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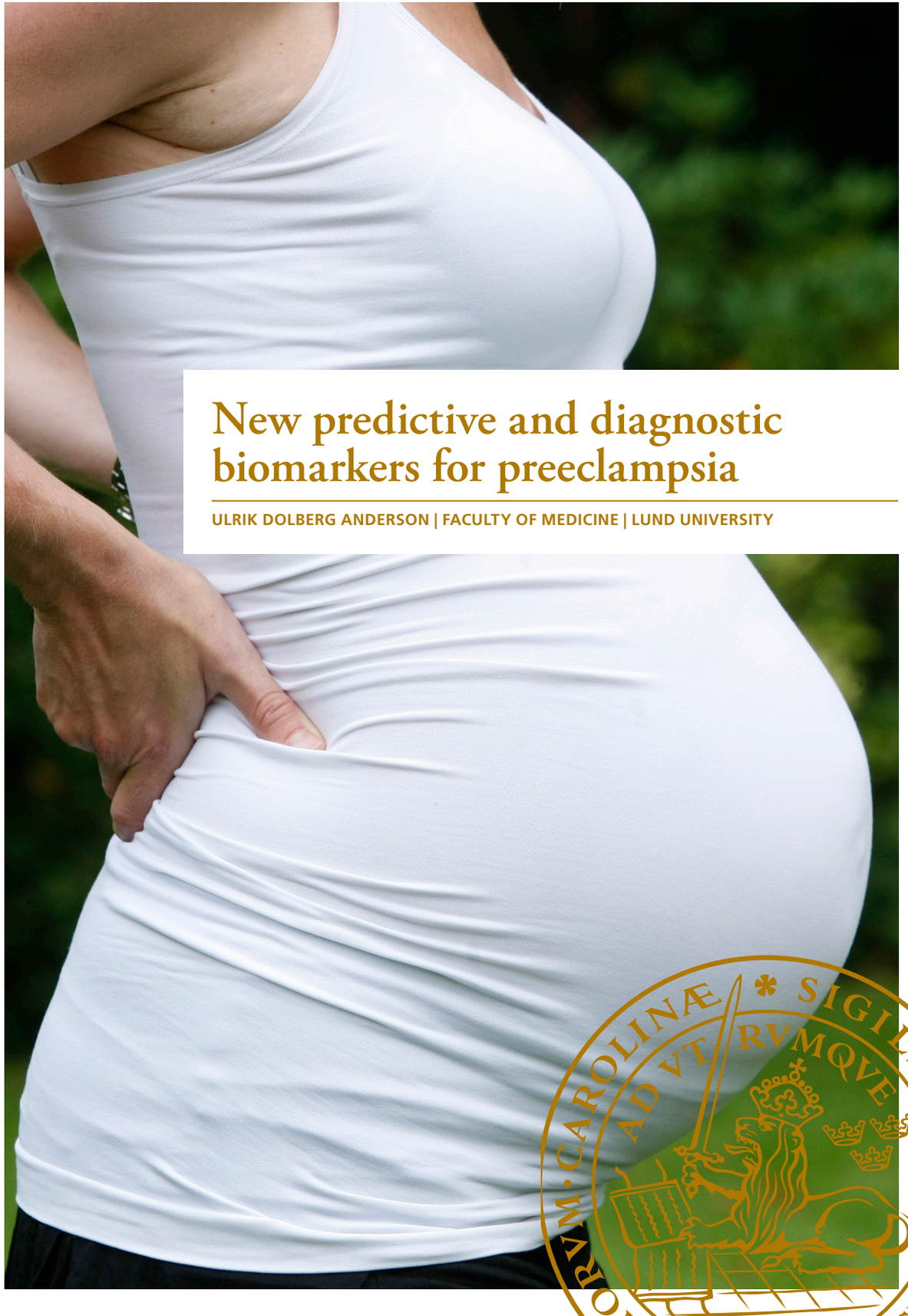

New predictive and diagnostic biomarkers for preeclampsia

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New predictive and diagnostic biomarkers for preeclampsia

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New predictive and diagnostic biomarkers for preeclampsia

Ulrik Dolberg Anderson
Department of Obstetrics and Gynecology
Skåne's University Hospital

LUND
UNIVERSITY

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Abstract Preeclampsia is a serious pregnancy complication that affects 3-8% of all pregnancies leading to maternal- and fetal morbidity and mortality. The etiology is still not known in detail. Previous findings have shown an up regulation of the genes coding for fetal hemoglobin (HbF) in preeclamptic placentas. The cell-free HbF protein was shown to be accumulating in the vascular lumen, to induce oxidative stress, which damages the blood-placenta-barrier, causing a leak into the maternal blood circulation. Unbound cell-free HbF is highly reactive and toxic and therefore normally being scavenged by haptoglobin, hemopexin (Hpx) and α_1-microglobulin (A1M). In the maternal circulation cell-free HbF causes general endothelial- and organ damage.

This thesis describes the role of placentally derived HbF in the etiology of preeclampsia and how it can be used as a biomarker for PE. The five articles also describe the role of hemoglobin- and heme scavenging proteins in prediction and diagnosis of preeclampsia and HbF as a causal factor for renal injury in preeclampsia.

The results in paper I and II show that the maternal HbF plasma concentration is increased as early as the first trimester in women who subsequently develop preeclampsia. Furthermore increased circulating concentrations of A1M and decreased levels of hemopexin were found. The results indicate that HbF, A1M and Hpx can be used as predictive biomarkers as early as the first trimester with 60% prediction rate at 5% false positive rate.

The results in paper III and IV indicate that the constant increased level of HbF strain the scavenging proteins and gradually deplete them. The HbF plasma levels were four fold increased in patients diagnosed with PE compared to controls. Furthermore, the A1M levels were increased and the levels of Hpx, Hpx enzymatic activity and Haptoglobin were significantly lower in patients with preeclampsia.

In both paper III and IV HbF, heme and scavenging proteins were evaluated as biomarkers that support the diagnosis of PE. The results showed that the combination of these biomarkers could detect up to 84% of the PE patients at a false positive rate of 10% in term pregnancy. It was further concluded that HbF and hemoglobin- and heme scavengers potentially can be used to support the diagnosis of PE. In addition, both HO-1 and Hpx activity correlated with the maternal blood pressure and hence the severity of PE.

It is known that excessive amounts of cell-free Hb lead to endotheliosis and kidney injuries. In paper IV it was shown that there was increased urinary concentrations of podocyte specific extracellular vesicles (EVs) in women diagnosed with PE. The excessive circulating concentrations of HbF in women with PE was correlated to the concentration of urinary podocyte specific EVs and proteinuria. It was concluded that increased circulating concentrations of HbF in combination with low scavenging capacity might cause significant damage to the renal podocytes.

In conclusion the thesis present free HbF and its' scavenging proteins as new important players in the PE etiology. Furthermore, it was shown that HbF and the heme scavenging proteins could be used as predictive- and diagnostic biomarkers for PE as early as the first trimester of pregnancy.

Key words HbF, A1M, preeclampsia, biomarkers, prediction, diagnosis, haptoglobin, hemopexin, HO-1

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New predictive and diagnostic biomarkers for preeclampsia

Ulrik Dolberg Anderson

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“The greatest enemy of knowledge is not ignorance; it is the illusion of knowledge”

Stephen Hawkings

Thesis at a glance

	Study area	Analyses /Cohort	Results	Conclusion(s)
Paper I	Prediction of preeclampsia	HbF, A1M, Total Hb 96 pregnancy serum samples (60 PE) collected in week 10-16 in London, UK	Increased HbF and A1M levels in patients who subsequently developed PE. Combined prediction rate 69% at 5% false positive rate.	HbF and A1M are potential predictive biomarkers for PE.
Paper II	Prediction of and early pathogenic mechanisms of preeclampsia	HbF, A1M, Hp, Hpx, maternal characteristics Uterine artery Doppler ultrasound 433 pregnancy serum samples (86 PE) collected in London, UK	Increased HbF and A1M levels and low Hpx levels in patients who subsequently developed PE. Combined sensitivity of 60% at 95% specificity.	Increased circulating levels of HbF strain the endogenous hemoglobin and heme scavenging systems. HbF, A1M and Hpx are potential predictive biomarkers for PE either alone or in combination with maternal characteristics and/or uterine artery Doppler ultrasound.
Paper III	Diagnosis and pathogenesis of preeclampsia	HbF, A1M, Hp, Hpx, CD163, Hb-Hp complex, Total Hb 145 term plasma samples (98 PE) collected in Lund, Sweden	Increased HbF and A1M and lower Hp and Hpx in patients with PE. HbF, A1M and Hpx diagnose 69 % of all PE at 5% false positive rate. Biomarkers are predictive of maternal and fetal outcomes.	Increased levels of HbF strain and deplete heme- and hemoglobin-scavenging proteins. HbF, A1M, Hp and Hpx are potential diagnostic biomarkers for PE.
Paper IV	Diagnosis and pathogenesis of preeclampsia	Hpx activity, free heme, HO-1 135 term plasma samples (89 PE) collected in Lund, Sweden	Decreased Hpx activity in late onset PE. Increased heme concentration and lower HO-1 concentration in PE. HO-1 correlated to both systolic- and diastolic blood pressure. Combined diagnostic rate of 84 % at 10 % FPR.	HO-1, heme and Hpx-a could be used as biomarkers supporting the diagnosis of PE.
Paper V	Kidney damaging effects of HbF in patients with preeclampsia	Podocyte specific proteins on urinary EVs, P-creatinine, P-Cystatin C, P, uric acid 92 term urine and plasma samples (49 PE) collected in Lund, Sweden	Increased urinary concentration of podocyte specific EVs in PE. P-HbF concentration positively correlated to the concentration of EVs and proteinuria.	Renal injury in patients with PE is associated to podocyte specific EVs, which is suggested to be HbF-induced injury.

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I. Anderson UD, Olsson MG, Rutardottir S, Centlow M, Kristensen KH, Isberg PE, Thilaganathan B, Åkerström B, and Hansson SR.
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Submitted manuscript

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PLOS ONE, 2015, *Manuscript in press.*

IV. Anderson UD, Jälmby M, Faas MM, and Hansson SR.
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Manuscript.

V. Gilani SI, Anderson UD, Jayachandran M, Weissgerber TL, Zand L, White WM, Milic N, Grande JP, Nath KA, Gram M, Åkerström B, Hansson SR, and Garovic VD.
Urinary Extracellular Vesicles Positive for Podocyte-specific Proteins are Associated with Renal Injury in Preeclampsia
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Anderson UD, Olsson MG, Kristensen KH, Åkerström B, and Hansson SR.
Review: Biochemical markers to predict preeclampsia.
Placenta. 2012;33 Suppl:S42-7.

Anderson UD, Gram M, Åkerström B and Hansson SR.
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Current Hypertension Reports. 2015;17(9):584.

Abbreviations

A1M	alpha-1-microglobulin
ASA	acetylsalicylic acid
AUC	area under the curve
CD 163	cluster of differentiation 163
DR	detection rate (diagnostic rate)
ELISA	enzyme-linked sorbent assay
EVs	extracellular vesicles
EVTs	extravillous trophoblast cells
FPR	false positive rate
HbF	cell-free fetal hemoglobin
Hb–Hp	haptoglobin-bound hemoglobin
HO-1	heme oxygenase 1
Hp	haptoglobin
Hpx	hemopexin
IUGR	intrauterine growth restriction
IVF	*in vitro* fertilization
MAP	mean arterial pressure
MOM	multiple of the median
NK cells	natural killer cells
PE	preeclampsia
PAPP-A	pregnancy-associated plasma protein A
PI	pulsatility index
PlGF	placental growth factor

PR	prediction rate
RI	resistance index
RIA	radioimmunoassay
PlGF	placental growth factor
ROC	receiver operational curve
sFlt-1	soluble FMS-like tyrosine kinase 1
UtAD	uterine artery Doppler ultrasound

Abstract

Preeclampsia is a serious pregnancy complication that affects 3-8% of all pregnancies leading to maternal- and fetal morbidity and mortality. The etiology is still not known in detail. Previous findings have shown an up regulation of the genes coding for fetal hemoglobin (HbF) in preeclamptic placentas. The cell-free HbF protein was shown to be accumulating in the vascular lumen, to induce oxidative stress, which damages the blood-placenta-barrier, causing a leak into the maternal blood circulation. Unbound cell-free HbF is highly reactive and toxic and therefore normally being scavenged by haptoglobin, hemopexin (Hpx) and α_1-microglobulin (A1M). In the maternal circulation cell-free HbF causes general endothelial- and organ damage.

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In both paper III and IV HbF, heme and scavenging proteins were evaluated as biomarkers that support the diagnosis of PE. The results showed that the combination of these biomarkers could detect up to 84% of the PE patients at a false positive rate of 10% in term pregnancy. It was further concluded that HbF and hemoglobin- and heme scavengers potentially can be used to support the diagnosis of PE. In addition, both HO-1 and Hpx activity correlated with the maternal blood pressure and hence the severity of PE.

It is known that excessive amounts of cell-free Hb lead to endotheliosis and kidney injuries. In paper IV it was shown that there was increased urinary concentrations of podocyte specific extracellular vesicles (EVs) in women diagnosed with PE. The excessive circulating concentrations of HbF in women with PE was correlated to the concentration of urinary podocyte specific EVs and proteinuria. It was concluded that increased circulating concentrations of HbF in combination with low scavenging capacity might cause significant damage to the renal podocytes.

In conclusion the thesis present free HbF and its' scavenging proteins as new important players in the PE etiology. Furthermore, it was shown that HbF and the heme scavenging proteins could be used as predictive- and diagnostic biomarkers for PE as early as the first trimester of pregnancy.

Preeclampsia

Epidemiology

Every year, over half a million women die worldwide of pregnancy-related complications. Ninety-nine per cent of these deaths occur in low- and middle-income countries [1]. Complications of preeclampsia (PE) and eclampsia account for between 9–26% of these deaths [1]. PE is also a major cause of fetal morbidity and mortality, accounting for up to 75 000 maternal and approximately 500 000 perinatal deaths each year, especially in developing countries [2, 3]. Preeclampsia occurs in 3–8% of all pregnancies, with large regional differences in incidence [4]. In developing countries with limited access to high-quality clinical care and no established antenatal maternity care system, the death rates are as high as 15% among patients with PE, but in the Western world with its high-quality maternal care and surveillance of maternal blood pressure throughout pregnancy, the mortality rate is as low as 0–1.8% [3]. The incidence of PE is now slowly increasing even in high-income countries, probably due to the increased incidence of obesity [5].

Definitions

Preeclampsia is classified as a syndrome because it is defined by clinical findings and not by a particular pathophysiology. The International Society for the Study of Hypertension in Pregnancy (ISSHP) define PE as *de novo* hypertension after 20 weeks of gestation in combination with significant proteinuria, defining hypertension as a blood pressure ≥ 140/90 and significant proteinuria as ≥ 300 mg/24 hours [6]. This definition is in line with the definitions given by the National Institute for Health and Clinical Excellence (NICE guidelines), but other national or international organizations have different definitions [7-10].

Lately, however, there has been discussion of whether manifestations of maternal organ dysfunction could replace proteinuria in some cases. Proteinuria is a relatively unstable biomarker because it has great day-to-day variation, and there are certain inaccuracies in measurement, depending on the method used [11]. The new definitions enable

clinicians to distinguish between PE with severe organ dysfunction (but without proteinuria) and gestational hypertension (GH). The latest update from the ISSHP from 2014 [12] has therefore expanded the definition to include the following:

De novo hypertension and the coexistence of one or more of the following new-onset conditions:

(1) Proteinuria (spot urine protein/creatinine ≥ 30 mg/mmol or ≥ 300 mg/day or at least 1 g/L (2+) on a dipstick).

(2) Other maternal organ dysfunction:

- Renal insufficiency (creatinine ≥ 90 mmol/L).
- Liver involvement (elevated transaminases—at least twice the upper limit of normal ± right upper quadrant, or epigastric pain).
- Neurological complications. Examples include eclampsia, altered mental state, blindness, stroke, or—more commonly—hyperreflexia when accompanied by clonus, severe headaches when accompanied by clonus, or persistent visual scotomata.
- Hematological complications (thrombocytopenia—platelet count below 150 000/µL, DIC, or hemolysis).

(3) Utero-placental dysfunction.

- Intrauterine growth restriction [12].

The American Congress of Obstetricians and Gynecologists (ACOG) support this new definition. In their latest *Task Force on Hypertension in Pregnancy* from 2013, the definition of PE is similar to that given by the ISSHP [7]. ACOG, however, has chosen different definitions for when to diagnose thrombocytopenia (<100 000/µL) and renal insufficiency (creatinine > 1.1 mg/dL, ISSHP > 90 mmol/L=1.02 mg/dL). In addition, pulmonary edema has also been added as an organ failure in the ACOG definition of severe PE [7].

Etiology

The two-stage model

Preeclampsia is usually described as a two-stage syndrome (Fig. 1) [13]. The first stage is characterized by shallow invasion of fetal trophoblast cells into the decidua and inadequate modification of the spiral arteries [14]. This leads to uneven blood flow to the placenta, and thus to placental stress. In response, the damaged placenta releases a number of factors and placental debris. Among these, fragments of syncytiotrophoblast cells, basal membrane, microparticles, microRNA, and fetal DNA have been detected in the maternal circulation where they cause inflammation and endothelial injuries [15-22].

The second stage of PE is characterized by the maternal disease, presenting with elevated blood pressure and proteinuria (Fig. 1) [13]. It has been suggested that a factor, X, links stages 1 and 2, but more likely there are several factors contributing to the PE etiology. These potential factors contribute to the maternal inflammation, endothelial damage, and clinical findings of PE—elevated blood pressure and proteinuria.

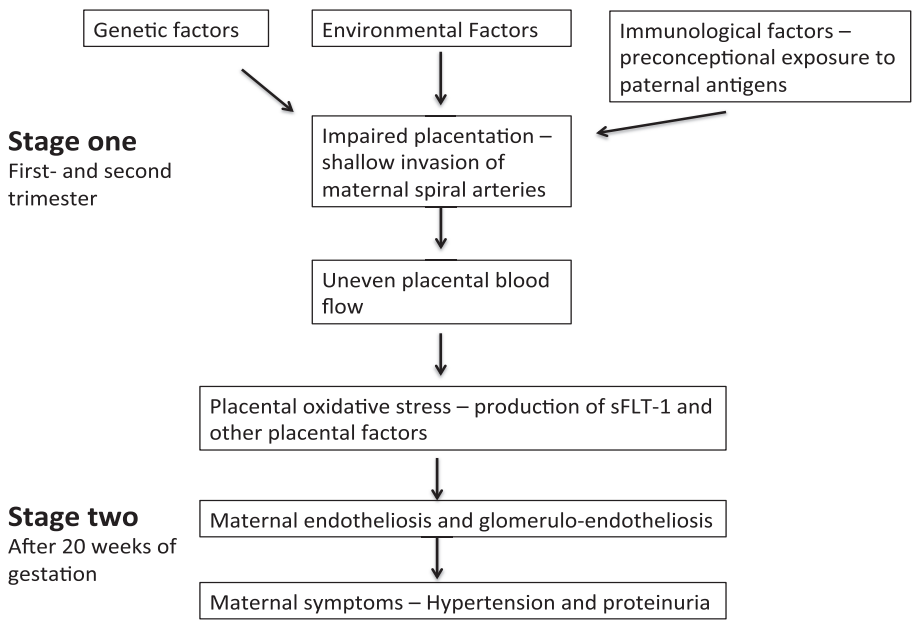

Figure 1 The two-stage model of preeclampsia

Placenta in normal pregnancy and PE

Approximately six days after conception the blastocyst implants in the maternal endometrium. The syncytio-trophoblast cells insert finger-like structures into the maternal decidua—the primary villi [23]. The core of the primary villi consists of cytotrophoblast cells. The primary villi pass through secondary and tertiary stages before reaching the stage of mature villi [23].

