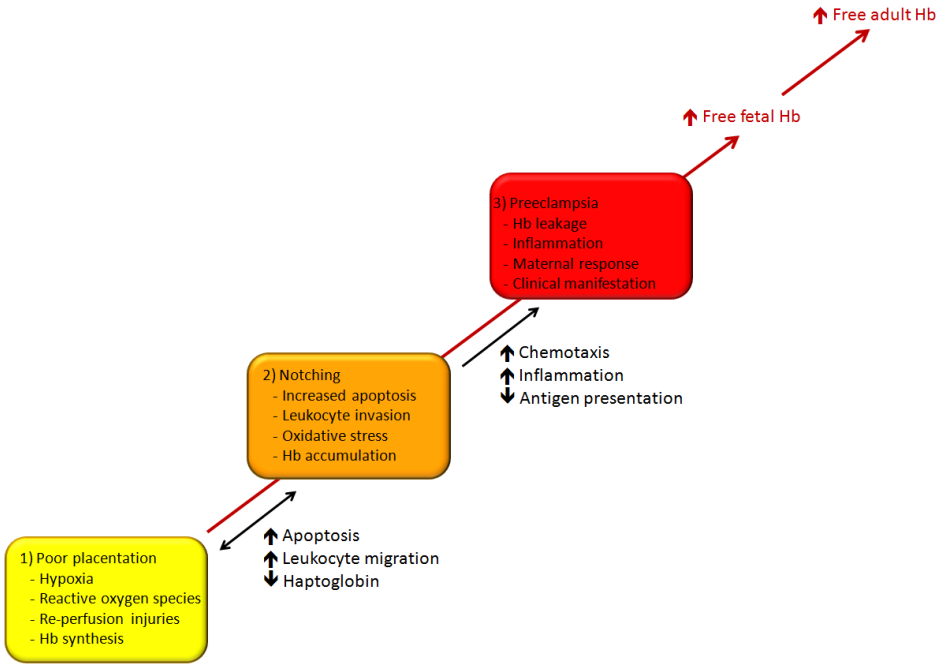

Figure 7. The proposed three-stage model of PE. Preeclampsia begins with the previously described abnormal placentation causing a hypoxic environment and re-perfusion injuries. The second stage, the notch placenta, is characterized by apoptosis and a beginning invasion of leukocytes. The accumulation of inflammatory cells may cause inflammation in the notch placenta, driving the progression into PE. In notch, where there was no manifest of PE, showed increased expression of antigen presenting genes which may prevent inflammation and progression to PE. Levels of fetal hemoglobin may be induced by the hypoxia in stage one and then accumulate as pregnancy progress. In the PE placenta the fetal Hb may damage the blood-placenta barrier and leak into the maternal circulation, where the fetal Hb disrupts maternal erythrocytes, causing release of adult Hb which may aggravate the pathophysiological effects of free Hb.

The control group significantly separated from the three PE groups, all sharing similar profiles. However, the initial analysis was inconclusive and therefore, further categorization by hierarchical clustering was done. Both the EPE and LPE groups divided into two subgroups respectively. These novel subgroups displayed an intricate relationship, showing both distinct differences and similarities. A more refined picture was revealed when the subgroups of EPE and LPE were analyzed, demonstrating key differences in their plasma protein profiles. PIN further differentiated from the EPE group supporting the hypothesis that the maternal immune response may play a central role in the progression from notch to PE and/or IUGR.

Methodological considerations

Limitations of gene expression studies

Gene expression is a field undergoing rapid development. It is only in the last few years that whole genome array results have been published (51, 59). In fact, more than half of the studies done to date were undertaken with less than 5,000 genes on the microarray chips. Furthermore, several arrays studies examining PE have only used genes associated with a certain biological function such as tumorigenesis or apoptosis. Needless to say, there is great variation in the genes present on different microarray chips. A PE associated subtraction library was created in Paper I in order to compensate for the possibility that important genes related to the PE pathophysiology were missing in the commercially available gene sets.

Due to the extensive costs of microarray analysis the number of samples in each group is generally low (median – 6, range – 2-11). Furthermore, some studies use pooled samples to minimize individual variability and costs. Although variability may be decreased, this method may also negate or enhance the expression of important genes. Gestational age is a parameter that is hard to match in PE. Since the only curative treatment for PE is delivery, the gestational age of patients with severe PE often is significantly lower than the control group delivered at term. A few previous studies have used uncomplicated pregnancies with pre-term delivery or IUGR as control groups. However, pre-term deliveries are not uncomplicated per definition and IUGR appears to have its own inflammatory profile. Thus, there is no clear answer in terms of how to match for gestational age. A compensation for this weakness is to have as homogenous groups as possible. Whereas some studies divide their groups into late and early onset PE, mixed groups are most commonly used. To the best of our knowledge, bilateral notching as a defined group has for the first time been profiled.