Extravillous cytotrophoblast cells (EVTs) invade deeper into the maternal decidua [24]. These EVTs present fetal (paternal) alloantigens on their surfaces. Maternal natural killer cells (NK cells) and T cells in the maternal decidua react to paternal alloantigens presented by the EVTs. The maternal immune system may be adapted to these antigens, as NK cells are exposed to paternal alloantigens presented by sperm cells [24]. In fact, antenatal sperm exposure has been shown to modify the risk of developing PE [25, 26]. Women with a long exposure time to paternal alloantigens presented by sperm cells have a reduced risk of PE [27]. The method of exposure may even play a role—since pregestational oral ingestion of seminal fluid has also been found to reduce the incidence of PE [28]. In contrast, for women who become pregnant shortly after meeting a new partner, or where couples have always used barrier contraception methods, the risk of developing PE is increased [25, 29]. Women who have more children with the same partner progressively reduce their risk of PE with each further pregnancy due to immune adaptation. This also means that if a woman changes partner between two pregnancies, her risk of PE is increased compared to the risk for a nullipara [4].

In the maternal decidua, the NK cells and T cells recognize the paternal alloantigens presented by the EVTs and release trophic factors (cytokines etc.) that promote the invasion of the EVTs deeper into the maternal decidua [30]. The EVTs invade the smooth muscle wall of the maternal spiral arteries and modify the wall by removing the smooth muscle cell layer, thereby remodeling the spiral arteries, making them wider and allowing a low-resistance flow. The remodeling of the spiral arteries begins at 8–10 weeks of gestation and continues to approximately 18–20 weeks of gestation. It leads to a constant, low-velocity, low-resistance blood flow into the placenta's intravillous space, which may be clinically measured by Doppler ultrasound [31-34]. In patients who subsequently develop PE, the invasion and remodeling of the spiral arteries is inadequate. It has been suggested that the maternal NK cells and T cells do not recognize the fetal/paternal alloantigens on the surface of the EVTs and therefore do not release sufficient amounts of trophic factor to promote deep invasion of maternal spiral arteries [30, 31]. This maladaptation to the fetal/paternal alloantigens could explain why the risk of PE is increased for young age, for short interval between first coitus and pregnancy, and for sperm or egg donation. The shallow invasion of the maternal spiral arteries causes the smooth muscle of the spiral arteries to keep their

ability to contract, which leads to uneven blood flow through the spiral arteries and into the placenta. The blood flow has a higher velocity through the narrow arteries, which mediate mechanical damage to the placenta. The uneven blood flow induces placental oxidative stress and placental endoplasmic reticulum stress [24, 30, 35].

Clinically, PE is a very heterogeneous condition with a broad range of symptoms and clinical manifestations [36]. The clinical presentation ranges from mild cases in late pregnancy to severe cases with early onset and intrauterine growth restriction (IUGR). Most likely, the heterogeneity is caused by variations in placental involvement, which in combination with maternal constitutional factors change the physiological capacity to handle pregnancy-related stress [24]. Severe PE cases present with high blood pressure ≥ 160/110 and proteinuria and/or (multiple) organ dysfunction [7, 37]. Maternal complications in patients with severe PE depend on the severity of the organ failure.

PE is characterized by general maternal endothelial dysfunction and inflammation, activation of the coagulation system, and hemoconcentration—these last may include cerebral edema with cramps (eclampsia), pulmonary edema, disseminated intravascular coagulation (DIC), heart failure, stroke/cerebral hemorrhage, and maternal death [24]. Fetal complications are often based on placental dysfunction and manifest as intrauterine growth restriction (IUGR), abruption of the placenta, and fetal death [38].

Eclampsia complicates about 1–2% of cases of severe preeclampsia [39]. The general seizures are caused by cerebral edema and are defined by tonic–clonic seizures in a pregnant or newly delivered woman that cannot be attributed to other causes (stroke, cerebral hemorrhage, epilepsy etc.). It is difficult to predict eclampsia. Many patients, however, have had prodromal signs or symptoms such as blurry vision or severe headache up to a week before the first seizure [40].

The kidney in preeclampsia

During normal pregnancy the kidneys increase in size and are reported to be up to 30% larger in volume, while the glomerular filtration rate (GFR) is increased by up to 40–50% [41]. This means that the normal values used for creatinine in clinical treatment are lower in pregnancy, and that renal insufficiency could be disguised within the normal range of non-pregnant women [42]. Creatinine has been shown to be a stable biomarker during pregnancy, but normal values should be assumed to be lower than for non-pregnant women. Cystatin C has been suggested as a marker of the GFR in pregnancy and also as a marker of the impaired kidney function seen in PE [43]. Increased cystatin C synthesis has also been shown in EVTs of placentas from preeclamptic women, which could explain part of the increased cystatin C levels in the

maternal circulation of women with PE [44]. Increased levels of cystatin C also reflect the degree of endotheliosis in normal, hypertensive, and preeclamptic pregnancies [45]. However, when using cystatin C as a marker of the GFR in pregnancy, it should be remembered that cystatin C increases during the third trimester of normal pregnancies [46]. Furthermore, a study has shown that that cystatin C does not correlate with inulin clearance during late pregnancy and post partum [47]. This is a matter of debate, as other studies have shown a correlation between iohexol clearance and cystatin C levels in both pregnant and non-pregnant women, and have concluded that cystatin C reflects the GFR very well even in the third trimester of pregnancy[48].

In PE, the maternal systemic inflammatory response is activated, and the endothelia are an important component in the systemic inflammatory network [24]. This is also true of the glomerular endothelia of the kidneys. Inflammation and activation of the kidneys' glomerular endothelial cells—glomerular endotheliosis—has been described in 100% of the PE cases in a study of kidney biopsies from pregnancies complicated with PE and gestational hypertension [49]. A different study examined glomerular lesions in samples from the Dutch pathology register [50]: renal samples from 11 women who died from PE were compared with 25 normotensive controls (who died for other reasons during pregnancy) and with non-pregnant controls either with (n=14) or without chronic hypertension (n=13). This study also found characteristic glomerular lesions and glomerular endotheliosis in most renal sections of the PE cases. The total number of podocytes per glomerulus was a fairly consistent between the PE cases and the controls, but signs of increased podocyte turnover were found in the PE cases. This indicated that the mechanisms of podocyte replacement might play a role in the renal pathology of PE which leads to proteinuria in PE [50]. However, even in normal pregnancies the systemic inflammatory network is activated, and low-grade signs of glomerular endotheliosis are reported in 12–42% of normotensive, healthy pregnancies [49, 50].

The glomerular endothelium consists of podocytes, which create a barrier to protein loss from the blood to the urine. The podocytes interact through specialized junctions called glomerular slit diaphragms [51]. This forms the main filtration barrier of the glomerulus. Mature podocytes loose their mitotic capacity, and thus loss of podocytes, due to either apoptosis or detachment from the glomerular basal membrane, could consequently lead to a disturbance in the glomerular filtration membrane and thus proteinuria [52, 53]. It is thought that podocytes are replaced by recruitment of parietal epithelial cells that migrate and differentiate to podocytes in the glomeruli [50, 54]. In a relatively small study of 15 PE cases and 16 healthy controls it was shown that in PE pregnancies at term, podocytes or podocyte fragments are detectable in maternal urine, with a 100% sensitivity and specificity [55]. This has been interpreted as a result of the glomerular endotheliosis seen in PE [55]. It has therefore been speculated that the excretion of podocytes into the urine could be caused by the imbalance between pro- and anti-angiogenic factors in PE. Podocytes need pro-angiogenic stimulation to

maintain homeostasis, but if the pro-angiogenic factors are inhibited by anti-angiogenic factors such as sFlt-1 they become inflamed and begin to shed into the urine [55]. Studies have shown that—much like the circulating angiogenic/anti-angiogenic imbalance—shedding of podocytes into the urine occurs earlier in the course of the glomerular disease of PE than proteinuria does. Podocyturia has been studied in the second trimester of pregnancy, showing that, before the onset of clinical symptoms, podocyturia is present in 100% of the patients who subsequently developed PE and in 0% of the healthy control patients [56]. Podocyturia was therefore suggested as a highly sensitive and specific biomarker of PE in the second trimester of pregnancy [56].

Long-term risks to the mother after preeclampsia

Preeclampsia is described as a systemic inflammation in the mother, in which the maternal endothelium plays an important role because it is inflammatorily activated. The maternal endotheliosis leads to long-term damage to the endothelium, increasing the risk of endothelial-related diseases later in life for both mother and child. Since the diagnosis of PE has varied over time, it is difficult to construct studies that investigate these long-term consequences for the mother and child. Most published studies therefore present relatively short follow-up times. There are some published studies that address this topic, however: two meta-analyses [57, 58] and a cohort study [59]. One of the meta-analyses shows an increased risk of maternal cardiac disease (RR 2.33, 95% CI: 1.95–2.78)), cerebrovascular disease (RR 2.03, 1.54–2.67), and cardiovascular mortality (RR 2.29, 1.73–3.04) [57]. Meta-regression revealed a graded relationship between the severity of PE and eclampsia and the risk of cardiac disease (mild PE: RR 2.00, 1.83–2.19; moderate PE: RR 2.99, 2.51–3.58; severe PE: RR 5.36, 3.96–7.27; $P < 0.0001$) [57].

The CHAMPS study was a retrospective cohort study of 1.03 million women which showed a 2.5-fold increased risk of cardiovascular disease later in life in women who had experienced maternal placental syndrome (PE, IUGR, placental abruption) [60].

Common for most of these studies is a relatively short follow-up time, which creates a problem, since such conditions as cardiac diseases, stroke, and Type 2 diabetes usually do not occur until after 50 years of age, 15–30 years after the patients have suffered from PE. Studies from the Danish National Patient Registry involving almost 800 000 patients have shown an increased risk of death from cardiovascular causes and increased risk of Type 2 diabetes [59]. A very recent study with a follow-up of up to 24 years finds that previous pregnancy with PE is an independent risk factor for long-term maternal atherosclerotic morbidity [61]. The risk is more substantial for patients with severe and recurrent episodes of PE [61].

Cell-free fetal hemoglobin as a new etiological factor

The placenta is central in the pathophysiology of PE. In order to compare placentas from PE and normal pregnancies, techniques such as proteomics and genomics were used, revealing fetal Hb to be a potential factor linking stages 1 and 2 in PE [62]. Gene microarrays and proteomic techniques were used to try to identify placental genes related to PE. A subtraction library was created and approximately 750 genes associated with the development of PE were identified [62]. Custom microarray chips were created and an analysis was made of a cohort of term PE placentas (n=10), term PE with bilateral uterine artery notching, cases with bilateral notching but without PE (n=5), and a control group of normal, normotensive, term pregnancies (n=15). The results showed the up-regulated expression of genes coding for α_2 and γ-chains of fetal hemoglobin in the PE placentas. The proteomics and *in situ* hybridization analyses revealed an accumulation of free HbF in the placental vascular lumen [62]. The cells expressing HbF were identified as hematopoietic stem cells located close to the vascular lumen (Fig. 2) [62]. Further array studies showed differentially expressed genes related to inflammation, apoptosis, and oxidative stress in the PE placentas [63-65].

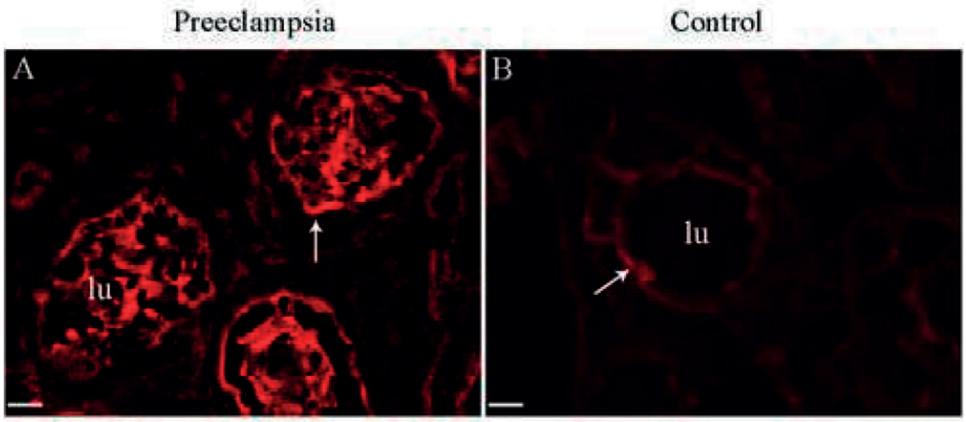

Figure 2 Increased expression of HbF in hematopoietic stem cell of the placeta. HbF accumulates in the placental capillary lumens. (Modified from Centlow et al 20008).

Hemoglobin toxicity

Cell-free hemoglobin and its metabolites, free heme and ferrous iron (Fe^{++}), are toxic to tissue in general because they induce oxidative stress (OS) [66-69]. Oxidative stress is defined as an imbalance between reactive oxidative compounds and the physiological antioxidative defense mechanisms. These reactive oxidative compounds could be free oxygen radicals, or peroxides [70]. Antioxidants are molecules that inhibit oxidation. They are classified into two groups: enzymatic and non-enzymatic antioxidants. An example of an enzymatic antioxidant is heme oxygenase (HO), which catalyzes the catabolization of heme into biliverdin, carbon monoxide (CO), and free iron [71, 72]. An example of a non-enzymatic antioxidant could be glutathione, an endogenous antioxidant that neutralizes free radicals and reactive oxygen species [73]. The heme scavenger alpha-1-microglobulin (A1M) is an example of a protein that has both enzymatic and non-enzymatic antioxidant properties [74-76].

In PE, oxidative stress has been described both in the placenta and in the maternal circulation [77, 78]. In addition, the concentrations of circulating antioxidants are reduced in women with PE [79, 80]. Extracellular unbound hemoglobin (cell-free Hb) induces oxidative stress at a very high level. The strong oxidative properties of cell-free Hb are mainly attributed to the redox activity of the iron atom [69]. The cell Hb binds strongly to the vasodilator nitric oxide (NO), which leads to vasoconstriction and thus to increased blood pressure [69]. Cell-free Hb with bound oxygen (OxyHb) generates free oxygen radicals spontaneously [81]. Free heme has direct effects on inflammatory pathways and is able to induce both neutrophils and cytokine synthesis [82]. Through

these mechanisms, cell-free Hb and its metabolites induce oxidative stress, vasoconstriction, hemolysis, and endothelial damage in both the maternal vascular bed and the kidneys [81].

The dual placental perfusion model

To evaluate the toxic effects of free Hb in the placenta, May et al. set up the dual placental perfusion model [83]. In a cotelydon of a newborn placenta the fetal and maternal circulations were reestablished. Cell-free Hb was added to the fetal circulation to mimic the situation in the PE placenta [62]. The cell-free Hb induced damage to the placenta much like that seen in PE placentas. This was verified with electron microscopy; in which extended cellular and matrix damage was seen [83]. This damage to the feto–maternal blood barrier caused leaking of cell-free Hb into the maternal circulation, where an increase in perfusion pressure was observed. If A1M was added to the maternal side, the damaging effects of cell-free Hb were reversed—no damage was seen in electron microscopy and no cell-free Hb leaked over the feto–maternal blood barrier. The conclusions of this study were that cell-free Hb induced the same damage in the placenta as seen in PE.

The pregnant ewe preeclampsia model

Wester-Rosenlöf et al. hypothesized that the increased circulating concentration of HbF seen in PE could possibly overwhelm the natural physiological scavenging mechanisms for cell-free Hb, and that treatment with the heme scavenger A1M could ameliorate the damaging effects of HbF [84]. Therefore a simulated *in vivo* PE model in pregnant ewes was set up. This model was built on starvation of sheep, which induces hemolysis and increases the circulating concentrations of free hemoglobin [85-87]. The use of A1M as a potential drug was tested in the model. A1M scavenges the circulating Hb and its breakdown product heme, and thereby reduces HbF-induced physiological effects that could potentially lead to PE. The original model involves starving the pregnant ewes for 96 hours, which causes hemolysis and leads to development of a PE-like disorder [85, 86]. The number of test animals was fifteen, of which eleven sheep were starved for 36 hours to induce PE-like symptoms and four served as healthy, well-fed controls. After starvation, the sheep were fed and observed for an additional 72 hours. Five of the animals were treated with A1M directly after starving; the remaining six animals placebo treated. The animals were observed for blood pressure, proteinuria, blood cell distribution, and clinical and inflammation markers in plasma. Before termination, the utero-placental blood flow was measured with Doppler ultrasound.

Renal function was assessed by Ficoll sieving [84]. The PE-like sheep showed signs of hemolysis revealed by the plasma markers. Furthermore, structural damage was seen in both the placenta and the kidneys, combined with an impaired renal function. The group of sheep treated with A1M did not show these pathological injuries and no side effects were observed in the treated group.

The conclusions of this study were that A1M had a positive impact in the starvation ewe PE model. Furthermore, A1M was suggested as a potential treatment for PE in humans.

The rabbit model

Nääv et al. presented findings from a study of nineteen pregnant rabbits where PE-like symptoms were induced by injection of free HbF [66]. The experiment was initiated at gestational day 20 and continued until the rabbits were terminated at gestational day 29. Five rabbits served as controls and were only injected with buffer. PE-like symptoms were induced in all other rabbits by injection of rabbit HbF (20 mg/kg) on days 1, 3, 5, 7, and 9 of the experiment. Eight rabbits were injected only with HbF and six rabbits were injected with both HbF and 6 mg/kg of A1M. Urine was collected daily and plasma samples were taken at day 1, 5, and 10 of the experiment. Blood pressure was measured daily. The animals were anesthetized before termination and their kidney function was estimated by Ficoll sieving, and organ samples and fetuses were collected for further analysis. The blood was analyzed for a range of biomarkers, including blood cell counts and markers for liver and renal function. Biopsies from the kidneys and the placentas were examined with electron microscopy. RNA expression of the gene coding for heme oxygenase 1 (HO-1) was measured in biopsies from the liver, kidney, and placenta. The model did not result in any measurable blood pressure increase in any of the animals injected with HbF. Pregnancy outcome measures (number of pups, live-born pups, weight of pups) were equal among all examined groups. A statistically significant decrease in kidney function was shown in the rabbits treated with HbF alone, but this decrease was not present in the rabbits also treated with A1M. Likewise, electron microscopy of the kidneys demonstrated structural damage in the groups treated with HbF. As a consequence of the HbF exposure, both intra- and extracellular damage was observed in the medulla and the cortex. The animals treated with both HbF and A1M showed normal renal morphology. The renal structural damage induced by HbF was therefore reversed by A1M. Electron microscopy of the placentas showed structural changes in the group treated with HbF alone that were not present in the control group or in the group treated with HbF and A1M. These structural placental injuries include a loss of extracellular matrix proteins with an almost complete loss of collagen fibers, and damage to the blood–placenta barrier.

This rabbit-based PE model therefore suggested that HbF injected into pregnant rabbits induces PE-like kidney and placental injuries. Furthermore, injection of the heme and radical scavenger A1M shows protective properties against these kidney and placental injuries. This rabbit-based study therefore supports the concept of using A1M as a potential therapy for PE in humans.

Free hemoglobin, oxidative stress, and scavenger systems

A range of physiological defense mechanisms has evolved to protect the body against the harmful effects of free Hb. The physiological defense system consists of several scavenging and catabolic pathways that eliminate free Hb from the circulation. Cell-free Hb is primarily bound by haptoglobin (Hp), while the Hb metabolite, heme, is bound to proteins such as hemopexin, albumin, and A1M and is also catabolized by heme oxygenase. The main physiological protection pathways against free heme are given in Fig. 3.

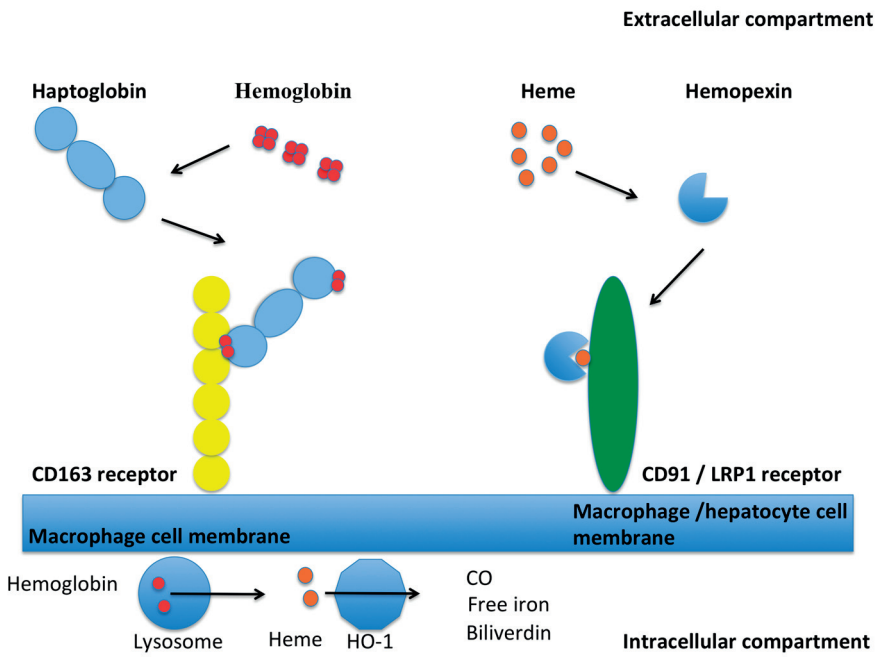

Figure 3 The hemoglobin- and heme degradation pathways through haptoglobin and hemopexin.

Haptoglobin

Haptoglobin is a circulating glycoprotein primarily produced by hepatocytes in the liver. It is the most important scavenger of cell-free Hb [88, 89]. Free Hb tightly binds to Hp in an interaction which is almost irreversible [90]. The Hb–Hp complex is then transported to the CD 163 receptor presented by monocytes or macrophages (Fig. 3), where the Hb–Hp complex is taken up by the macrophages by endocytosis [67, 68, 91, 92]. In the intracellular compartment, hemoglobin is catabolized to heme by lysosomes and heme is then further reduced to biliverdin, CO, and free iron by HO-1 (Fig. 3) (described in detail below) [91].

Haptoglobin's Hb-scavenging properties are highly rate limited by its concentration. Accelerated hemolysis or long-term exposure to increased levels of free Hb depletes Hp and increases Hb-induced injuries [88].

Hemopexin

Like Hp, hemopexin (Hpx) is a circulating glycoprotein that actively takes part in the innate physiological defense against cell-free Hb [91]. Hpx is the heme-binding protein with the highest heme affinity, and it binds heme at an equimolar ratio [93]. Hpx is mainly synthesized in the liver, but also in cells such as neurons, astrocytes, photoreceptor cells of the retina, fibroblast-like cells of the peripheral nervous system, renal mesangial cells, and skeletal muscle cells [94, 95]. The Hpx–heme complex is transported to the liver where the hepatocytes take up the Hpx–heme complex with the help of the LDL-receptor-related protein 1 (LRP1 receptor), also known as the CD91 receptor system [91, 96, 97]. In this way, Hpx is involved in the recycling of iron by facilitating the recovery and re-uptake of iron in the liver [91]. Furthermore, the Hpx–heme complex is taken up by macrophages through the CD91 receptor system with a mechanism similar to the uptake of the Hb–Hp complex by the CD 163 receptor (Fig. 3).

α_1-microglobulin

α_1-microglobulin (A1M) is a plasma- and extravascular protein that provides protection through its ability to bind and neutralize free heme and radicals [75, 98, 99]. Several *in vitro* and *in vivo* studies have shown that A1M protects cells and tissues in conditions with increased concentrations of extracellular Hb, heme, and reactive oxidative species (ROS) [100]. In PE, A1M expression in liver and placenta cells has been shown to be up-regulated following exposure to Hb, heme, and ROS [100, 101]. Furthermore, the serum concentration of A1M has also been shown to be significantly elevated in

maternal blood in the first trimester for patients who subsequently develop PE and in term pregnancies with manifest PE [100, 102].

Heme oxygenase 1

The first step in the heme catabolism toward biliverdin, carbon monoxide, and free iron is catalyzed the rate-limiting enzyme HO-1 [103]. Biliverdin is further reduced to bilirubin. This process mainly takes place in the intracellular compartment after macrophagic uptake of hemoglobin (via the Haptoglobin-CD163 pathway) or heme (via the Hemopexin-CD91 receptor pathway) (Fig. 3).

HO-1 has been shown to possess cytoprotective as well as anti-inflammatory and anti-apoptotic properties. It also acts as a cell proliferation regulator [104-106]. Furthermore, HO-1 modulates both the innate and adaptive immune systems [104]. In the placenta HO-1 is highly expressed, and it is involved in placentation, spiral artery remodeling, angiogenesis and placental blood pressure regulation [107].

Screening and diagnosis of preeclampsia

In Western countries, the trend is toward general and early pregnancy screening for malformations and chromosomal abnormalities. Screening for Down's syndrome at the end of the first trimester is a good example of a combination of ultrasound scanning and the use of biochemical markers that has sufficiently high sensitivity and specificity to be used for general screening [108]. Indeed, many Western countries have established general screening for Down's syndrome at 11+0–13+6 weeks of gestation—although not Sweden, where the combined screening is only offered to women aged 33 and over. Since many patients undergo screening procedures at this stage of pregnancy it could also be a suitable time to screen for PE [109]. Furthermore, if PE prophylaxis with low dose ASA proves to be effective it should be initiated at this early stage of pregnancy in hogh risk pregnancies [110-112].

Since biomarkers and ultrasound are low-risk, non-invasive procedures, these kinds of examinations are safe and appropriate for early pregnancy screening.

Why screen for PE?

Kypros Nicolaides at King's College Hospital, London, has suggested that clinicians use the new screening methods to think in a different way and turn the pyramid of prenatal care upside down [113, 114]. This means that instead of increasing the number of prenatal care visits toward the end of the pregnancy, effective screening at the beginning of pregnancy would lead to fewer unnecessary visits and more focused prenatal care (Fig. 4). Effective first trimester screening for PE would allow clinicians to invert the maternal care pyramid, allowing them to focus on the high-risk pregnancies [113, 114]. Low-risk pregnancies may consequently attend a standard care program with fewer visits. This strategy would allow for more accurate monitoring of high-risk cases and prophylactic treatment with low dose ASA. A reduction in the number of pregnancies complicated with PE would probably also lead to fewer long-term complications and lower risk factors for both mother and child [57-60].

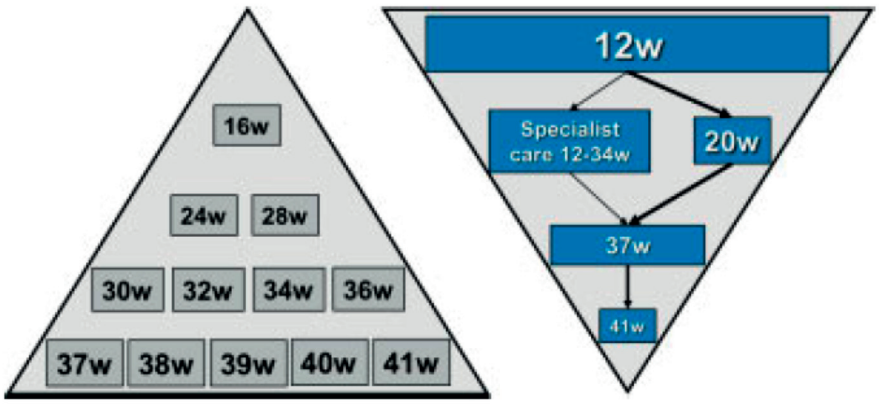

Figure 4 The pyramid of prenatal care in the past (left) and the future (right). Modified from Nikolaides 2011

Over the last decade, advanced methods such as genomics, proteomics, and metabolomics have been made more widely available for clinical research. In the search for the PE etiology, several new pathways and biochemical factors have been described [115, 116]. Many of the described biochemical factors are measurable in maternal blood and have therefore been evaluated as biomarkers for the prediction and diagnosis of PE. These include serum and plasma markers of placental function, endothelial dysfunction, renal dysfunction, general metabolic status, oxidative stress, and hemolysis and inflammatory markers.

Several maternal clinical characteristics have been identified as risk factors for the development of PE. Heredity is an important risk factor: in particular, a maternal family history of PE increases the risk [117]. Several studies indicate that certain genes increase the risk of PE [118-121]. The most important maternal risk characteristics are ethnicity, age, parity, multiple pregnancy, IVF pregnancy, and a history of severe or early onset PE in previous pregnancies [4]. In addition, maternal constitutional factors increase the risk of developing PE. These include systemic disorders such as obesity, Type 2 diabetes, essential hypertension, renal disease, antiphospholipid syndrome, and certain autoimmune diseases, particularly systemic lupus erythematosus [4, 122-128]. Maternal pregestational obesity is one of the strongest potentially modifiable risk factors for preeclampsia [129]. There is a dose–response relationship between

pregestational body mass index (BMI) and the risk of the woman experiencing either mild or severe preeclampsia [129, 130].

Lately, it has been shown that maternal cardiovascular dysfunction is also a severe risk factor for both early and late-onset PE [131-133]. However, none of these maternal risk factors alone or in combination predict PE sufficiently well. In combination, maternal risk characteristics are reported to show a prediction rate (PR) of about 30% at a 5% false positive rate (FPR)—and with a higher rate for early onset PE than late-onset PE [134, 135].

The definitions are a-changin'

Globally, the ISSHP definitions for PE are still not an entirely accepted classification [6, 12]. Different organizations have slightly different definitions; however, there is a move toward the increased use of biomarkers to define and diagnose the syndrome [7, 12]. Recently, the ACOG and ISSHP have changed their definition of severe PE regarding proteinuria: in the absence of proteinuria, biomarkers of renal dysfunction may form part of the definition instead [7, 12]. This places very high demands on the biomarkers regarding reproducibility.

The WHO statement

The World Health Organization (WHO) has produced a statement defining a set of properties to which predictive biomarkers intended for screening should ideally conform [136]. The definitions may be summed up as follows. Ideally, the biomarkers should:

(1) Play a central role in the pathogenesis and be specific to the condition.

(2) Appear early or before the clinical manifestations.

(3) Be easy and cheap to measure in maternal blood or urine.

(4) Show a high sensitivity and specificity.

(5) Correlate with the severity of the condition.

(6) Be non-detected or expressed at very low levels in normal pregnancies.

Biochemical markers

Few biochemical markers have proven sufficiently specific and sensitive as single markers to predict or diagnose PE [116, 137]. Researchers have therefore developed a range of algorithms for PE prediction. These algorithms include clinical measurements such as Doppler ultrasound and maternal clinical risk factors to further enhance the prediction capacity at a low FPR [116, 137].

The main predictive biochemical markers for PE are markers that reflect placenta function. Dozens of markers have been tested for their predictive value, but the best documented are pregnancy-associated plasma protein A (PAPP-A), placental growth factor (PlGF), and soluble FMS-like tyrosinkinase 1 (sFlt-1) [115, 116]. Other markers such as PP13 and human choriogonadotropin (HCG) have also been examined [138-141].

PAPP-A

PAPP-A is a glycoprotein synthesized in the placenta which has been evaluated as a biochemical marker to be measured in pregnancy for the last three decades [142]. During normal pregnancy its concentration in plasma progressively increases. PAPP-A is widely used in combination with HCG and the ultrasound parameter nuchal translucency thickness to assess the risk of Down's syndrome [108, 143]. PAPP-A's function is not completely clear, but it has been suggested that PAPP-A is implicated in placental development. Besides being used in aneuploidy screening, low levels of PAPP-A are associated with the development of all placental syndromes: PE, IUGR, placental abruption and stillbirth [144, 145]. As a single biomarker for PE, PAPP-A only predicts 22% of early onset PE at the end of the first trimester of pregnancy at an FPR of 5%. In combination with uterine artery Doppler ultrasound (UtAD), the PR for early onset PE increases, reaching PR 62.5% at 5% FPR, but it still only predicts 32% of all PE at 5% FPR [146].

Placental growth factor and soluble FMS-like tyrosinkinase 1

The pro- and anti-angiogenic proteins PlGF, sFlt-1, and VEGF are well described as predictive biomarkers for PE in both the first and second trimester of pregnancy [116, 137]. It is well documented that these proteins play a central role in the pathogenesis of PE, and that a pro-/anti-angiogenic imbalance is crucial for the transition from stage 1 to stage 2 of PE [13, 30, 147, 148]. The anti–angiogenic protein sFlt-1 binds to inactive pro-angiogenic proteins, and decreased angiogenic stimulation of the maternal endothelium then leads to endotheliosis of the maternal vascular bed [35, 149, 150].

This endotheliosis is present even in the endothelium of the maternal glomeruli, leading to glomerular endotheliosis, podocyte injury, and, eventually, proteinuria, which are the hallmarks of PE [151].

The level of placental growth factor (PlGF) has been shown to be lower in patients who subsequently develop PE as early as 11–13+6 weeks of gestation, and it has therefore been included in several prediction algorithms. In contrast, published results concerning first and early second trimester levels of sFlt-1 have disagreed, and sFlt-1 is usually considered not to have any clinical value as a predictive biomarker for PE in early pregnancy [152-154]. However, sFlt-1 is significantly elevated in the second trimester of pregnancy and is therefore considered useful for PE prediction from week 20 and onward [152, 155, 156]. As a single biomarker, PlGF has a 47% PR at 5% FPR [134]. Today, especially PlGF is used as part of several larger prediction algorithms, where it has been shown to increase the overall prediction rate, as listed in Table I [153, 157-159].

Biophysical markers

MAP

Mean arterial pressure (MAP) is defined as the average arterial pressure during one cardiac cycle and can easily be measured with standardized blood pressure gauges [160]. MAP is significantly elevated as early as the end of the first trimester in patients who subsequently develop PE and is therefore incorporated into many prediction algorithms in order to increase the prediction rate [115, 116, 160]. The increased MAP in patients who subsequently develop PE probably reflects a reduced elasticity of the maternal arteries combined with increased vasoconstriction [160]. MAP is therefore a measure of a maternal conditional factor that increases the risk of developing PE.

Doppler ultrasound

Almost all first and second trimester prediction algorithms include uterine artery Doppler ultrasound (UtAD) measured as either a pulsatility index (PI) or a resistance index (RI). Furthermore, diastolic notching is used as a sign of increased vascular resistance and reduced vascular elasticity. A high first trimester PI is reversible, however, and a high PI can appear at the end of the first trimester in pregnant women with a normal placentation [34]. Therefore, first and early second trimester UtAD have a relatively low positive predictive value of approximately 21% at 5% FPR [34]. In contrast, a normal PI by the end of first trimester is highly predictive for a normal

placentation as these women have a less than 1% risk of subsequent development of PE. Normal PI has therefore a high negative predictive value [34]. Several studies have been published listing reference values for PI by the end of the first and second trimesters [32, 161]. Based on these publications, it has been concluded that Doppler ultrasound alone should not be used as a first trimester prediction method for PE, but it may be valuable as part of other predictive algorithms that also include plasma biomarkers.

Prediction algorithms

Because PE is a syndrome, and not a disease with one well-described pathogenesis, it has not been possible to predict PE with a single biomarker [116, 137]. Therefore, the most recent research on prediction biomarkers has predominately focused on prediction algorithms that combine maternal risk characteristics with one or more biophysical markers (UtAD and/or MAP) and several plasma or serum biochemical markers. To date, there is no general acceptance of these algorithms in clinical practice [115, 116]. Since prediction rates are presented in several different ways, it is difficult to compare the results from different prediction models directly. The statistical methods used vary; the prediction rates are often published at different FPR [116, 137].

The algorithms showing the best predictive values are listed in Table I (modified from Anderson et al. 2015) [115]. The prediction algorithms mainly focus on markers reflecting placental function (or dysfunction). Even though several studies use prediction algorithms that are quite similar in their choice of biomarkers, the prediction rates differ considerably between different studies [153, 157-159, 162].

Kenny et al. evaluated a prediction model in a low-risk cohort containing 5 690 patients, part of the SCOPE cohort [157]. The final prediction model suggested in the study contained the risk factors; maternal characteristics, MAP, UtAD- RI, and PlGF. The results showed a prediction rate of 44% in the validation cohort and 67% in the test cohort for early onset PE at 5% FPR (Table I).

Akolekar et al. published a prediction algorithm from a larger cohort containing 58 884 low-risk patients studied at 11+0–13+6 weeks of gestation [158]. The model included maternal characteristics, MAP, UtAD PI, PAPP-A, and PlGF. The prediction rate was 93% for early onset PE and 38% for all PE at 5% fixed FPR. This model showed the highest predictive capacity for early onset PE—i.e. PE with a dominating placenta pathology—but less efficiency in the prediction of term PE [134, 146, 163]. Crovetto et al. used the anti-angiogenic protein sFlt-1 instead of PAPP-A in an otherwise identical model to that presented by Akolekar et al. [153]. In a cohort of 9 462 patients, plasma samples were collected at 8–11 weeks of gestation and UtAD measured at 11+0–

13+6 weeks of gestation. The PR was 88% for early onset PE and 68% for late-onset PE at 5% FPR. These results are remarkable, because sFlt-1 is usually not used in early pregnancy prediction algorithms—several studies have shown that sFlt-1 levels do not rise until later in the second trimester in patients who subsequently develop PE [152]. This is, however, a matter of debate, as other studies have shown increased levels of sFlt-1 as early as the beginning of the second trimester of pregnancy [153]. Parra-Cordero et al. presented a prediction model with PlGF as the only biomarker, combined with maternal characteristics and UtAD PI [159], finding lower prediction rates compared to the other models: 47% for early onset PE and 29% for late-onset PE, at 10% fixed FPR.

In general the above-mentioned prediction algorithms predict early onset PE better than late-onset or term PE (Table I).

Author /journal / year	Cohort	Markers	Prediction rate /FPR
Kenny L et al. *Hypertension* 2014	*Low risk cohort* 5690 Patients 278 PE 14 – 16 weeks	PlGF MAP Maternal characteristics UtAD RI	All PE: 17 - 22 %* ePE: 44 - 67 %** tPE: 6 -19 %** FPR: 5%
Skråstad RB et al *BJOG* 2014	*Nulliparous women* 541 Patients 11+0 – 13 +6 weeks	FMF model: Predictor model:	All PE: 40% Preterm PE: 80% All PE 30 % FPR 10%
Crovetto et al. *Prenatal diagnosis* 2015	*Low risk cohort* 9462 Patients 303 PE Plasma sampling 8 - 11 weeks UtAD 11 -13+6 weeks	Maternal characteristics MAP UtAD PI PlGF sFlt-1	ePE: 87.7% lPE: 68.3% FPR: 5%
Akolekar et al. *Fetal diagnosis and Therapy* 2013	*Low risk cohort* 58,884 Patients 1426 PE 11+0 – 13 +6 weeks	Maternal characteristics MAP UtAD PI PlGF PAPP-A	All PE: 38% ePE: 93% ptPE: 61% FPR: 5%
Parra-Cordero M et al. *Ultrasound in Obstetrics and Gynecology* 2013	*Not normal cohort* 359 Patients 70 PE 11+0 – 13 +6 weeks	Maternal characteristics UtAD PI PlGF	ePE: 47% lPE: 29% FPR: 10%

Table 1 Prediction algorithms shown with prediction rates and false positive rates.

ePE = early onset PE (≤34+0 weeks), lPE= late onset PE (>34+0 weeks), ptPE = preterm PE (<37+0 weeks), tPE = term PE (≥37+0 wee

*: Training cohort and validation cohort.
**: Slightly different prediction models than for all PE.

HbF as a diagnostic biomarker in maternal plasma

Olsson et al. developed an enzyme-linked immunosorbent assay (ELISA) method specific for cell-free HbF [100]. In a pilot study, plasma HbF was measured for 30 plasma control pregnancies and 30 patients diagnosed with PE. In addition, the total cell-free Hb concentration and levels of the heme scavenger α_1-microglobulin (A1M) and the hemoglobin scavenger haptoglobin were measured. Of these, HbF, total Hb, and A1M were significantly increased in the patients with PE and haptoglobin was significantly decreased. Further evaluations were needed in larger cohorts and this requirement formed the basis for the studies in this thesis.

Thesis

General aims

The general aims of this thesis were to study the role of cell-free HbF and the specific scavenger systems for hemoglobin and heme in patients with PE. The secondary aims were to evaluate these proteins as potential predictive and diagnostic biomarkers for PE and to study HbF's correlation to renal injury.

Specific aims

Paper I: The aim of this study was to measure the concentrations of HbF, total cell-free Hb, and A1M in maternal serum at the end of the first trimester of pregnancy, and to evaluate these proteins as predictive biomarkers for PE.

Paper II: The aim of this study was to verify the findings presented in Paper I. In addition to HbF and A1M, the endogenous hemoglobin- and heme-scavenging proteins haptoglobin and hemopexin were evaluated as potential predictive biomarkers for PE in combination with maternal characteristics and/or maternal UtAD. These biomarkers were also tested as potential biomarkers for prediction in the subgroups early, late, and term PE.