Although microarray is a powerful technique it has its limitations. In the experiments the gene expression may not only be dependent on PE. Several external environmental factors such as diet, parity, stress, and medications can alter the transcription of genes in the placenta, contributing to the inter-individual variation. Placental tissue sampling can also affect gene expression results. The placenta itself is a very heterogeneous organ, which makes it hard to select comparable areas between patients. It has been shown that gene expression differs within the areas of the placenta (103). Moreover, samples may be compromised by infarction and necrosis, which also affects gene expression. Samples included in the thesis were always sampled from a central portion of the placenta and care was taken to avoid infarctic areas.

The microarray technique itself also introduces variables. Extraction and labeling of samples as well as the hybridization may affect the results. The Lowess normalization compensates for labeling variation. To make up for variation in hybridization, the subtracted cDNAs were

printed in triplicate allowing exclusion of spots with high variation without losing information for a specific gene. There are no guidelines on how to compensate for multi-variable testing in microarrays. When analyzing microarrays, several genes are compared in the same experiment, which requires statistical compensation for multi-variable testing. To adjust for this, a false discovery based modified t-test was used in the array studies. The advantage of this method is that it not only provides the statistical p-value but also the accuracy of the statistical calculations by estimating the chance that each gene may be a false positive. To avoid relying on one method, genes of interest were verified by means of real-time PCR.

Limitations in proteomics studies

Few proteomics studies have been performed on the PE placenta. In general, the main problem with the study of the proteome is the dynamic range of protein expression which can range over as many as 8-12 magnitudes, meaning that low abundance proteins may remain undetected in the proteomics analysis (104). The low abundance proteins may of course also be of biological and pathophysiological importance. There is today no proteomic platform that adequately identifies and quantifies the entire proteome of an organ or organism. It has been suggested that dividing the proteome into smaller fractions, such as mitochondrial or trophoblast membrane fractions, can address the problems associated with the dynamic range. This approach has been shown to enrich low abundance proteins about 20 fold (105).

In plasma the high abundance proteins such as immunoglobulins, albumin and transferrin, make up approximately 95% of the total protein mass (106). Removing the high abundance proteins would allow closer examination of the low abundance proteins. However, most high abundance proteins interact in complexes with other proteins and removing them may cause a loss of important complexes. Therefore, antibody microarrays were used in Paper V to specifically screen for inflammatory markers.

The limitation of the 2D-PAGE platform in placenta is the complexity of the organ itself. Since the placenta contains many different cell types, it is hard to obtain a homogenous extract of trophoblasts. The dynamic range in the placenta is hard to overcome, which was confirmed in a study by Mine et al (107). Although over 100 out of 180 proteins expressed on their placenta 2D-PAGE were identified, the human proteome is suggested to contain up to 1,000,000 proteins. Furthermore, they were unable to identify the differentially expressed proteins between PE and controls due to the low abundance of proteins in the spots highlighting the issue of dynamic range. Moreover, the placenta contains both fetal and maternal blood, making it hard to define if it is the fetal or maternal proteins being examined. In an attempt to maximize the number of spots on 2D-PAGE, the methods for sample preparation were optimized (Paper III). 2D-PAGE-analysis using optimized protocols resulted in 604 spots on the PE gels and 747 spots on the control gels, indicating that optimizing the extraction protocols further enriched the proteins in the placenta

Summary

Preeclampsia is a serious pregnancy disorder defined by hypertension and proteinuria. PE is believed to be a two-stage disease beginning with an abnormal placentation causing underperfusion of the placenta. The second step, the maternal systemic response, is characterized by general vascular inflammation and endothelial dysfunction. The etiology of PE is unclear although some factors such hypoxia, oxidative stress, apoptosis, inflammation and angiogenetic factors are important mechanisms in PE pathophysiology.

Using a subtraction cDNA library to create PE associated microarray chips, increased expression of hemoglobin γ and α chains were revealed in the PE placenta. Analysis of Hbγ protein expression with immunohistochemistry confirmed accumulation of free fetal Hb, particularly in the vascular lumen. Free hemoglobin is a harmful molecule capable of causing endothelial damage and inflammation, two hallmarks of PE.