Paper III: The aims of Paper III were to study the physiological response and Hb defense mechanisms resulting from long-term exposure to cell-free Hb. We hypothesized that consumption of the Hb and heme scavengers may determine when the onset occurs and/or the eventual severity of the disease. The plasma concentrations of HbF, total Hb, and Hp-bound Hb (Hp–Hb), together with the Hb and heme scavengers Hp, Hpx, A1M, and CD 163, were studied as possible diagnostic biomarkers for PE.

Paper IV: The aim was to investigate the role of the hemoglobin-/heme-degrading pathways in patients with PE; specifically, to investigate how HbF and heme impact the scavenger systems HO-1 and hemopexin enzymatic activity in patients with PE. A secondary aim was to evaluate these proteins as potential biomarkers supporting PE diagnosis.

Paper V: The aim was to show that renal injury of PE (podocyte damage) is associated with the presence of extracellular vesicles (EVs) of podocyte origin in the urine. Furthermore, that cell-free HbF in maternal plasma could represent a causative factor for the podocyte injury seen in PE.

Materials and methods

Cohorts.

All results presented in the thesis are from serum, plasma, and urine collected at either St George's University Hospitals, London (papers I and II), or the Department of Obstetrics and Gynecology, Skåne University Hospital in Lund and Malmö, Sweden (papers III, IV and V).

Ethical approval was given by the local ethical boards at Lund University and St George's University Hospitals. All samples were collected after oral and written consent was obtained from the patients.

In total, three different cohorts were used;

1) The first cohort, 96 patients (Paper I), was selected at St George's University Hospitals. Serum samples were collected during the first visit to the antenatal care unit. Initially, the cohort consisted of 100 patients, but four patients were excluded due to the exclusion criteria pre-gestational Type 1 diabetes and pre-gestational hypertension. This cohort consisted of serum samples from patients collected at 11–16 weeks of gestation. Sixty of the patients developed PE later in pregnancy and 36 patients served as healthy controls.

2) The second cohort, 433 patients (Paper II), was also included at St George's University Hospitals during the years 2006–2008. The serum samples were collected at 6–20 weeks of gestation and uterine artery Doppler ultrasound indices (pulsatility index and notching) from early pregnancy for each patient. In total, 86 of the patients subsequently developed PE and 347 healthy pregnancies served as normal controls.

3) The third cohort, 150 patients (papers III, IV and V), was collected at Skåne University Hospital during the years 2005–2011. A plasma and a urine sample were collected from each patient within 24 hours prior to delivery. The cohort was originally constructed for Paper III and consisted of 100 PE patients and 50 controls. However five patients were excluded due to lack of information in the patients' charts or the exclusion criteria pre-gestational diabetes or gestational diabetes. The final cohort had 98 PE patients and 47 healthy controls.

The results in Paper IV are also based on the cohort used in Paper III. In total, 135 patients were included, of which 89 had PE and 46 were normal pregnancies.

In Paper V only a part of the original cohort from Paper III was used since some of the cases in the cohort lacked urine samples. In total, 92 patients were included in this study—49 PE cases and 43 controls.

Specific methods used for analysis

Cell free fetal hemoglobin

During this thesis work, the ELISA methods used to analyze HbF were further developed at our laboratory. Different antibodies were used in the various studies. The ELISAs for papers I and II were sandwich ELISAs based on polyclonal antibodies, as has previously been described in detail by Olsson et al. [100]. These ELISAs measured all cell-free tetrameric HbF, including the cell-free HbF bound to haptoglobin.

For papers III, IV, and V, a new ELISA based on a mouse monoclonal antibody was developed in-house. This ELISA only measured cell-free unbound tetrameric HbF. The details of this analysis are described in Paper III.

Total Hb

In papers I, II, and III, total Hb was measured by an ELISA developed in-house using polyclonal antibodies against adult Hb. The method was described in detail by Olsson et al. [100].

The concentration of total Hb described in Paper III was determined using a Human Hb ELISA Quantification Kit from Genway Biotech Inc. (San Diego, CA). The analysis was performed according to the manufacturer's instructions and the absorbance was read at 450 nm using a Wallac 1420 multilabel counter.

A1M

A1M was measured with an in-house RIA method, as previously described by Olsson et al. [100]. Briefly, the analysis was performed by mixing goat antiserum against human A1M ("Halvan"; diluted 1:6000) with ^{125}I-labelled A1M (≈ 0.05 pg/ml) and unknown patient samples or calibrator A1M concentrations. After incubation overnight at RT, antibody-bound antigen was precipitated, after which the ^{125}I-activity

of the pellets was measured in a Wallac Wizard 1470 gamma counter (Perkin Elmer Life Sciences). The same method was used in all papers.

Haptoglobin–HbF complex

The Hp–HbF complex was measured with a sandwich ELISA developed in-house for Paper III. The ELISA displayed a high preference for Hp–HbF compared to uncomplexed HbF (≥10x higher recovery of a Hp–HbF calibrator series compared to a HbF calibrator series with the same molar content of HbF). No cross-reactivity was observed with Hp or adult Hb. The method is described in detail in Paper III. Briefly, ninety-six-well microtiter plates were coated with anti-HbF antibodies overnight at RT. Wells were blocked for 2 hours using blocking buffer, followed by an incubation with Hp–HbF calibrator or the patient samples for 2 hours at RT. HRP-conjugated anti-Hb antibodies were added and incubated for 2 hours at RT. Finally, a ready-to-use TMB (Life Technologies) substrate solution was added. The reaction was stopped after 30 minutes and the absorbance was read at 450 nm using a Wallac 1420 multilabel counter (Perkin Elmer Life Sciences).

Hemopexin

In papers II and III, the maternal serum/plasma concentrations of Hpx in maternal plasma or serum were determined using the Human Hpx ELISA Kit from Genway Biotech Inc. The analysis was performed according to the manufacturer's instructions and the absorbance was read at 450 nm using a Wallac 1420 multilabel counter.

Hemopexin activity

Plasma Hpx activity was measured in EDTA plasma samples using the Hx-MCA substrate (synthesized by Pepscan, Lelystad, the Netherlands). The plasma samples (40 µl) were diluted 1:4 with the substrate solution (0.2M Tris + 0.9% NaCl pH 7.6, substrate concentration 80 µM/L) to a final volume of 200 µl. The emission was measured at 460 nm on a Varioskan spectrophotometer (Thermo Fisher) at 37 °C. The Hx activity was measured after 0 min, 30 min (Hx30), 60 min (Hx60), and 24 hours. The measured value represented the total amount of serine catabolized by Hx at the given point in time. If the value was <5 after 24 hours, the activity was considered "very low" and the samples were excluded from further analysis. The analysis of the area under the curve was based on Hx30 and Hx60 measurements (HxAUC) as previously described in detail by Bakker et al. [164].

Haptoglobin

In papers II and III, the concentrations of Hp in maternal plasma or serum were determined with the Human Hp ELISA Quantification Kit from Genway Biotech Inc. The analyses were performed according to the manufacturer's instructions and the absorbances were read at 450 nm using a Wallac 1420 multilabel counter.

CD-163

In Paper III, the concentration of CD163 in maternal plasma was determined using a Human CD163 Duo Set from R&D Systems (Abingdon, UK). The analysis was performed according to the manufacturer's instructions and the absorbance was read at 450 nm using a Wallac 1420 multilabel counter.

Heme oxygenase 1

In Paper IV, the concentration of HO-1 in maternal plasma was determined with the human HO-1 ELISA kit (Enzo Life Sciences Inc., Farmingdale, New York) according to the manufacturer's instructions.

Heme

In Paper IV, free heme concentration was determined with the QuantiChrom Heme Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions.

Plasma uric acid, creatinine, and cystatin C concentrations in Paper V were measured by standard methods on a Cobas 6000 (Roche Diagnostics Limited, Rotkreuz, Switzerland) at the Clinical Chemistry Laboratory at Skåne University Hospital.

Characterization and quantification of urinary EVs

Frozen urine samples were thawed in a 37 °C water bath for 5 min. Each sample was thawed twice: the first thaw to perform a concentration check; the second thaw to stain with a predetermined set of antibodies for flow cytometric analysis using a method, which has been described previously [165]. Each urine sample was stained with annexin-V, nephrin, podocin, and synaptopodin.

A digital flow cytometer (FACSCanto TM) was used to perform the analysis of urinary EVs. The flow cytometer settings and gates for recording events and analysis have been published recently [165]. The absolute counts of single and double fluorescently labeled urinary EVs were expressed as urinary EV/μL of urine using the standardized method previously described [165, 166]. The number of podocyte protein-specific positive EVs was normalized to annexin+ EVs. The number of EVs was not expressed as a ratio to urine creatinine concentration because podocytes are situated on the outer aspect of the glomerular basement membrane, and, notably, EVs originating from podocytes are not subject to glomerular filtration. In contrast, urine metabolites that are commonly normalized to the creatinine concentration in the respective urine sample originate in the blood and are filtered in the urine, and thereby need to be factored for urine creatinine concentration so as to control for the concentration or dilution of the urine. In addition, we calculated the ratio between nephrin+ EVs and podocin+ EVs, as previous studies have indicated that the nephrin mRNA to podocin mRNA ratio may serve as a marker of disease activity or progression [167].

Summary of results

First trimester prediction—a pilot study (Paper I)

The aim of this study was to measure the concentrations of HbF, total cell-free Hb, and A1M in maternal serum at the end of first trimester of pregnancy, and evaluate these proteins as predictive biomarkers for PE. Cell-free HbF, total Hb and A1M were measured. The ratio between HbF and total Hb (HbF ratio=HbF concentration/total Hb concentration) was calculated to account for hemolysis. The results were analyzed with a stepwise logistic regression model. This statistical method was used to calculate statistically significant differences between the groups and to calculate the odds ratios for the risk of developing PE. Receiver operational curve analysis (ROC curve analysis) was performed based on the logistic regression probabilities. The ROC analysis was used to calculate the sensitivity (prediction rate) of the biomarkers as predictive biomarkers at different screen positive rates.

There were significantly higher concentrations of HbF ($p<0.001$) and A1M ($p<0.001$) in the PE group (Table 1.1). The difference in the total Hb concentration was not significant between PE and control groups ($p=0.26$). The HbF ratio was significantly higher in the PE group ($p<0.001$). The odds ratios showed increased risk for PE with increasing concentration of HbF and A1M (Table 1.1). The ROC curve analysis showed 55% sensitivity at 5% fixed screen positive rate for the HbF ratio and 17% at 5% screen positive rate for A1M. In combination, the two biomarkers showed a combined sensitivity of 69% at 5% screen positive rate. The optimal sensitivity (defined as the part of the ROC curve that was closest to the upper left-hand corner) for the combination of HbF ratio and A1M was 90% sensitivity at 23% screen positive rate.

Serum concentrations of fetal hemoglobin, α_1-microglobulin, total hemoglobin, and fetal hemoglobin ratio

Variable	PE (SD)	Controls (SD)	*P* value	Odds ratio[a] (95% CI)
HbF (μg/mL)	1.38 (1.53)	0.45 (0.65)	< .0001	2.4 (1.2–5.0)
A1M (μg/mL)	24.77 (5.37)	20.50 (4.26)	< .0001	1.2 (1.1–1.4)
Total Hb (μg/mL)	177.90 (62.72)	198.34 (102.10)	.26	1.0 (1.0–1.0)
HbF ratio[b]	0.0079 (0.0073)	0.0020 (0.0018)	< .0001	166.7 (8.5–3256.8)[c]

Mean concentration of HbF, A1M, and total Hb in PE cases and controls. Binary logistic regression was used to determine significance. All odds ratios are adjusted for ethnicity and gestational age at sampling.

A1M, α_1-microglobulin; *CI*, confidence interval; *Hb*, hemoglobin; *HbF*, fetal hemoglobin; *PE*, preeclampsia.

[a] Odds ratios are calculated as per 1-U change (μg/mL for HbF, A1M, and total Hb, but U for HbF ratio); [b] HbF ratio = HbF/total Hb concentration; [c] Odds ratio calculations based on HbF ratio × 100–this is due to very low values of HbF ratio.

Anderson. HbF and A1M, potential predictive biomarkers for preeclampsia. Am J Obstet Gynecol 2011.

Table 1.1

This proof-of-principles study clearly showed elevated serum levels of HbF and A1M at the end of the first trimester and beginning of the second trimester in women who subsequently developed PE. The results of the ROC curve analysis indicated a possibility of using HbF and A1M as predictive serum biomarkers for the subsequent development of PE. The sensitivity of the presented set of biomarkers fully match other potential first trimester predictive biomarkers of PE (see prediction algorithms section) and shows one of the highest sensitivities for first-trimester PE prediction [116].

First-trimester prediction—a verification study (Paper II)

The aim of this study was to verify the findings in Paper I in a larger cohort. In addition to HbF and A1M, the endogenous Hb- and heme-scavenging proteins haptoglobin and hemopexin were evaluated as potential predictive biomarkers for PE in combination with maternal characteristics and/or maternal UtAD. Furthermore, these biomarkers were evaluated as potential biomarkers for prediction of the subgroups early-, late-, and term PE.

Plasma levels for HbF, total Hb, A1M, hemopexin, and haptoglobin were analyzed as described in the material and methods section. All the patients were examined with UtAD at approximately the same time as the serum sampling was performed [34]. The pulsatility index (PI) obtained from the UtAD examinations were transformed to multiples of the median (MoM) values according to normal PI values published by Valeauthar et al. [32]. Group comparison between the study groups was performed using the ANOVA test (Table 2.1). Logistic regression models were developed and ROC curve analyses were performed to evaluate the predictive potential of the biomarkers. All biomarkers were tested individually and in different combinations with one another. The UtAD PI values and the maternal risk factors were added to the algorithm in order to find the optimal prediction model.

The stepwise logistic regression method has been criticized for its way of calculating prediction values. The method was originally developed to reveal causality rather than prediction [168, 169]. Therefore, three different logistic regression models were compared; stepwise, Lasso, and boosted tree regression [170].

Serum HbF ($p=0.02$) and A1M ($p=0.03$) levels were significantly higher in the PE group compared to the controls (Table 2.1). The Hpx concentration was lower in the PE group compared to controls ($p=0.05$). The UtAD PI MoM values were significantly higher in the PE group compared to controls ($p<0.0001$).

Biomarker	Controls N=347 (95%CI)	Preeclampsia N=86 (95%CI)
HbF (μg/ml)	5.6 (4.2-7.4)	10.8 (5.2-16.5) p=0.02
A1M (μg /ml)	15.5 (14.9-16.1)	17.3 (15.5-19.2) p=0.03
Total Hb (μg /ml)	297 (257-337)	258 (160-358) p=0.47 **NS**
Haptoglobin (μg /ml)	971 (915-1028)	1102 (991-1131) p=0.089 **NS**
Hemopexin (μg /ml)	1143 (1111-1175)	1062 (992-1132) p=0.05
UtAD PI MoM	0.98 (0.92-0.99)	1.18 (1.04-1.31) p<0.0001

Table 2.1 Mean concentrations with 95% confidence interval of the biochemical markers cell-free fetal hemoglobin (HbF), α_1-microglobulin (A1M), total cell-free hemoglobin (Total Hb), haptoglobin, hemopexin and Uterine artery Doppler ultrasound Pulsatility Index (UtAD PI) *Multiples of the Median* (MoM). P-values were calculated with one-way ANOVA as compared to the control group.

The logistic regression and ROC curve analysis revealed relatively poor predictive values for the individual biomarkers: HbF 15%, A1M 19%, and Hpx 17% at 90% fixed specificity). In combination, the biomarkers showed a sensitivity of 33% at 90% specificity. The maternal risk factor characteristics; ethnicity, number of previous

pregnancies (gravidae), number of previous deliveries (parity), maternal BMI, maternal diabetes, and maternal hypertension were each significantly associated to the development of PE in the logistic regression analysis. The maternal risk factor characteristics combined showed 60% collective PR at 90% specificity. The UtAD PI MoM values showed similar prediction values as the biomarkers alone, with a 25% sensitivity at 90% specificity.

The early, late, and term PE subgroup analyses revealed elevated levels of HbF in all PE groups. The A1M levels were only significantly elevated in the late-onset and term groups. The Hpx concentrations were lower in all the PE groups; however, they were only significantly lowered in the early onset PE group. Haptoglobin and total Hb did not show any significant differences. The UtAD PI values were only significantly elevated in the early onset group ($p<0.00001$). The late-onset and term PE groups did not show any significant differences ($p=0.06$ and $p=0.35$ respectively).

The logistic regression and ROC curve analyses for the early, late, and term PE groups showed a sensitivity for HbF of 23% at 90% specificity in the late-onset PE group and 19% sensitivity at 90% specificity in term PE. A1M was statistically significantly elevated in the late-onset and term groups ($p=0.01$ and $p=0.003$). Hpx was only statistically significantly decreased for the early onset group, and showed a sensitivity of 32% at 90% specificity. The UtAD values performed best in the early onset group with a sensitivity of 57% at 90% specificity, but were also statistically significant in the late-onset group ($p=0.025$) (this p-value applies only to the logistic regression analysis). However, UtaD PI in the term PE group was not significantly elevated ($p=0.36$). None of the biomarkers were statistically significant when combined with one another, with maternal characteristics, or with UtAD values in any of the PE subgroups.

The comparison of the three different logistic regression models was based on AUC of the ROC curves. The stepwise method and the Lasso methods gave similar results in the training cohort, with AUCs 0.76 and 0.77 respectively. The boosting regression had a much higher AUC of 0.93. In the test cohort, stepwise and Lasso also performed quite similarly with AUCs of 0.68 and 0.70, but the boosting regression predicted at a much lower level with an AUC of 0.63. The stepwise method was therefore chosen for the calculations of the results in Paper II.

This validating study confirms the findings in Paper I that HbF and A1M are elevated in maternal serum from women who subsequently develop PE [102]. Furthermore, the findings indicate that already at this early stage of pregnancy the increased circulating levels of HbF place a strain on the endogenous hemoglobin and heme scavenging systems—hemopexin is consumed and depleted, and therefore circulates at lower levels in pregnancies destined to develop PE. The results indicate that HbF, A1M, and Hpx are potential biomarkers for first trimester prediction of PE, either in combination with one another, or in combination with maternal characteristics and/or Doppler ultrasound indices.

HbF and heme scavenging systems as biomarkers for PE (Paper III)

The specific aim of this paper was to study the physiological response of the Hb defense mechanisms caused by long-term exposure to cell-free hemoglobin. Papers I and II show that women who develop PE later in pregnancy have increased levels of circulating free HbF as early as the first trimester of pregnancy. The clinical manifestations occur in the second or third trimester as early or late-onset PE. We hypothesized that long exposure time to high levels of HbF may consume heme scavenger proteins leading to increased toxicity and thereby more severe clinical manifestations. The plasma concentrations of HbF, total Hb, and Hp-bound Hb (Hp–Hb), together with the Hb and heme scavengers Hp, Hpx, A1M, and CD 163 were studied as potential diagnostic biomarkers for PE.

There was fourfold increase in HbF concentration in patients with PE ($p=0.01$) (Table 3.1). This increase was seen in both early and late-onset PE. In accordance with results presented by Olsson et al., the A1M concentration was higher in the all-PE group [100]. In contrast, the Hp concentration was significantly lower in plasma from the PE group ($p<0.00001$). The CD163 concentration was not significantly different between the groups. The hemopexin concentration was significantly lower in both early and late-onset PE ($p<0.0001$). Correlation analysis revealed a significant inverse correlation between HbF and Hp ($r=-0.335$, p⊠0.0001). This correlation was even stronger when analyzing only the PE group.

Biomarker	Normal pregnancy (Control; n=47)	Preeclampsia (n=98)	Early onset PE[1] (n=22)	Late onset PE[2] (n=73)
HbF (ng/ml)	3.85 (2.51-5.20)	15.26 (7.0-23.6) p=0.01	18.72 (1.6-39.05) p=0.006	14.60 (5.10- 24.0) p=0.17
HbF-Hp (µg/ml)	0.59 (0.003-1.18)	0.61 (0.31-0.90) p=0.018	1.07 (-0.10-2.24) p=0.15	0.48 (0.29-0.66) p=0.02
Total-Hb (µg/ml)	277 (232-321)	285 (238-331) p=0.53	290 (152-430) p=0.80	284 (237-331) p=0.73
Hp (mg/ml)	1.17 (1.04-1.30)	0.97 (0.75-1.19) p=<0.0001	1.34 (0.39-2.30) p=0.067	0.89 (0.77-1.02) p=0.001
CD 163 (µg/ml)	461 (408-512)	485 (445-527) p=0.37	433 (324-543) p=0.35	508 (465-551) p=0.07
Hpx (mg/ml)	0.93 (0.88-0.98)	0.69 (0.66-0.73) p=<0.0001	0.69 (0.61-0.77) p<0.0001	0.69 (0.65-0.73) p<0.0001
A1M (µg/ml)	29.93 (27.89-31.97)	33.50 (31.90-35.10) p=0.035	34.07 (30.31-37.83) p=0.26	33.70 (31.90-35.50) p=0.03

[1] Early onset PE was defined as diagnosis before 34+0 weeks of gestation.

[2] Late onset PE was defined as gestational week > 34+0.

Table 3. The mean concentrations of the biomarkers in the PE group and normal pregnancies (controls). Statistical comparison vs. controls. Significance was calculated with non-parametric statistics (Mann-Whitney). Values are mean values with (95%CI). A p-value <0.05 was considered significant.

Furthermore, the Hpx concentration was correlated to both systolic and diastolic blood pressure ($p<0.00001$). None of the other biomarkers were correlated to blood pressure. The logistic regression model/ROC analysis revealed that Hpx detected PE better than any of the other biomarkers with 64% diagnostic detection rate at 5% FPR. The combination of HbF, A1M, Hp, and Hpx diagnosed PE somewhat better, with 69% DR at 5% FPR.

In addition, the logistic regression model/ROC analysis revealed a potential for predicting fetal and maternal outcomes. HbF, Hp, and Hpx each showed an association with "Admission to NICU", but the regression model was not significant when the biomarkers were combined. Hpx and CD163 showed significant association with premature delivery, both individually and in combination. Hpx showed a significant association with the risk of Cesarean section.

The main findings in this study were that the increased levels of HbF seem to strain and deplete heme- and hemoglobin-scavenging proteins. The biomarkers in combination show a clear potential to be used as clinical tools to predict the severity of PE and to predict specific clinical outcomes.

Hemopexin activity, HO-1, and free heme in PE (Paper IV)

To further investigate the importance of HbF in PE pathology and its impact on the hemoglobin and heme scavenging systems, this study was designed as a follow up to Paper III. Previous publications on hemopexin enzymatic activity (Hpx activity) have shown decreased Hpx activity in patients with PE [171]. Furthermore, Hpx activity might influence the renin–angiotensin system in a way that increases blood pressure when Hpx activity is decreased [172, 173].

The aim was to investigate the role of the Hb- and heme-degrading pathways in patients with PE; specifically, to investigate how HbF and heme impact the scavenger systems HO-1 and hemopexin's enzymatic activity in these patients. A secondary aim was to evaluate these proteins as potential biomarkers for supporting a PE diagnosis. The plasma samples were sent blinded to be analyzed for hemopexin activity at 30 min (Hxa30) and 60 min (Hxa60), and the AUC (HxaAUC) was calculated (for the specific methods used see the serum/plasma/urine analysis section). The samples were also analyzed for heme and HO-1 at the research laboratory at the BMC Biomedical Center in Lund, Sweden. The HbF, total Hb, and hemopexin concentrations were included obtained from paper III and included in the correlation- and ROC analyses. Samples with low hemopexin activity after 24 hours of incubation, 8 controls and 3 PE, were excluded from further analysis due to low plasma sample quality or technical problems.

The results showed significantly lower Hpx activity in the PE groups compared to controls for Hx30 ($p=0.02$), Hx60 ($p=0.05$), and for the HxAUC ($p=0.02$). However,

when analyzing early and late-onset PE, the early onset group (Hx30=0.81) showed identical values to the control group (Hx30=0.80). In contrast, the late-onset group showed an even more marked decrease in the Hx activity (Hx30=0.54, p=0.007). The heme concentration was significantly higher in patients with PE compared to controls (75.03 μM vs. 59.86 μM p=0.01). The concentrations were higher both in early (69.54 μM, p=0.26) and late-onset PE (77.55 μM, p=0.002). The HO-1 concentration was significantly lower in the PE group compared to the controls (4.48 ng/ml vs. 5.29 ng/ml p=0.03). Both early (4.67 ng/ml, p=0.02) and late-onset PE (4.42 ng/ml, p=0.01) showed significantly lower HO-1 concentrations.

The correlation analysis did not reveal any correlation between Hpx activity and the Hpx concentration. There were no significant correlations between Hx30 (p=0.82) and heme, or between heme and HO-1 (p=0.08), nor was there significant correlation between HO-1 concentration and Hx30 (p=0.92). The heme concentration did significantly correlate to the total Hb concentration (correlation coefficient=0.18, p= 0.002). Hx30 was significantly correlated to the diastolic blood pressure (p=0.04). HO-1 levels were inversely correlated with statistical significance to both systolic (p=0.01, correlation coefficient=-0.15) and diastolic blood pressures (p=0.003, correlation coefficient=-0.25).

The results from the logistic regression and ROC analysis revealed a diagnostic potential for HbF, Hpx activity, heme, and HO-1. The combination of HbF, Hx30, Hx concentration, heme, and HO-1 together showed 84% DR at 10% FPR, AUC=0.93. The main findings in this paper confirmed previously published results that Hpx activity is decreased in plasma from patients with PE [171]. Furthermore, the Hpx and HO-1 concentrations were reduced in PE, whereas heme levels were increased. The results from Paper III show that HbF is increased in PE. This, together with the increased heme concentrations in patients with PE, suggests a depletion of the protective Hb and heme scavenging and degradation systems. This is supported by the low HO-1 concentration and low Hpx activities found in the plasma of patients with PE. Measurements of these potential diagnostic biomarkers for PE could, in the future, lead to the more precise diagnosis of PE.

HbF and kidney injuries (Paper V)

This study was performed in collaboration with Vesna Garovic at the Mayo Clinic, Rochester, MN. The aim was to show that renal injury in PE (podocyte damage) is associated with the presence of extracellular vesicles (EVs) of podocyte origin in the urine; also, to investigate whether cell-free HbF in maternal plasma represents a causative factor for the podocyte injury seen in PE.

In the cohort described above, the plasma concentrations of cystatin C, creatinine, and uric acid were measured as a clinical routine. The urine samples were analyzed blind at

the Mayo clinic for EVs (a measure of podocyte injury). Podocyte specificity was assessed by the concentrations of EVs that stained for podocyte-specific proteins (annexin, nephrin, podocin, and synaptodin) by flow cytometry, as previously described [165]. Furthermore, the results were correlated with the HbF, A1M, Hp, and Hpx data obtained in Paper III.

The plasma results showed significantly reduced renal function in PE patients ($p<0.001$), elevated levels of HbF ($p=0.015$) and A1M ($p=0.036$), and decreased levels of Hp ($p<0.001$) and Hpx ($p<0.001$) (data from Paper III). The urine analysis revealed increased annexin- ($p=0.008$), nephrin- ($p<0.001$) and podocin-positive EVs ($p=0.037$) and an increased ratio of nephrin$^+$ EVs to podocin$^+$ EVs in the PE group. The ROC curve analysis of the EV results revealed the sensitivity for diagnosing PE. The best performing measure for diagnosing PE was the ratio of nephrin$^+$ EVs to podocin$^+$ EVs, showing 91.8% sensitivity and 86% specificity.

Furthermore, there were positive correlations between HbF and the degree of proteinuria, and between HbF and the ratio of nephrin$^+$ EVs to podocin$^+$ EVs, which suggests that HbF is damaging the podocytes.

The conclusion of this study was that PE is associated with an elevated urinary nephrin$^+$ EVs to podocin$^+$ EVs ratio, and its results suggest that HbF is a potential factor for PE-induced renal (podocyte) injuries.

Discussion

HbF – a new etiological factor

In this thesis an alternative hypothesis for the pathogenesis of PE is presented. The hypothesis is based on previous findings presented by Centlow et al. showing an up regulated placental synthesis of fetal hemoglobin in the placenta of patients with PE at term [62]. The placenta is already known to be a potential hematopoietic organ during fetal life [174, 175].

A schematic summarizing model is presented in Fig. 5, where the model is fitted into a modified two-stage model. In papers I and II, it was shown that maternal serum HbF concentrations are already increased at the end of the first trimester of pregnancy in patients who subsequently develop PE. This could indicate that HbF synthesis in the placenta already is up regulated at the end of the first trimester of pregnancy in this patient group. Increased placental HbF levels damage the feto–maternal barrier, and as a result HbF leaks out into the maternal circulation. As a physiological response to this, the liver up regulates its synthesis and release of the heme scavenger A1M, and so the circulating concentration of A1M is measurably increased in patients who subsequently develop PE (papers I and II). This is in line with previous *in vitro* findings that A1M synthesis and release from the liver is up regulated as a consequence of increased cell-free Hb levels [74, 101]. As a sign of depletion of Hb and heme scavengers, Hpx concentration is decreased in early pregnancy in the serum of patients who subsequently develop PE (Paper II). Previous findings by Olsson et al. show increased concentration of HbF in PE plasma in term pregnancy as well. At this stage of pregnancy a correlation with the blood pressure could be shown [100]. This indicates that the up regulation of placental HbF synthesis continues until term.

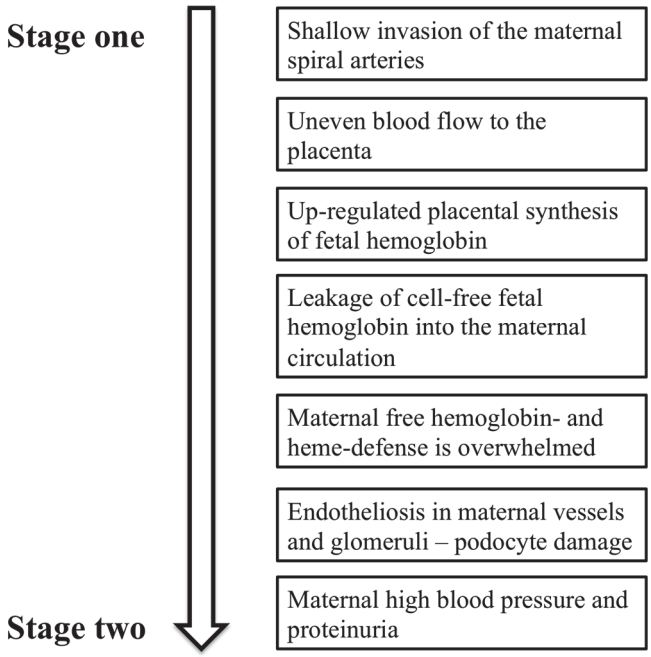

Figure 5 A modified two-stage model based on the HbF-hypothesis.

Papers III and IV confirmed that circulating concentrations of HbF are increased in patients with PE. Increased circulating concentrations of HbF throughout pregnancy strain Hb- and heme-scavenging physiological protection mechanisms and slowly deplete the Hb and heme-scavenging proteins concerned. It was shown in Paper III that circulating concentrations of haptoglobin and hemopexin are decreased in patients with diagnosed PE—probably as a consequence of increased maternal HbF load which leads to depletion of haptoglobin and hemopexin. In Paper IV it was shown that there are increased circulating levels of free heme and lower levels of circulating HO-1 in maternal plasma in patients with PE. It was also shown there was a decreased level of Hpx activity in patients with late-onset PE. The increase in heme concentration is probably a direct effect of increased concentrations of HbF. The heme degradation enzyme HO-1 is primarily an intracellular protein (see Introduction and Fig. 3) but it is also found in small amounts circulating in the blood. The finding of lower circulating levels of HO-1 in patients with PE is most likely directly caused by the increased levels of circulating heme. The increased heme load is probably also the direct depletion

mechanism behind the lower concentration of hemopexin (Paper III) and contributes to lower Hpx activity (Paper IV).

Once the protection proteins are depleted due to increased HbF and heme loads throughout pregnancy, the toxicity of HbF and heme increase causing the mother's clinical symptoms (Fig. 5). The innate capacity of the maternal Hb and heme scavenger and enzyme systems may be important constitutional factors that determine how and when the clinical symptoms present in stage two of PE. The more the systems are strained and proteins depleted, the more severe the clinical symptoms will be.

HbF and the depletion of Hb- and heme scavengers affect blood pressure in several ways. Several of the heme- and Hb-scavenging proteins also serve as regulators of other systems, such as inflammation, blood vessel contractility, the renin–angiotensin system (see Paper IV), decidual spiral artery remodeling, and endothelial homeostasis. Furthermore, cell-free Hb binds strongly to the vasodilator nitric oxide (NO), which leads to vasoconstriction and consequently to increased blood pressure. Hpx activity has an important impact on the RAS system and depletion of Hpx leads to increased maternal vascular contractility and a consequent increase in blood pressure. Heme oxygenase 1 seems to play a crucial role in the placental regulation of inflammation, blood pressure, remodeling of maternal spiral arteries, and cell proliferation [104-107]. Its depletion due to excessive heme load could therefore lead to disturbances in the regulatory mechanisms mediated by HO-1. This could lead to increased placental inflammation, impaired remodeling of the maternal spiral arteries, and decreased circulating amounts of CO, which lead to vasoconstriction and add to the increase in maternal blood pressure.

Continuously increased circulating levels of HbF also affect the kidneys. The findings in paper IV describes a significant correlation between HbF concentrations and podocyte-specific urinary EVs indicating that the podocyte damage seen in PE, to a certain extent, is caused by cell-free Hb. However, whether HbF is a direct causal agent will need to be evaluated in further studies that focus on the specific injurious effects of HbF on podocytes. The theory is supported by *in vivo* studies in sheep- and rabbit PE models, that show Hb induced kidney damage as previously described [66, 84].

New biomarkers

Prediction

This thesis present a new set of potential predictive and diagnostic biomarkers. The results from Paper I reveal a prediction rate of 69% at 5% false positives, which matches some of the best first trimester prediction algorithms published to date [115, 116]. The findings in the larger cohort in Paper II did not confirm these prediction rates; the combination of all biomarkers and maternal characteristics had a prediction rate of 60% at 10% FPR. However, a very interesting finding in Paper II was that the prediction is not markedly improved by the addition of maternal risk-factor characteristics or UtAD.

Previously described predictive biomarkers primarily predict early onset and severe PE [115, 116]. These are also the groups of PE that respond best to prophylactic treatment with ASA [110-112, 176]. However, this group only represents 20% of PE patients. Therefore, biomarkers that predict late-onset and term PE are definitely needed, along with a more efficient prophylaxis strategies for this group of patients. This thesis presents a set of biomarkers that not only predict early onset PE, but also other subtypes of PE as shown in papers I and II. In addition, treatment with Hb and heme scavengers such as A1M could offer an alternative prophylaxis strategy to ASA and as a therapeutic substance. The therapeutic potential has been shown in different animal PE-models [66, 84] and is to be validated further before being applied as a potential human (prophylactic) treatment of PE.

Diagnosis

The results in papers III, IV, and V indicate a large potential for HbF and Hb, heme scavengers, and urinary EVs as potential diagnostic biomarkers for PE. Paper III shows a combined diagnostic detection rate of 69% at 5% false positives, and Paper IV shows a combined diagnostic detection rate of Hpx activity, Hpx, HO-1, heme, and HbF of 84% at 10% FPR.

The need for new and more specific biomarkers has increased in recent years, since the ACOG and ISSHP have changed their definition of severe PE to include biomarkers in the absence of proteinuria (see introduction section) [7, 12]. The Hpx activity, HO-1, and HbF all correlate to the patient's blood pressure and thereby to the severity of PE. This could be of clinical value, for example in aiding the clinicians deciding the optimal time to deliver women with PE.

Methodological considerations

The three cohorts used in the studies included in this thesis are all constructed case-control cohorts with an unnaturally higher number of PE patients than normal (3-8%). This most likely has an influence on the specific prediction or detection rates obtained. The lower the prevalence, the higher becomes the number of expected false positives. This means that in these cohorts, with their unnaturally high prevalence of PE, an unnaturally low number of false positives could be expected. To get more accurate prediction or detection rates the findings need to be validated in larger cohorts with a normal prevalence of PE.

The cohorts of papers III, IV, and V are practically the same cohort. The original number of 145 included patients in the cohort for Paper III was reduced by 11 for Paper IV for technical reasons—these 11 patients showed "extremely low hemopexin activity" after 24 hour of incubation and were therefore excluded from further analysis—and since these patients were excluded, there is also a difference in mean HbF concentration between reported in papers III and IV.

When measuring free Hb in plasma or serum, a potential confounding factor is hemolysis. This places high demands on the blood sampling technique, as well as the handling of the samples until spun and frozen. The samples used in this thesis have been handled according to prevailing standards, but minor hemolysis must be taken into account. In Paper I, the ratio between cell-free HbF and total free Hb was calculated to avoid hemolysis as a confounding factor.

During the work of this thesis, new ELISA methods were being developed. The ELISA methods used for the analysis of HbF changed from a polyclonal antibody ELISA that measured all cell-free HbF (both unbound and bound to haptoglobin) to a monoclonal that only measured unbound HbF. This is an important factor explaining why the levels of HbF vary between the different cohorts.

Several different antibodies against HbF have been tested during the developmental work and much effort has been made to create a stable ELISA

Statistical considerations

Logistic regression methods are often used for validation of biomarkers for diagnostic or prediction outcomes. Several logistic regression methods have been developed for various purposes, but recently the most-used method—the stepwise method—has been criticized for overestimating the prediction capacity of biomarkers [168, 169]. It has been remarked that the stepwise method was not constructed for prediction purposes, but for causality [168, 169]. Possible alternative methods include Lasso/LARS and

boosted regression, and two of these methods were tested in Paper II as alternatives when building prediction models. The results indicated equality between the stepwise and Lasso methods, but the boosted regression method performed very differently between the test and trial cohorts. For our study in Paper II, we therefore stayed with the stepwise method. However, boosted regression might be the chosen method in future studies of larger cohorts.

Future directions

Further evaluations of the above-mentioned new HbF etiology hypothesis are indeed needed. Consequently, further research needs to proceed in two directions. First, a mechanistic direction is needed to clarify the pathogenesis at a more detailed level. What leads to the increase in placental HbF synthesis in the placenta in women with PE? Is there a deregulated hematopoiesis in this patient group? The second direction is biomarker research. The work presented in this thesis indicates that HbF, Hb and heme scavengers are potential biomarkers for PE. These results need to be verified in larger prospective cohorts with a normal prevalence of PE patients before they can be implemented for clinical use. If it does prove possible to set up a prediction model that is applicable in clinical practice, it should be followed by an effective prophylactic strategy. This prophylactic strategy could possibly be based on hemoglobin scavengers such as haptoglobin, or heme scavengers such as A1M.

Main conclusions:

(*i*) The concentration of HbF is increased in the maternal blood in women with both early and late onset PE and can be measured as early as the first trimester.

(*ii*) HbF in combination with A1M and hemopexin are potential predictive biomarkers for PE, either as-is, or in combination with maternal characteristics or Doppler ultrasound.

(*iii*) In late pregnancy, HbF, A1M, haptoglobin, and hemopexin are potential plasma biomarkers that in combination could support the diagnosis of PE, indicate severity of PE, and predict adverse maternal and fetal outcomes.

(*iv*) Podocyte-specific urinary extracellular vesicles are a measure of podocyte injury in PE and a potential biomarker of PE in urine. HbF may be a contributing causal factor in this injury.

Populärvetenskaplig sammanfattning.

Preeklampsi, eller havandeskapsförgiftning, är ett tillstånd, som årligen drabbar 8-9 miljoner gravida kvinnor i världen. Sjukdomen orsakar ett stort lidande och bidrar, framför allt i utvecklingsländer, till den höga foster- och mödramortaliteten.. I Sverige drabbas ca 3-6% av alla gravida vilket motsvarar ca 5000 kvinnor per år.