2D-PAGE was used to compare the protein profile of the PE and normal placenta. In order to gain maximum resolution and reproducibility, optimized protein extraction protocols were developed. Lysing placenta samples with urea/CHAPS and precipitating with dichloromethanol gave the best protein separation. Removing glycogen from the extracted placental samples was crucial for separation. 2D-PAGE showed 28 proteins increased and 23 decreased in the PE placenta of which two proteins were successfully identified. Apolipoprotein A1 (APOA1) was increased 1.63 times and tropomyosin 1 absent in the PE placenta. The accumulation of APOA1 was verified by means of western blot.

There was increased expression of genes related to movement of inflammatory cells in the PE placenta. The high risk group with notching revealed increased expression of genes related to apoptosis and antigen presentation but no genes related to inflammation. Hence the notch group had a profile similar to the early PE placenta. Based on this, PE is suggested to be a three-stage disease where the notch placenta is a reversible middle stage in the PE progression. Accumulation of inflammatory cells in the notch placenta may cause inflammation that drives the pathophysiology into PE. In the notch without PE placenta, the increase in genes associated with antigen presentation may offer protection against immune reaction and inflammation thereby preventing progression into PE (Figure 7).

Fetal hemoglobin (HbF) consists of two γ and two α hemoglobin chains. Normally, HbF is present in very low levels in adult blood. In PE, there was eight fold increase of free HbF in the maternal plasma. Free Hb and its metabolites have systemic effects such as increasing hypertension. Free HbF may therefore play a role in the systemic endothelial dysfunction and inflammation seen in PE. Higher levels of plasma HbF may in the future serve as a diagnostic marker for PE.

PE is hard to define based on hypertension and proteinuria, rather time of onset seem to better reflect how severe PE manifests. To specifically profile the maternal inflammatory response in PE, antibody microarrays were used to compare changes in maternal inflammatory markers in early and late onset PE (EPE and LPE respectively), PE with notching and IUGR (PIN) and controls. The results underlined the complexity of the maternal response in PE. The different signatures suggest that the maternal immune response may play a central role in PE manifests.

Conclusions

- Fetal hemoglobin is over-expressed in the PE placenta. Cells expressing Hb may either be intrinsic to the placenta or of fetal origin.
- The hemoglobin scavenger haptoglobin showed decreased expression in the notch placenta suggesting that impaired Hb scavenging may contribute to the Hb accumulation
- Increased levels of plasma HbF may serve as a diagnostic marker for PE.
- PE is suggested to be a three-stage disease with notch as a reversible middle stage.
- Increased expression of antigen presenting genes may protect against inflammation and thereby preventing PE
- The severity and type of immune response may determine the different PE manifestations
- Dividing PE into early and late onset may be too simplified to describe the complex maternal immune response in PE
- By comparing different lysis and precipitation methods in PE, we identified three steps that may be crucial for 2D-PAGE: 1) removal of glycogen by centrifugation 2) the choice of lysis buffer affects the protein contents in the extracted sample. Lysing with urea/CHAPS appear to result in more low-molecular weight proteins 3) 2D-PAGE expression is affected by which precipitation method that is used.

Future directions

- To confirm Hb's pathophsyiological effects on the placenta using the dual *in vitro* placenta perfusion model
- To confirm Hb's systemic pathophsyiological effects using an established animal model
- To develop free fetal Hb as a diagnostic marker for PE
- To evaluate alpha-1-microglobulin, a high affinity heme scavenger, as potential treatment for PE

Populärvetenskaplig sammanfattning

Bakgrund

Havandeskapsförgiftning – preeklampsi (PE) – är en graviditetssjukdom som årligen drabbar 8 000 000 kvinnor i världen. PE karakteriseras av högt blodtryck och läckage av äggvita i urinen. PE yttrar sig med ett flertal diffusa symptom, bland annat huvudvärk, svullnad och buksmärtor. Det finns idag ingen säker diagnosmetod, ej heller något botemedel mot PE, endast symptomatisk behandling med blodtryckssänkande medel finns tillgängligt. I svåra fall av PE utvecklas epileptiska kramper – eklampsi – vilket kan vara fatalt för både mor och barn. Den enda behandlingen för PE är förlossning varvid moderkakan – placentan – avlägsnas. Hur och varför PE uppkommer är fortfarande okänt, dock anses placentan spela en central roll i sjukdomsförloppet eftersom alla symptom försvinner när moderkakan avlägsnas.