Preeklampsi kännetecknas av högt blodtryck och äggvita- (protein)-läckage i urinen, som uppstår efter graviditetsvecka 20. Symptomen är mycket diffusa och sjukdomens svårighetsgrad kan variera mycket mellan patienterna. Det finns idag ingen egentlig bot, endast symptomlindrande blodtryckssänkande behandling finns att tillgå. Enda sättet att bota tillståndet är, att avbryta graviditeten genom att sätta igång förlossningen. Moderkakan eller placentan anses vara central för sjukdomens uppkomst, en teori som stöds av det faktum att kvinnan tillfrisknar efter att placentan har avlägsnats i samband med förlossning. I de svåra preeklampsifallen måste graviditeten avslutas före fullgången graviditet (40 veckor), vilket medför att preeklampsi orsakar ca 15 % av alla för tidiga, s.k. prematura förlossningar.

Trots intensiv forskning är orsaken till sjukdomen till stor del okänd. Uppkomsten av preeklampsi förklaras oftast med tvåstegsmodellen; där steg 1 kännetecknas av förändringar i placentan som uppstår när denna bildas tidigt i graviditeten, och steg 2 beskriver de kliniska symptom som uppstår efter graviditetsvecka 20.

Under graviditeten har placentan en central roll för fostrets utveckling. Utöver att försörja fostret med syre och näringsämnen, är placentan även ett blodbildande organ, som hjälper fostret med att bilda röda blodkroppar och fosterhemoglobin (HbF) fram tills det att fostrets benmärg tar över produktionen.

Tidigare forskning har visat att det bildas för mycket fosterhemoglobin i placentor hos kvinnor med preeklampsi. Fritt fosterhemoglobin skadar moderkakan så att det läcker över till mammans blodcirkulation. Det fria HbF skadar mammans blodkärl och njurar, och bidrar därmed till utveckling av högt blodtryck och proteinläckage i urinen. Kroppen har olika skyddsmekanismer mot fritt hemoglobinet och dess giftiga nedbrytningsprodukt heme. Proteinet haptoglobin kan binda och eliminera fritt hemoglobin. Hemopexin och α1-microglobulin (A1M) är protein som kan binda och eliminera fritt heme, som i sin tur bryts ner av enzymet heme oxygenase 1 (HO-1). Dessa naturliga skyddsmekanismer kan under en kort tid hantera förhöjda nivåer av

HbF, men efter flera veckors exponering, som vid preeklampsi, förbrukas skyddsproteinerna.

Syftet med detta avhandlingsprojekt har varit i att studera om HbF och ovan nämnda skyddsproteiner kan fungera som sjukdomsmarkörer, s.k. biomarkörer, för preeklampsi.

Avhandlingen består av fem artiklar. Artiklarna I och II beskriver resultat från blodprover tagna i tidig graviditet hos mödrar som senare utvecklade preeklampsi. Syftet var att studera hur väl biomarkörerna kunde förutsäga vilka patienter, som kom att utveckla preeklampsi senare i graviditeten. Artikel I beskriver resultat från 96 kvinnor där blodprover togs i graviditetsvecka 10-16. Biomarkörerna HbF- och A1M, var förhöjda i mammans blod redan i tidig graviditet hos de som senare utvecklade preeklampsi jämfört med friska. Med hjälp av dessa biomarkörer kunde preeklampsi förutsägas eller predikteras i 69 % av patienterna. I artikel II beskrivs 433 undersökta kvinnor där blodproverna insamlades mellan graviditetsveckorna 6-20. Förutom HbF och A1M mättes även skyddsproteinerna haptoglobin och hemopexin. På samma sätt som i studie I, var HbF och A1M nivåerna förhöjda. Skyddsproteinet hemopexin var lågt hos de kvinnor som senare utvecklade preeklampsi. Sänkningen tolkades som ett tecken på att hemopexinet förbrukades på grund av de långvarigt höga nivåerna av HbF.

Artikel III beskriver hur HbF, påverkar skyddsproteinerna hos kvinnor som fått diagnosen preeklampsi i senare delen av graviditeten. Totalt analyserades 145 blodprover, som insamlats ett dygn innan förlossning, med avseende på HbF, A1M, haptoglobin, hemopexin, hemoglobin-haptoglobin-komplexet och CD163. Återigen påvisades förhöjda nivåer av HbF och A1M vid preeklampsi samt lägre nivåer av haptoglobin och hemopexin. Vidare kunde ett samband mellan hemopexin-nivån korreleras till kvinnans blodtryck, och därmed ge en indikation om sjukdomens allvarlighetsgrad. I framtiden skulle denna biomarkör kunna vara vägledande för hur förlossningsläkaren skall ta hand om patienten, t.ex. avgöra när förlossning skall sättas igång.

Artikel IV är en fortsättning på artikel III. I tillägg mättes även aktiviteten av det enzym som bryter ner det giftiga ämnet heme. Resultaten visade en minskad aktivitet hos de kvinnor som utvecklade preeklampsi sent i graviditeten, dvs. efter graviditetsvecka 34. Som en konsekvens av minskad nedbrytning kunde förhöjda nivåer av heme påvisas hos dessa kvinnor.

Resultaten i studierna I-IV visade en ökning av HbF och en minskning av skyddsproteinerna liksom en minskad nedbrytning av heme vid preeklampsi. Nettoeffekten blir en ökad nivå av giftiga ämnen i mammans blod. Njurarna tar skada vid preeklampsi, bland annat pga. av dessa hemoglobinrelaterade komponenter. I artikel V studerades därför sambandet mellan njurskada och HbF nivåerna. Graden av

njurpåverkan/skada mättes i såväl mammans blod som urin. Med en ny analysmetod kunde små fragment från njurarna, så kallade extracellulära vesiklar (EVs), mätas i urinen. Mängden partiklar i urinen kunde sedan korreleras till nivåerna av HbF, A1M, haptoglobin och hemopexin beskrivna i artikel III. Resultaten indikerar att fritt HbF kan vara en direkt orsak till de typiska njurskador som ses vid preeklampsi.

Sammanfattningsvis visar resultaten ökade nivåer av HbF och minskade nivåer av skyddsproteiner i mammans blod redan i tidig graviditet, hos de kvinnor som senare utvecklade preeklampsi. Som en konsekvens tar njurarna skada av det giftiga HbF och nedbrytningsprodukten heme. Vidare har vi visat att man, genom att mäta dessa biomarkörer tidig i graviditet kan förutsäga, vilka kvinnor som har ökad risk att utveckla preeklampsi. Nivåerna av biomarkörerna avspeglar i senare delen av graviditeten även sjukdomens allvarlighetsgrad. Detta kan i framtiden användas som ett kliniskt hjälpmedel för att bedöma när graviditeten skall avslutas, vilket i sin tur kan minska antalet för tidigt födda barn.

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Paper I

OBSTETRICS

Fetal hemoglobin and α_1-microglobulin as first- and early second-trimester predictive biomarkers for preeclampsia

Ulrik Dolberg Anderson, MD; Magnus G. Olsson, PhD; Sigurbjörg Rutardóttir, MSc; Magnus Centlow, PhD; Karl Heby Kristensen, MD, PhD; Per Erik Isberg, BSc; Baskaran Thilaganathan, MD, PhD; Bo Åkerström, PhD; Stefan R. Hansson, MD, PhD

OBJECTIVE: The aim of this study was to evaluate fetal hemoglobin (HbF) and α_1-microglobulin (A1M) in maternal serum as first-trimester biomarkers for preeclampsia (PE).

STUDY DESIGN: The design was a case-control study. We included 96 patients in the first trimester of pregnancy (60 with PE and 36 controls). Venous serum samples were analyzed for HbF and total hemoglobin (Hb) by enzyme-linked immunosorbent assay and for A1M by radioimmunoassay. Sensitivity and specificity was calculated by logistic regression and receiver operating characteristic curve analysis.

RESULTS: The HbF/Hb ratio and A1M concentration were significantly elevated in serum from women with subsequent development of PE ($P < .0001$). The optimal sensitivity and specificity was obtained using the biomarkers in combination; 69% sensitivity for a 5% screen positive rate and 90% sensitivity for a 23% screen positive rate.

CONCLUSION: The study suggests that HbF/Hb ratio in combination with A1M is predictive biomarkers for PE.

Key words: α_1-microglobulin, first-trimester biomarker, free fetal hemoglobin, prediction preeclampsia

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Preeclampsia (PE) affects up to 7% of all pregnancies and is an important factor of maternal morbidity and mortality.[1,2] The clinical manifestations of PE appear in the second to third trimester. Early-onset PE, the more severe form, appears before a gestational age (GA) of 34 weeks.[2,3] The symptoms and objective findings are often diverse, such as headache, blurry vision, epigastralgia, and edema. Hypertension and proteinurea are not only hallmarks of the disease but are also integral to the diagnosis.[4] Clinically, PE spans vary broadly, from mild cases with few subjective symptoms, only mild hypertension and little proteinurea, to life-threatening cases with severe hypertension and marked proteinurea often complicated with some degree of renal failure and the worst cases with seizures.

Although there are clinical parameters used for diagnosing PE and a very clear definition of the diagnosis, there are still some difficulties in predicting which patients will develop the most severe cases of the disease. Neither the amount of proteinurea nor the level of hypertension is a very good predictor for, for example, HELLP syndrome or eclampsia.[5,6]

Due to the fact that there are no established biomarkers for predicting and/or diagnosing PE, great effort has lately been put into this field. However, finding a good biomarker is a major challenge. Many of the suggested markers need to be combined with each other and/or evaluated in combination with Doppler ultrasound to improve the diagnostic accuracy. To date, several have been suggested, but none are accepted as being useful biomarkers for the clinical prediction or diagnosis of PE.[7-20] Two antiangiogenic factors are promising, showing a significant association with PE: soluble fms-like tyrosine kinase 1 (sFlt) and soluble endoglin.[8,14,15,17,21,22] These markers have been shown to be particularly useful in the second and third trimester, but their value in the first trimester is still to be determined.

Our recent studies have indicated the involvement of hemoglobin (Hb)-induced oxidative stress in the development of PE. Increased local synthesis of fetal Hb (HbF) by cells in the placenta was indicated by an up-regulation of the HbF genes and the accumulation of HbF in the term PE placenta.[23] Free Hb, ie, outside the red blood cell, and its metabolites heme and iron induce oxidative stress by formation of reactive oxygen species.[24] In fact, free heme, bilirubin, and biliverdin have been identified among 14 metabolites in a metabolomic signature of PE using first-trimester plasma.[20] The oxidative stress may damage the blood-placenta barrier, leading to leakage of HbF into the maternal circulation and eventually cause elevated

From the Department of Obstetrics and Gynecology, Clinical Sciences (Drs Anderson, Centlow, and Hansson); the Division of Infection Medicine (Drs Olsson and Åkerström and Ms Rutardóttir); and the Department of Statistics (Mr Isberg), Lund University Hospital, Lund University, Lund and the Department of Obstetrics and Gynecology, Malmö University Hospital, Malmö (Dr Kristensen), Sweden, and the Division of Clinical Development Sciences, Department of Obstetrics and Gynaecology, St Georges University of London, London, England, UK (Dr Thilaganathan).

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Reprints: Ulrik Dolberg Anderson, MD, Lund University, Tornavägen 10, 221 84 Lund, Sweden. ulrik.dolberg_anderson@med.lu.se.

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TABLE 1
Demographics of cases and controls

Demographic	Normal pregnancy, control (n = 36)	PE (n = 60)
GA at delivery[a]	39.9 (39.5–40.3)	36.8 (35.7–37.8)
GA at sampling, wk[b]	12.9 (12.2–13.6)	14.1 (13.5–14.7)
Birthweight, g[c]	3484 (3281–3687)	2752 (2484–3019)
Ethnicity[d]		
Caucasian	29	31
Asian	4	11
Afro-Caribbean	2	15
Mixed	1	4
Preterm[e]	0 (0%)	20 (33.3%)

Data for GA at delivery, at time of sampling, birthweight, parity, and preterm delivery. Values are shown as mean (95% confidence interval) or number (%). One-way analysis of variance was used to calculate significance.
GA, gestational age; *PE*, preeclampsia.
[a] $P = .0001$; [b] $P = .18$; [c] $P = .001$ after adjusting for GA with general linear univariate model; [d] $P = .01$; [e] Preterm defined as delivered <37+0 wk, 7 patients with PE delivered <34+0 wk.
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levels in the maternal plasma or serum. Indeed, increased levels of HbF, total Hb, and markers for oxidative stress, as well as the heme-scavenger and antioxidant endogenous protein α_1-microglobulin (A1M), were found to be elevated at term in both plasma and placenta from women with PE.[25-27]

The aim of the present study was to measure the concentrations of HbF, total Hb, and A1M in first-trimester serum samples to evaluate their value as predictive biomarkers for PE.

MATERIALS AND METHODS

Patients and demographics

The study was designed as a case-control study. Originally a total of 100 women were included in the study. Exclusion criteria were diabetes, prepregnancy hypertension, and premature delivery. Four controls were excluded due to these criteria, as 2 had type 1 diabetes and 2 others had essential hypertension. In the end a total of 96 women were included, 60 women who subsequently developed PE (cases) and 36 with normal uncomplicated pregnancies (controls). Characteristics are shown in Table 1. Of the 60 cases, 20 cases delivered at a GA of <37+0 weeks, of which 7 delivered <34+0 weeks. All controls delivered >37+0 weeks.

The patients were recruited with ethical permission, as part of an ongoing prospective study of first-trimester ultrasound and serum markers for PE in women attending a routine antenatal care visit at St. Georges Hospital Obstetric Unit, London. GA was calculated from the last menstrual period and confirmed by ultrasound crown-rump-length measurement. A maternal venous serum sample was collected at a GA of 11-16 weeks for analysis of HbF, total Hb, and A1M. The venous blood was collected into a 5-mL Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ), without additives to allow clotting, and centrifugated at 2000*g* at room temperature for 10 minutes. The serum was stored at −80°C until further analysis. The samples were transported from Britain to Sweden in a container with carbon-dioxide ice, and they were all still frozen on arrival.

All pregnancy outcomes were obtained from the main delivery suite database and checked for each individual patient. The International Society for the Study of Hypertension in Pregnancy definition for PE was used: 2 readings of blood pressure >140/90 mm Hg at least 4 hours apart, and proteinurea, ≥300 mg in 24 hours, or 2 readings of at least 2+ on dipstick analysis of midstream or catheter urine specimens, if no 24-hour urine collection was available.[4] Normal pregnancy was defined as delivery after a GA of 37+0 weeks and normal blood pressure. The control samples were chosen as consecutive cases from the same time period as the cases who met the selection criteria.

Measurement of total Hb, HbF, and A1M

HbF was measured with a sandwich enzyme-linked immunosorbent assay. The sensitivity was 5 ng/mL, with an interassay coefficient of variation (CV) of <7.99% and an intraassay CV of <2.21%. A1M concentrations were determined by a radioimmunoassay. The sensitivity was 1.95 ng/mL, with an interassay CV of <4.05% and intraassay CV of <6.46%. The total Hb concentration in serum was measured by a competitive enzyme-linked immunosorbent assay, using antibodies against adult Hb. The sensitivity was 40 ng/mL, with an interassay CV of <5.31% and intraassay CV of <2.04%. All 3 methods were previously described by Olsson et al.[28] The ratio HbF/total Hb was calculated and is referred to as HbF ratio.

Statistical analysis

Statistical computer software Statistical Package for the Social Sciences (SPSS), version 17.0 (SPSS Inc, Chicago, IL) for Apple computers (Apple Inc, Cupertino, CA) was used to analyze the data. The probability of developing PE was analyzed in relation to the HbF ratio and/or the A1M levels using a binary logistic regression analysis with a likelihood ratio test. Odds ratio (OR) was calculated for the biomarkers. All ORs were adjusted for GA at sampling and ethnicity. Since the HbF ratio showed low values, they were multiplied by 100 before the OR was calculated. A significance level of .05 was used in all tests.

Receiver operating characteristic (ROC) curves for HbF, A1M, HbF ratio, and the combination of HbF ratio and A1M were done based on the logistic regression results. The area under the curve was calculated from the ROC curves. The logistic regression analysis allowed us to test sensitivity at different screen posit-

TABLE 2
Serum concentrations of fetal hemoglobin, α_1-microglobulin, total hemoglobin, and fetal hemoglobin ratio

Variable	PE (SD)	Controls (SD)	*P* value	Odds ratio[a] (95% CI)
HbF (μg/mL)	1.38 (1.53)	0.45 (0.65)	< .0001	2.4 (1.2–5.0)
A1M (μg/mL)	24.77 (5.37)	20.50 (4.26)	< .0001	1.2 (1.1–1.4)
Total Hb (μg/mL)	177.90 (62.72)	198.34 (102.10)	.26	1.0 (1.0–1.0)
HbF ratio[b]	0.0079 (0.0073)	0.0020 (0.0018)	< .0001	166.7 (8.5–3256.8)[c]

Mean concentration of HbF, A1M, and total Hb in PE cases and controls. Binary logistic regression was used to determine significance. All odds ratios are adjusted for ethnicity and gestational age at sampling.
A1M, α_1-microglobulin; *CI*, confidence interval; *Hb*, hemoglobin; *HbF*, fetal hemoglobin; *PE*, preeclampsia.
[a] Odds ratios are calculated as per 1-U change (μg/mL for HbF, A1M, and total Hb, but U for HbF ratio); [b] HbF ratio = HbF/total Hb concentration; [c] Odds ratio calculations based on HbF ratio × 100 –this is due to very low values of HbF ratio.
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ive rates. The optimal sensitivity level was evaluated. The linear correlations among the 3 biomarkers were evaluated by bivariate correlation analysis calculating the Pearson correlation coefficients.

RESULTS

The study groups

The demographic data of the included cases and the controls are shown in Table 1. There was an expected, significant difference in gestational length at the time of delivery. There was a significant difference in birthweight after adjustment for GA ($P = .001$). The PE blood samples were, by coincidence, on average collected 8 days later than the control samples ($P = .03$).

HbF ratio and A1M serum levels

A high concentration of total Hb was seen in all samples, suggesting a certain degree of hemolysis. The high levels were verified by spectrophotometry (data not shown). However, there was no significant difference in total Hb concentration between the examined groups, 178 μg/mL in PE and 198 μg/mL in the controls ($P = .232$) (Table 2). The mean concentration of HbF was 1.38 μg/mL in PE and 0.45 μg/mL in the controls. Since background hemolysis may contribute to the HbF values, and since HbF levels significantly correlated to Hb total levels, the HbF ratio (HbF/total Hb) was calculated. The mean HbF ratio was significantly elevated in PE compared to controls ($P < .0001$) (Table 2). The mean A1M level was significantly elevated in PE (24 μg/mL) compared to controls (21 μg/mL) (Table 2) ($P = .0001$).

Correlation analysis

Neither the HbF levels nor the HbF ratio levels were significantly correlated to the A1M levels ($P = .17$, $r = 0.16$). However, HbF levels significantly correlated to total Hb levels ($P = .001$, $r = 0.35$), a correlation that was even stronger in the PE cases ($P = .005$, $r = 0.39$). The levels of total Hb and A1M were not significantly correlated ($P = .61$, $r = -0.055$).

Correlation of the HbF ratio to GA

The HbF ratio and A1M levels were tested for correlation to GA using Pearson correlation coefficients. This correlation analysis was done for controls, for PE, and for all the patients together, but no correlation to GA was observed between 10-16 weeks for neither HbF ratio ($P = .96$, $r = 0.01$) nor A1M ($P = 0.24$, $r = 0.12$) in either of the groups.

Logistic regression and ROC curves

Logistic regression was used for calculating significance and ORs (Table 2). ROC curves were drawn (Figure) and the sensitivity and specificity for the HbF ratio, A1M, and their combination were obtained. The results were analyzed at 4 different cutoff values as presented in Table 3. The area under the curve was 0.82 for the HbF ratio, 0.75 for A1M, and 0.89 for the combination of the HbF ratio and A1M. The combination of the 2 markers showed the highest values of prediction, with an optimal sensitivity of 90% at 23% screen positive rate (Table 3).

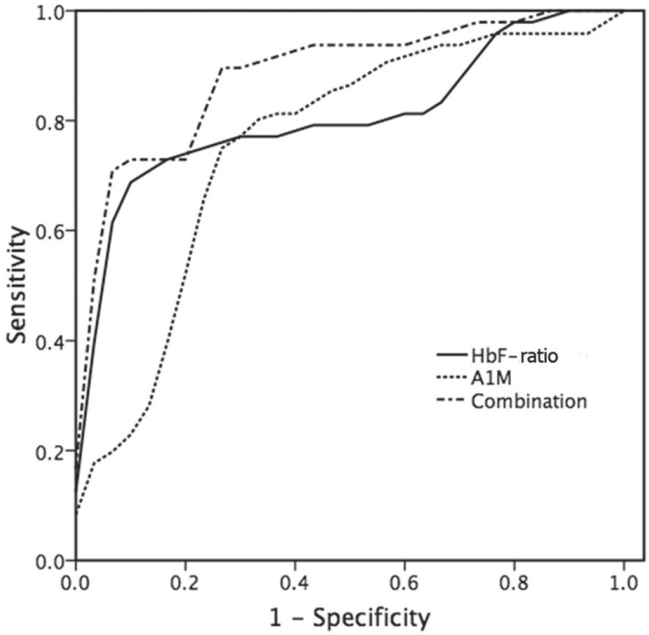
COMMENT

The aim of this study was to evaluate maternal serum concentrations of HbF and A1M as first-trimester markers for subsequent development of PE. The results clearly show that the serum concentrations of HbF and A1M, alone or in combination, were significantly different between the study groups. Based on the optimal sensitivity of 90%, these markers have the potential of being used as first-trimester markers for the subsequent development of PE.

A1M is an important endogenous scavenger of heme.[25,29,30] Its expression has been shown to be up-regulated in response to free Hb and reactive oxygen species.[31] A1M is synthesized and secreted mostly from the liver and rapidly distributed to different tissues, where it is found in the extravascular compartments both in free form and as high molecular–weight complexes bound to IgA,[32] albumin, and prothrombin.[33] The biological and metabolical link between Hb and A1M is further supported by our current results, since both HbF and A1M were elevated in the first-trimester samples. The simultaneous increase of A1M and Hb has also been shown in PE in term pregnancies.[34]

PE is generally believed to develop in 2 stages, where the first stage is characterized by defective placentation,[1,6,35-37] which leads to uneven blood perfusion, ischemic reperfusion injuries, and increased oxidative stress in the placenta.[38] The second stage of PE, the maternal syndrome, is characterized by a general vascular dysfunction based on severe endothelial damage that eventually causes vasoconstriction, general vascular inflammation, and multiple organ dys-

FIGURE
Receiver operating characteristic curves



Receiver operating characteristic curves showing sensitivity and specificity for fetal hemoglobin (HbF) ratio, α_1-microglobulin (A1M), and combined markers. Area under curve is 0.82 for HbF ratio, 0.75 for A1M, and 0.89 for combination

Anderson. HbF and A1M, potential predictive biomarkers for preeclampsia. Am J Obstet Gynecol 2011.

TABLE 3
Sensitivity and specificity values for fetal hemoglobin ratio, α_1-microglobulin levels, and combination of 2 parameters

Screen positive rate	HbF ratio sensitivity[a]	A1M sensitivity	HbF ratio combined with A1M sensitivity[b]
5%	55%	17%	69%
10%	69%	25%	73%
20%	76%	48%	73%
30%	78%	73%	90%
Optimal[c]	78% sensitivity 26% screen positive rate	77% sensitivity 35% screen positive rate	90% sensitivity 23% screen positive rate

A1M, α_1-microglobulin; *HbF*, fetal hemoglobin.

[a] HbF ratio = HbF/total hemoglobin concentration; [b] Based on logistic regression including both parameters; [c] Optimal sensitivity is chosen by authors from coordinates list of receiver operating characteristic curve and represents as high a sensitivity as possible for as low a screen positive rate as possible.

Anderson. HbF and A1M, potential predictive biomarkers for preeclampsia. Am J Obstet Gynecol 2011.

function.[39] The link between the 2 stages is not known but based on our previous data[23,28,34] and the present results, we hypothesize that leakage of free HbF may play an important role in the pathogenesis of PE.

Our data indicate that the combination of the HbF ratio and A1M levels may be used as first-trimester biomarkers for screening of PE. Several studies have shown a prediction rate around 50%, particularly for early onset PE, using Doppler ultrasound.[17,18]

The predictive values obtained for the HbF ratio and A1M levels, especially in combination, compare favorably with most other promising PE biomarkers. Furthermore, HbF ratio and A1M levels are also useful for PE diagnosis and determination of PE severity in term pregnancies.[28] The antiangiogenic marker sFlt is a well-described potential PE marker when used in the second and third trimester. However, in the first trimester, sFlt levels are unchanged compared to normal pregnancies, making it less useful for prediction of PE.[7] The placenta growth factor has also been proposed as a PE marker, but available data show conflicting results.[8,15,40] Soluble endoglin has a lower screening efficiency in early pregnancy, when compared to the predictive values obtained when the combination sFlt/placenta growth factor were used.[41]

Even though the combined measurements of HbF ratio and A1M levels give good prognostic values, there are some caveats to the methodology. During blood sampling, a low grade of hemolysis may occur. The levels of total Hb in the noncell fraction indicate that some degree of hemolysis occurred in many of our samples, possibly due to the fact that the samples were allowed to coagulate before centrifugation. To correct for the contribution of HbF derived from lysed maternal red blood cells, the ratio HbF: total Hb (HbF ratio) was used. Even though care should be taken to minimize technical hemolysis during sample processing, this often happens. By using the HbF ratio, most samples can be evaluated without risk of a false-positive prediction or diagnosis.

In summary, we present new data demonstrating that HbF/A1M measurement in early pregnancy may be developed into a useful first-trimester screening tool for prediction of women who subsequently will develop PE. To our knowledge the combination of HbF ratio and A1M in either plasma or serum may offer one of the most effective sets of early predictive biomarkers hereto described for PE. ■

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Paper II

Fetal hemoglobin, α_1-microglobulin and hemopexin are predictive first trimester biomarkers for preeclampsia

Ulrik Dolberg Anderson[1a,b*+], Magnus Gram[2+], Jonas Ranstam[3], Basky Thilaganathan[4], Bo Åkerström[2#] and Stefan R. Hansson[1a,b#]

[1a] Section of Obstetrics and Gynecology, Department of Clinical Sciences Lund, Lund University, Sweden

[1b]Skåne University Hospital, Malmö/Lund, Sweden

[2]Department of Clinical Sciences, Lund, Infection Medicine, Lund University, Sweden

[3]Department of Clinical Sciences, RC Syd, Lund University, Sweden

[4]Fetal Medicine Unit, St. George's University Hospital, London, United Kingdom

+: Contributed equally to the manuscript

#: Contributed equally to the manuscript

*Corresponding author:

ulrik.dolberg_anderson@med.lu.se

Department of Obstetrics and Gynecology

University Hospital Skåne
S-21466 Malmö, Sweden

Abstract

Preeclampsia is a syndrome that complicates 3-8% of all pregnancies. Recent research shows that overproduction of cell-free fetal hemoglobin in the placenta in the preeclamptic placenta might be a new etiological factor. In this study maternal serum levels of cell-free fetal hemoglobin and the endogenous hemoglobin/heme scavenging systems were evaluated as predictive biomarkers for preeclampsia in combination with uterine artery Doppler ultrasound. The study was designed as a case-control study and included 433 women in early pregnancy (mean 13.7 weeks of gestation) of which 86 subsequently developed preeclampsia and 347 were included as controls. The serum concentrations of cell-free fetal hemoglobin, total cell-free hemoglobin, the heme-scavenger hemopexin, the hemoglobin scavenger haptoglobin and the heme- and radical-scavenger α_1-microglobulin were measured. All patients were examined with uterine artery Doppler ultrasound Pulsatility index and notching was recorded. Logistic regression models were developed, which included the biomarkers, ultrasound indices and maternal risk factors. There were significantly higher serum concentrations of cell-free fetal hemoglobin and α_1-microglobulin and significantly lower serum concentrations of hemopexin in patients who later developed preeclampsia. The uterine artery Doppler ultrasound results showed significantly higher pulsatility index values in the preeclampsia group. The optimal prediction model was obtained by combining the biomarkers cell-free fetal hemoglobin, α_1-microglobulin and hemopexin in combination with the maternal characteristics parity, diabetes and pre-pregnancy hypertension and predicted 60% of preeclampsia cases at 95% specificity. The model predicts both early and late onset preeclampsia. The results confirm that women who subsequently develop preeclampsia have a

higher concentration of circulating cell-free fetal hemoglobin. Cell-free hemoglobin is known to be harmful to tissues and organs and increased amounts of cell-free fetal hemoglobin early in the pregnancy may strain the physiological protecting heme- and hemoglobin scavenging mechanisms; thereby contributing to the development preeclampsia. In summary, cell-free fetal hemoglobin in combination with α_1-microglobulin and/or hemopexin may therefore be potential predictive serum biomarkers for preeclampsia – either alone or in combination with maternal risk factors and/or uterine artery Doppler ultrasound.

Introduction

Preeclampsia is a pregnancy-related condition affecting up to 8 % of pregnancies worldwide [1]. The incidence varies according to geographical, social, economic and racial differences [2]. It has been estimated that preeclampsia or complications of the condition account for more than 50,000 maternal deaths worldwide each year [1].

Clinical manifestations appear after 20 weeks of gestation. Although the diagnostic criteria are clear [3] it has been difficult to find a way to predict the disease as early as the first trimester of pregnancy or to predict which women that will develop severe preeclampsia and/or eclampsia.

The details of the pathophysiology remain elusive but recent research has improved the understanding of the condition markedly. Preeclampsia is described to develop in two stages [4, 5]. The first stage is characterized by a defect placentation [6]. The extravillous trophoblast cells do not remodel the spiral arteries in the maternal decidua properly and consequently fail to create a low-resistance even utero-placental blood flow [6, 7]. Uneven perfusion leads to oxidative stress in the placenta that contributes to the damage of the blood-placenta barrier and subsequent leakage between the fetal and maternal circulation. In fact, cell-free fetal DNA [8, 9], micro-particles [10] and cell-free fetal hemoglobin [11, 12] have been described in the maternal circulation of women with preeclampsia [13, 14]. The second stage of preeclampsia is characterized by the clinical manifestations, e.g. increased blood pressure and proteinuria detected after 20 weeks of gestation [4, 5].

The link between the stages 1 and 2 is still a main focus of modern preeclampsia research. Maternal constitutional factors such as obesity are important risk factors for late onset

preeclampsia in particular. Furthermore a new focus area is the role of maternal cardiac strain in the development of preeclampsia [14-17].

Results from gene- and proteome profiling studies have shown an over-production and accumulation of cell-free fetal hemoglobin in the preeclamptic placenta [18]. *Ex-vivo* studies using the dual-placental perfusion model have shown that cell-free hemoglobin causes damage to the blood-placenta barrier and leakage of cell-free hemoglobin into the maternal circulation [12, 19, 20]. Furthermore, cell-free fetal hemoglobin has been shown to appear early in pregnancy in women who subsequently develop preeclampsia and has therefore been suggested to be an important etiological factor and a potential biomarker for early detection of preeclampsia [11, 12, 21-23]. In term pregnancies cell-free fetal hemoglobin was shown to correlate to the severity of the disease, *i.e*. blood pressure [24].

Extracellular hemoglobin is in general toxic to tissues and organs [25, 26]. Hemoglobin and its metabolites heme and free iron are particularly reactive and generate free radicals which can cause cell- and tissue damage, oxidative stress, inflammation and vascular endothelial damage [26]. The human system has evolved several different scavenging proteins that protect the body from the toxicity of extracellular hemoglobin and heme. Haptoglobin, the most well investigated human hemoglobin clearance system, binds cell-free hemoglobin in the blood [26, 27]. The hemoglobin-haptoglobin complex subsequently bind to its receptor CD163 [28] which eliminates the complex from the blood. Hemopexin is a circulating plasma protein and is the major scavenger of free heme in the blood [29]. The hemopexin-heme complex is taken up by cells such as macrophages and hepatocytes, expressing the CD91 receptor, thus facilitating the heme clearance heme from the blood [25]. Previous *in vitro* results have indicated that decreased hemopexin *activity* may regulate blood pressure through the renin-angiotensin-system in patients with preeclampsia [30, 31].

α_1-microglobulin is a plasma- and extravascular protein that provides protection through its ability to bind and neutralize free heme and radicals [32-34]. Several *in vitro* and *in vivo* studies have shown that α_1-microglobulin protects cells and tissues in conditions with increased concentrations of extracellular hemoglobin, heme and reactive oxidative species [24]. [24]. In studies using liver- and placenta cells, α_1-microglobulin expression has been shown to be up-regulated following exposure to hemoglobin, heme and reactive oxygen species [24, 35]. Furthermore, the serum concentration of α_1-microglobulin has also been shown to be significantly elevated in maternal blood in the first trimester of patients who subsequently develop preeclampsia [22].

In the last decade a number of predictive biomarkers have been described for preeclampsia. Most of these markers reflect the placental pathology of preeclampsia and the pro-/anti-angiogenetic imbalance. Some of the most well investigated markers are: Pregnancy associated plasma protein A, placental protein 13, soluble endoglin, placental growth factor and soluble FMS-like tyrosin kinase 1 (sFlt-1) [11, 21, 22, 36-43]. In order to increase the sensitivity and specificity of the different markers, new algorithms have been developed that include several of these markers [21, 23]. Furthermore, these algorithms combine biomarkers with maternal risk characteristics such as parity, body mass index (BMI), maternal age, systemic disorders and clinical parameters such as mean artery blood pressure. In addition, uterine artery Doppler ultrasound can give an indication on the blood flow in the utero-placental unit. Pulsatility index, resistive index and diastolic notching are parameters used clinically to identify decreased blood flow and therefore added to increase the sensitivtity. To date, a number of algorithms have been suggested to predict preeclampsia but none are yet broadly accepted for clinical use [21, 23].

The aim of this study was to analyze the serum concentrations of cell-free fetal hemoglobin and the hemoglobin- and heme scavenging systems haptoglobin, hemopexin, and α_1-microglobulin in a larger cohort of uncomplicated pregnancies and pregnancies with subsequent development of preeclampsia. Their potential was evaluated as: a) first trimester biomarkers for prediction of preeclampsia and b) biomarkers for sub-classification of early-, late- and term-onset preeclampsia.

Materials and methods

Patients and samples

The study was approved by the local ethical committees at St Georges University Hospitals, London, UK. All participants signed a written informed consent prior to inclusion. Women attending a routine antenatal care visit at St. Georges Hospitals' Obstetric Unit were recruited from 2006 to 2008.

The gestational length was calculated from the last menstrual period and confirmed by ultrasound crown-rump-length measurement. Uterine artery Doppler ultrasound was measured as previously described [44]. A maternal venous blood sample was collected at 6-20 weeks of gestation (mean 13.7 weeks) in a 5 mL vacutainer tube (Becton Dickinson, Franklin Lakes, NJ, USA) without additives. After clotting, the samples were centrifuged at 2000xg at room temperature (RT) for 10 minutes and serum was separated and stored at -80°C until further analysis.

All pregnancy-outcome data was obtained from the main delivery suite database and checked for each individual patient. Preeclampsia was defined according to ISSHP definitions as: 2 readings of blood pressure ≥140/90 mm Hg at least 4 hours apart, and proteinuria, ≥300mg in 24 hours, or 2 readings of at least +2 on dipstick analysis of midstream or catheter urine specimens if no 24-hour urine collection was available [3]. Early onset preeclampsia was

defined as preeclampsia with clinical manifestations before 34+0 weeks of gestation and late onset preeclampsia was defined as clinical manifestations after 34+0 weeks of gestation. Term preeclampsia was defined as preeclampsia with clinical manifestations between 37+0 to 42+0 weeks of gestation. Normal uncomplicated pregnancy was defined as delivery at or after 37+0 weeks of gestation with normal blood pressure. The uncomplicated pregnancy samples (controls) included in this study were randomly selected from samples collected during the same period. Uncomplicated pregnancy was confirmed after delivery.

Measurement of cell-free fetal hemoglobin, α_1-microglobulin, cell-free total hemoglobin, haptoglobin and hemopexin

Cell-free fetal hemoglobin concentration in the serum samples was measured with a sandwich ELISA as previously described [24]. Briefly, 96-wells plates were coated with affinity-purified rabbit anti-cell-free fetal hemoglobin (4 µg/ml in PBS) overnight at RT. In the second step, a standard series of cell-free fetal hemoglobin or the patient samples diluted in incubation buffer were incubated for 2 hours at RT. In the third step, HRP-conjugated affinity-purified rabbit anti-HbA antibodies, were added and incubated for 2 hours at RT. Finally, a ready-to-use 3,3′,5,5′-Tetramethylbenzidine (TMB, Life Technologies, Stockholm, Sweden) substrate solution was added. The reaction was stopped after 30 minutes using 1.0 M HCl and the absorbance was read at 450 nm using a Wallac 1420 Multilabel Counter (Perkin Elmer Life Sciences, Waltham, MA, USA).

The α_1-microglobulin concentrations were determined by a radioimmunoassay (RIA) as previously described (24). Briefly, RIA was performed by mixing goat antiserum against human α_1-microglobulin (“Halvan”; diluted 1:6000) with ^{125}I-labelled α_1-microglobulin (appr. 0.05 pg/ml) and unknown patient samples or calibrator α_1-microglobulin - concentrations. After incubating overnight at RT antibody-bound antigen was precipitated

after which the ^{125}I-activity of the pellets was measured in a Wallac Wizard 1470 gamma counter (Perkin Elmer Life Sciences).

The concentration of total hemoglobin was determined with the Human Hemoglobin ELISA Quantification Kit from Genway Biotech Inc. (San Diego, CA, USA). The analysis was performed according to the manufacturer's instructions and the absorbance was read at 450 nm using a Wallac 1420 Multilabel Counter.

The concentrations of haptoglobin in serum samples were determined using the Human Haptoglobin ELISA Quantification Kit as described by the manufacturer (Genway Biotech Inc.). The serum concentrations of hemopexin were determined using a Human Hemopexin ELISA Kit as described by the manufacturer (Genway Biotech Inc.).

Statistical analysis

Statistical computer software IBM Statistical Package for the Social Sciences (SPSS statistics) version 21.0 for Apple computers was used for all analyses. A p value ≤ 0.05 was considered significant. Significant differences between the groups for the biomarkers cell-free fetal hemoglobin, total hemoglobin, haptoglobin, hemopexin, and α_1-microglobulin were calculated with one-way ANOVA. The uterine artery Doppler ultrasound examination was performed at a significantly later time point in pregnancies complicated by preeclampsia compared to the control group and therefore the uterine artery Doppler ultrasound values were transformed into *Multiples of the Median* (MoM)-values according to median values given by Velauthar et al [45].

The prediction models based on the biomarkers and maternal characteristics were built on stepwise logistic regression. Separate analyses were performed for early onset preeclampsia and late onset preeclampsia. Receiver operation curves (ROC-curves) were obtained for all regression parameters and the parameters in combination. Their sensitivities at different specificity levels were calculated. The optimal sensitivity/specificity was defined as the point

of the ROC-curve closest to the upper left corner.

Results

Demographics

In total, 433 women were included of which 86 subsequently developed preeclampsia. As controls, 347 women with uncomplicated pregnancies and term delivery (>37 weeks of gestation) were included. The maternal characteristics are shown in Table 1. Of the 86 preeclamptic cases, 28 were delivered before 37+0 weeks of gestation; of which 17 delivered before 34+0 weeks of gestation. There was no statistically significant difference between the cases and control groups in terms of time of serum sampling.

There was a statistically significant difference in ethnicity in the control group as compared to the preeclampsia group, $p<0.000001$ (table 1). The preeclampsia group showed significantly higher pregnancy/parity rate compared to the control group. The BMI was significantly higher in the preeclampsia group compared to the control group. The uterine artery Doppler ultrasound examination was performed at a significantly later time point in pregnancies complicated by preeclampsia compared to the control group (mean gestation of 18.5 weeks in the preeclampsia group vs. 12.5 weeks of gestation in the control group, $p=0.0001$). The birth weight was significantly lower in the preeclampsia group as compared to the control group (3467g vs. 2716g, $p<0.0001$) . There were significantly more preterm deliveries in the preeclampsia group as compared to the control group. Furthermore, diabetes (type not specified) was diagnosed in three preeclampsia patients whereas none of the women in the control group was diagnosed with diabetes.