PE tros utvecklas i två steg. Det första steget beror på en ytlig inväxt av moderkakan i livmodern, vilket leder till en försämrad blodcirkulation i placentan. På grund av ojämnt blodflöde och ojämna syrenivåer, bildas fria syreradikaler som i sin tur orsakar skador på moderkakans celler – trofoblasterna – samt på blodkärlens väggar – endotel. Kärlskadorna framkallar en inflammatorisk reaktion som ytterligare förvärrar skadorna på och läckage uppstår. Detta läckage yttrar sig som äggvita i urinen när njurarnas blodkärl drabbas.

Det andra steget i PE är moderns reaktion på de skador som uppkommit i placentan. Det spekuleras i att faktorer (gift) utsöndras från placentan ut i moderns blod. Moderns symptom anses vara orsakade av inflammation och rubbad funktion i kroppens kärlväggar, som i sin tur orsakar svullnaden, högt blodtryck och äggviteläckage, typiska manifest vid PE.

Med hjälp av Doppler ultraljud kan man tidigt i graviditeten avgöra om blodgenomströmningen i moderkakan är försämrad. Ett ökat kärlmotstånd karakteriseras av ett specifikt ultraljudsmönster, kallat notch. En graviditet med notch-tecken i livmoderns båda kärl har en ökad risk att utveckla PE, dock drabbas inte alla. Detta innebär att placentan från kvinnor med notch som inte drabbas av PE kan ha försvarsmekanismer som skyddar mot utveckling av PE. En reducerad placentaperfusion kan ge upphov till minskad fostertillväxt, intrauterin tillväxthämning (IUGR). IUGR ses i ett av fyra PE fall och i en av fem graviditeter med notch.

Målsättning

Detta translationella projekt syftar till att klargöra de bakomliggande mekanismerna kring utvecklingen av PE. Då moderkakan anses vara av central betydelse för uppkomsten av PE, har gen- och proteinuttrycket studerats i placentan från PE, graviditeter med notch utan PE, notch som utvecklat PE samt friska kontroller. Projektets specifika målsättningar var att:

- Ta fram gener specifika för PE genom att subtrahera fram skillnader mellan PE och friska placentor och sedan skapa verktyg för att undersöka deras uttryck i placentor från PE.
- Skapa nya hypoteser för PE genom att undersöka skillnader i genuttrycket i placentor från olika kliniska undergrupper av PE
- Optimera metoder för proteinextraktion och med dessa undersöka placentas proteinprofil
- Studera den maternella inflammatoriska responsen vid PE med hjälp av ny teknologi

Resultat

Genuttryck i placentor från PE visade ökad produktion av fosterhemoglobin. Vidare var inflammatoriska gener överuttryckta i moderkakor från PE. Placentor från graviditeter med

notch-tecken som inte utvecklade PE visade högre uttryck av gener relaterade till oxidativ stress, ökad celldöd samt gener relaterade till immunförsvarets igenkänningsmekanism. Det ökade genuttrycket av fosterhemoglobin motsvarades även av en ansamling av proteinet hemoglobin i placentans blodkärl. De ökade nivåerna av fosterhemoglobin visades också läcka över till mammans blodcirkulation. Blodprovsmätningar visade åtta gånger högre nivåer av fosterhemoglobin i PE. Då fritt hemoglobin är känt för att vara vävnadsretande och inflammationsinducerande syftade arbete V till att studera det inflammatoriska svaret vid olika allvarlighetsgrader av PE. Detta gjordes med nyutvecklad teknik som bygger på antikroppsidentifiering av inflammatoriska markörer i blod. Antikroppsscreeningen visade att PE kan uppvisar olika grader av inflammation beroende på hur allvarligt sjukdomen yttrar sig. De svårare fallen av PE kunde särskiljas från de andra grupperna medans de mildare fallen mer liknade den normala kontrollgruppen. De PE-fall med notch-tecken och IUGR hade en egen unik inflammationsprofil.

Slutsatser

Resultaten antyder att fritt fosterhemoglobin kan vara en skadlig faktor som bildas i moderkakan vid PE och senare läcker över till moderns blodcirkulation och där kan orsaka skador på moderns blodkärl. Förutom nivåerna av Hb, tycks den maternella inflammationsresponsen spela en avgörande betydelse för hur tidigt och hur allvarligt PE utvecklar sig. Nivåerna av fritt Hb kan i framtiden möjligen användas för att förutspå, diagnostisera och bedöma allvarlighetsgraden av PE.

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