Biomarkers

The serum levels of the biomarkers cell-free fetal hemoglobin, haptoglobin, hemopexin, α_1-microglobulin and total hemoglobin are shown in Table 2. The mean concentration of cell-

free fetal hemoglobin in the preeclampsia group was significantly higher than in the control group (10.8 μg/ml vs. 5.6 μg/ml, p=0.02). The mean α_1-microglobulin concentration was also significantly increased (17.3 μg/ml vs. 15.5 μg/ml, p=0.03). The mean hemopexin concentration in the preeclampsia group was significantly lower (1062 μg/ml vs. 1143 μg/ml in the control group p=0.05). There was a higher haptoglobin concentration in the preeclampsia group (1102 μg/ml) as compared to the control group (971 μg/ml), however this was not significant (p=0.089). The uterine artery Doppler ultrasound MoM values were significantly higher in the preeclampsia group than the controls (1.18 vs. 0.95, p<0.0001).

Logistic regression analysis

The abilities of the biomarkers to predict preeclampsia were evaluated in logistic regression models. Corresponding ROC-curves were generated to calculate the prediction values. All biomarkers were individually tested as well as evaluated in combination to find the optimal predictive value. The results are outlined in Table 3 and the ROC-curves are shown in Figure 1 and 2. Despite a significantly increased serum cell-free fetal hemoglobin concentration in patients who subsequently developed preeclampsia, it displayed limited predictive value when used alone (sensitivity 15% at 90% specificity). α_1-microglobulin showed a similar prediction (sensitivity of 19% at 90% specificity). In combination however the biomarkers α_1-microglobulin, cell-free fetal hemoglobin, and hemopexin performed better (sensitivity of 33% at 90% specificity (optimal 66%/78%)).

All measures of maternal characteristics were tested alone and in combination using a logistic regression analysis to compare preeclampsia and controls. Furthermore, the biophysical parameters obtained from the uterine artery Doppler ultrasound (PI left, PI right, PI mean, diastolic notch right and left) were also evaluated in the logistic regression analysis. The maternal characteristics, *i.e.* ethnicity, number of previous pregnancies (gravidae), number of previous deliveries (parity), maternal BMI, maternal diabetes and maternal hypertension,

were each significantly associated to the development of preeclampsia in the logistic regression analysis. In the combined logistic regression model however, the combination of maternal ethnicity, BMI, diabetes and hypertension were the only parameters that significantly altered the sensitivity. All the maternal characteristics in combination showed a high sensitivity of 60% at 90% specificity (Table 3, Figure 2). The Uterine artery Doppler ultrasound parameters alone showed similar prediction to the biomarkers alone (sensitivity of 25 % at 90% specificity).

The combination of maternal characteristics (parity, diabetes, pre-pregnancy hypertension) and the biomarkers (cell-free fetal hemoglobin, α_1-microglobulin and hemopexin) increased the sensitivity to 62% at 90% specificity (Table 3, Figure 2) and the combination of uterine artery Doppler ultrasound values and maternal characteristics combined showed a similar but lower prediction rate (sensitivity 57% at 90% specificity). However, the combination of biomarkers, maternal characteristics and uterine artery Doppler ultrasound values did not show higher prediction rates (sensitivity 53% at 90% specificity) than the combinations uterine artery Doppler ultrasound/ biomarkers-, biomarkers/maternal characteristics- or uterine artery Doppler ultrasound/ maternal characteristics - combinations (Table 3).

Early-, late- and term-onset preeclampsia

The results elevated levels of cell-free fetal hemoglobin in both early- late- and term-onset preeclampsia groups (Table 4). The α_1-microglobulin levels were only significantly higher in the late and term onset groups (p=0.01 and p=0.016) (Table 4). The hemopexin protein concentration was lower in all groups as compared to the control group, but only statistically significant in the early onset preeclampsia group (p=0.04)(Table 4). The uterine artery Doppler ultrasound PI MoM was significantly elevated especially in the early onset group (1.63 vs. 0.95, p<0.00001). It was only marginally elevated in the late onset group but this difference was not statistically significant (1.06 vs. 0.95, p=0.06). In the term preeclampsia

group there was only a marginally elevated PI (PI=1) but this was not statistically significant (p=0.35). There were no significant differences for total hemoglobin or haptoglobin in either of the study groups.

The logistic regression models for early-, late- and term-onset preeclampsia showed a sensitivity for cell-free fetal hemoglobin of 23% at 90% specificity in the late onset preeclampsia group (Table 5) and 19% sensitivity at 90% specificity in term preeclampsia. α_1-microglobulin was statistically significantly elevated in the late- and term onset groups (24% sensitivity at 90% specificity, and p=0.003). Hemopexin was only statistically significantly decreased in the early onset group and showed a sensitivity of 32% at 90% specificity. The Uterine artery Doppler ultrasound values performed best in the early onset group with a 57% sensitivity at 90% specificity but was even statistically significant in the late onset group (p=0.025) however not in the term onset group (p=0.36). None of the biomarkers were statistically significant when combined with each other, with maternal characteristics or with uterine artery Doppler ultrasound values in either of the preeclampsia subgroups.

Discussion

The aim of this study was to validate previous findings showing that increased serum levels of cell-free fetal hemoglobin and α_1-microglobulin in the first trimester of pregnancy and evaluate their usefulness as biomarkers for early prediction of preeclampsia [22]. The cohort size in this study was larger and somewhat better reflecting a normal incidence of preeclampsia. In addition, the study evaluates the impact of cell-free fetal hemoglobin on the hemoglobin- and heme-scavenging proteins haptoglobin, hemopexin and α_1-microglobulin. The main finding in this paper confirms that both cell-free fetal hemoglobin and α_1-microglobulin are significantly elevated in first trimester serum in women who subsequently

developed preeclampsia (Table 2)[22] and that they have the potential of being useful as predictive first and early second trimester biomarkers for preeclampsia. Furthermore, the heme scavenging plasma protein hemopexin also showed predictive properties and was therefore suggested as an additional potential first trimester biomarker for preeclampsia. The uterine artery Doppler ultrasound indices primarily showed higher PI MoM values in the early onset group. This is in full concordance with previously published results [21, 45].

Even though cell-free fetal hemoglobin, α_1-microglobulin and hemopexin showed predictive ability as individual biomarkers, there was only a weak additional value when they were combined in the logistic regression models. This could be due to the fact that they are biologically linked to each other and therefore predict the clinical outcome in the same way. The optimal prediction model contained cell-free fetal hemoglobin, α_1-microglobulin and hemopexin in combination with the maternal characteristics; parity, diabetes and pre-pregnancy hypertension.

Compared to previously published results, the prediction capacity of these biomarkers is weaker [22]. The current cohort however, better reflects a normal population with fewer preeclampsia cases, which clearly influences the results. The results presented do however need to be confirmed in a cohort with normal prevalence of preeclampsia. A lower prevalence of preeclampsia would consequently lead to a higher number of false positives (i.e. lower specificity). Furthermore a lower prevalence would lead to a lower positive predictive value but a higher negative predictive value.

In contrast to this, the maternal characteristics were found to have a high prediction capacity, 60% sensitivity at 90% specificity (Table 3). Comparable data from previously published studies containing several additional parameters show sensitivity values of approximately 46% at 90% specificity for early onset preeclampsia [21]. The use of maternal characteristics

as prediction tool in a clinical setting is simple but requires software to calculate the patients' risks. The advantage of a prediction model solely based on biomarkers is the fixed cut off value for indication of high-risk.

Doppler ultrasound indices have been used in several first trimester prediction algorithms. Significantly higher PI and notching in patient who subsequently develop preeclampsia or IUGR have been shown at the end of the first trimester [47]. However, at this early stage of pregnancy the placenta is not fully developed and high PI and presence of diastolic notching could be physiological. After 18-20 weeks of gestation, when the placenta is fully developed, the remodeling of the maternal spiral arteries have been completed and the resistance in the uterine arteries are lower - clinically indicated by lower PI. Persistent high PI and notching is therefore considered a pathological sign after this time point in pregnancy. In the present study there was a significant difference regarding when in the pregnancy the uterine artery Doppler ultrasound values were obtained, earlier for the controls (mean gestation of 12.4 weeks) than for the preeclampsia group (mean gestation of 18.5 weeks). Since uterine artery resistance is known to decrease progressively during pregnancy transformation of the values to MoM-values was needed. In a perfect setting the cohort would have enough controls to make a normal PI median-values for each week of gestation. With a mean PI MoM of 0.95 in the control group it seems fair to use these previously published normal values to normalize our PI values. A disadvantage of using Doppler examination is that it requires expensive equipment and trained personnel. A biomarker model in combination with maternal characteristics would therefore be preferable for implementation in developing countries.

Several studies indicate differences in disease mechanisms for early- and late onset preeclampsia [21, 37, 39, 48]. To study the role of cell-free fetal hemoglobin and its toxicity in relation to onset of clinical manifestations, the cohort was subdivided into early-, late- and term onset preeclampsia. All the preeclampsia-groups showed significantly increased serum

levels of cell-free fetal hemoglobin. The concentration was highest in the early onset preeclampsia group. We have previously suggested that α_1-microglobulin concentrations rise as a response to increased cell-free fetal hemoglobin levels [22, 24], which is supported by the current findings for late- and term onset preeclampsia (Table 4) [22, 24]. A higher concentration of cell-free fetal hemoglobin was seen in early onset- compared to late onset preeclampsia. This suggests that a consumption of the hemoglobin- and heme-scavenging proteins takes place and may explain the lower levels of hemopexin in the early onset group where HbF is highest (Table 4). Most prediction algorithms that are based on placental and angiogenic markers such as pregnancy associated plasma protein A, placental growth factor and sFlt-1, mostly in combination with uterine artery Doppler ultrasound, primarily predict early onset preeclampsia [21, 43, 48-51]. Contrary to this, the set of biomarkers presented in the present study show potential to also predict late onset preeclampsia, which may have important clinical implications.

The etiological transition from stage 1 to 2 is probably multifactorial. It has often been suggested that several different pathologies could lead to the clinical manifestations that define preeclampsia, hypertension and proteinuria. Generally, early onset preeclampsia more often presents with placenta pathology and IUGR whereas late onset preeclampsia is more dependent on maternal constitutional factors. Assuming that overproduction of cell-free fetal hemoglobin occurs in both types of preeclampsia, it is more likely that an early onset preeclampsia will deplete the protective scavenger systems early in pregnancy, *i.e.* presenting with lower concentrations of scavengers as shown for hemopexin in the early onset preeclampsia group (Table 4).

It was however not possible to predict all preeclampsia patients with the current set of biomarkers. As a consequence it can be assumed that not all preeclamptic patients have a defect placental hematopoiesis. An ideal algorithm for the prediction of preeclampsia should therefore contain biochemical markers that reflect several types of pathology (placental or maternal) and maybe combined with biophysical markers that reflect different parts of the pathophysiological cascades seen in preeclampsia [21, 23].

Conclusions:

Cell-free fetal hemoglobin and α_1-microglobulin concentrations are elevated in maternal serum at the end of first trimester in patients who subsequently develop preeclampsia.

Maternal serum levels of cell-free fetal hemoglobin, α_1-microglobulin and hemopexin are potential predictive biomarkers for subsequent development of early- and late-onset preeclampsia. Furthermore, combining them with uterine artery Doppler ultrasound and/or maternal characteristics increased the sensitivity and specificity.

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	Control n=347	Preeclampsia n=86
Ethnic origin		
Caucasian (304)	252	41
South Asian (70)	36	18
Black (54)	20	21
East Asian (4)	3	0
Mixed (19)	13	2
Not known (38)	23	4
		p<0.000001*
Gravidae	1.46 (1.36-1.56)	2.73 (2.32-3.14)
		p<0.0001
Para	0.11 (0.06-0.16)	1.14 (0.78-1.14)
(Mean -95%CI)		p<0.0001
Body Mass Index	23.4 (22.9-23.9)	26.9 (25.5-28.35)
		p<0.0001
GA at ultrasound scanning	12.5 (12.4-12.6)	18.5 (17.5-19.5)
(Mean -95%CI)		p<0.0001
GA at blood sampling (Mean – 95%CI)	13.5 (13.3-13.8)	13.9(13.3-14.6)
		p=0.17 **NS**
Fetal gender		
Male	185	49
Female	161	36
		p=0.44 **NS***

Birth weight	3467 (3415-3520)	2716 (2485-2947)
		p<0.0001
Prematurity (%)	0%	28 (33%)
		p<0.0001
Mean GA at delivery	40.4 (40.3-40.5)	36.7 (35.7-37.8)
		p<0.0001
Diabetes		
Yes	0	3
No	346	83

Table 1 The maternal characteristics from in the different subgroups. P-values were calculated with one-way ANOVA except * which were calculated with Pearson's Chi-square. They were all calculated as compared to the control group.

GA = gestational age. BMI = body mass index. NS = not significant.

Biomarker	Controls N=347 (95%CI)	Preeclampsia N=86 (95%CI)
Cell-free fetal hemoglobin (µg/ml)	5.6 (4.2-7.4)	10.8 (5.2-16.5) p=0.02
α_1-microglobulin (µg /ml)	15.5 (14.9-16.1)	17.3 (15.5-19.2) p=0.03
Total cell-free hemoglobin (µg /ml)	297 (257-337)	258 (160-358) p=0.47 **NS**
Haptoglobin (µg /ml)	971 (915-1028)	1102 (991-1131) p=0.089 **NS**
Hemopexin (µg /ml)	1143 (1111-1175)	1062 (992-1132) p=0.05
Uterine artery Doppler ultrasound PI (MoM)	0.95 (0.92-0.99)	1.18 (1.04-1.31) p<0.0001

Table 2 Mean concentrations with 95% confidence interval of the biochemical markers cell-free fetal hemoglobin, α_1-microglobulin, total cell-free hemoglobin, haptoglobin, hemopexin

and Uterine artery Doppler ultrasound Pulsatility Index *Multiples of the Median* (MoM). P-values were calculated with one-way ANOVA as compared to the control group.

Prediction model	AUC (95% CI)	95%	90%	Optimal sensitivity	PPV	NPV
Cell-free fetal hemoglobin*	0.65 (0.58-0.71)	13%	15%	60%/65%	39%	81%
α_1-microglobulin *	0.58 (0.5-0.66)	7%	19%	57%/54%	26%	80%
Haptoglobin £	0.58 (0.5-0.66)	9%	17%	53%/62%	32%	81%
Hemopexin*	0.58 (0.5-0.66)	9%	17%	40%/72%	32%	81%
Uterine artery Doppler ultrasound PI MoM	0.60 (0.52-0.68)	18%	25%	48%/73%	47%	82%
HbF* + A1M* + Hp* + Hpx*	0.73 (0.66-0.8)	22%	33%	66%/78%	53%	83%
Maternal characteristics *§	0.85 (0.8-0.9)	52%	60%	73%/77%	73%	89%
UtAD* + Maternal characteristics*	0.82 (0.75-0.89)	51%	57%	78%/73%	72%	89%
UtAD* +	0.76	26%	42%	61%/76%	40%	80%

Biomarkers*	(0.68-0.83)					
Biomarkers + Maternal characteristics *¶	0.83 (0.75-0.91)	60%	62%	81%/74%	75%	91%
Biomarkers + Maternal characteristics + UtAD	0.79 (0.71-0.87)	47%	53%	71%/81%	70%	88%

Table 3 Sensitivities for the diagnosis *preeclampsia* at different specificity levels for each of the different biomarkers, the Uterine artery Doppler (UtAD) Pulsatility (PI) Multiples of the median (MoM) values, and the maternal characteristics. All prediction values are derived from Receiver operation curves (ROC-curves) based on stepwise logistic regression models.

AUC = area under the ROC-curve (see also figure 1 and 2).

PPV: Positive predictive value at 95% specificity

NPV: Negative predictive value at 95%specificity

£: p=0.089.

*: $p \leq 0.05$.

§: Maternal characteristics consisting of the parameters *Para, Diabetic, Pre-pregnancy hypertension* and *Body mass index (BMI)*.

¶: The final model consists of the parameters *Para, Diabetic, Pre-pregnancy hypertension, Hemopexin* and α_1-microglobulin . Other parameters were not significant in the collected model.

Biomarker	Controls N=346 (95%CI)	Early onset preeclampsia N=16 (95%CI)	Late onset preeclampsia N=64 (95%CI)	Term preeclampsia N= 58 (95%CI)
Cell-free fetal hemoglobin (µg/ml)	5.6 (4.2-7.4)	13.7 (-6.8-34.2) p=0.05	10.1 (4.8-15.4) p=0.04	11.3 (5-17.7) p=0.016
α_1-microglobulin (µg /ml)	15.5 (14.9-16.1)	15.4 (12.5-18.4) p=0.98 **NS**	17.8 (15.6-20) p=0.01	18.4 (15.9-21) p=0.003
Total cell-free hemoglobin (µg /ml)	297 (257-337)	154 (67-241) P=0.23 **NS**	280 (162-399) p=0.78 **NS**	313 (169-457) p=0.8 **NS**
Haptoglobin (µg /ml)	971 (915-1028)	1108 (673-1542) P=0.43 **NS**	1101 (943-1258) p=0.12 **NS**	1120 (927-1313) p=0.097 **NS**
Hemopexin (µg /ml)	1143 (1111-1175)	947 (757-1137) p=0.04	1085 (1009-1162) p=0.22 **NS**	1084 (997-1170) p=0.25 **NS**
Uterine artery Doppler ultrasound PI MoM	0.95 (0.92-0.99)	1.63 (1.2-2.06) p<0.00001	1.06 (0.94-1.19) p=0.06	1.0 (0.89-1.1) p=0.35 **NS**

Table 4 Mean concentrations of biomarkers in the sub-groups early onset preeclampsia (def.: delivery ≤ 34+0 weeks of gestation) and late onset preeclampsia (def.: delivery > 34+0 weeks of gestation) and term preeclampsia (def.: delivery ≥37+0). P-values were calculated with one-way ANOVA as compared to the control group.

NS: not statistically significant

Biomarker	Early onset (N=16) AUC (95% CI)	90%	80%	Late onset (N=64) AUC	90%	80%	Term PE (N=58) AUC	90%	80%
Cell-free fetal hemoglobin	0.60 £ (0.46-0.74)	6%	36%	0.66* (0.59-0.73)	23%	47%	0.68* (0.60-0.75)	19%	40%
α_1-microglobulin	**NS**			0.59* (0.51-0.68)	24%	33%	0.62* (0.52-0.71)	25%	28%
Hemopexin	0.70* (0.53-0.86)	32%	56%	**NS**			**NS**		
Uterine artery Doppler ultrasound PI MoM	0.78* (0.62-0.94)	57%	71%	0.55* (0.47-0.64)	18%	32%	**NS**		

Table 5 Sensitivities at different specificities for each of the significant biomarkers in the three subgroups early onset preeclampsia, late onset preeclampsia, and term preeclampsia. All values are derived from Receiver operation curves (ROC-curves) based on separate logistic regression models. AUC = area under the ROC-curve. None of the biomarkers were significant in combination with each other or with maternal characteristics in either of the groups.

£: $p = 0.14$.

*: $p \leq 0.05$.

Figure:

Figure 1

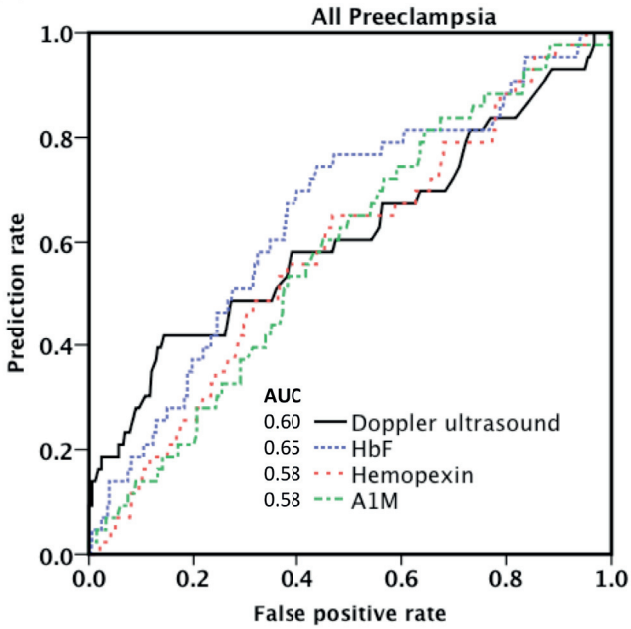

Figure 1: Receiver operation curves of cell-free fetal hemoglobin, α_1-microglobulin, haptoglobin and Doppler ultrasound PI as predictive biomarkers of all preeclampsia cases. All biomarkers are presented with area under the ROC-curve (AUC). Specific values are found in Table 3.

Figure 2

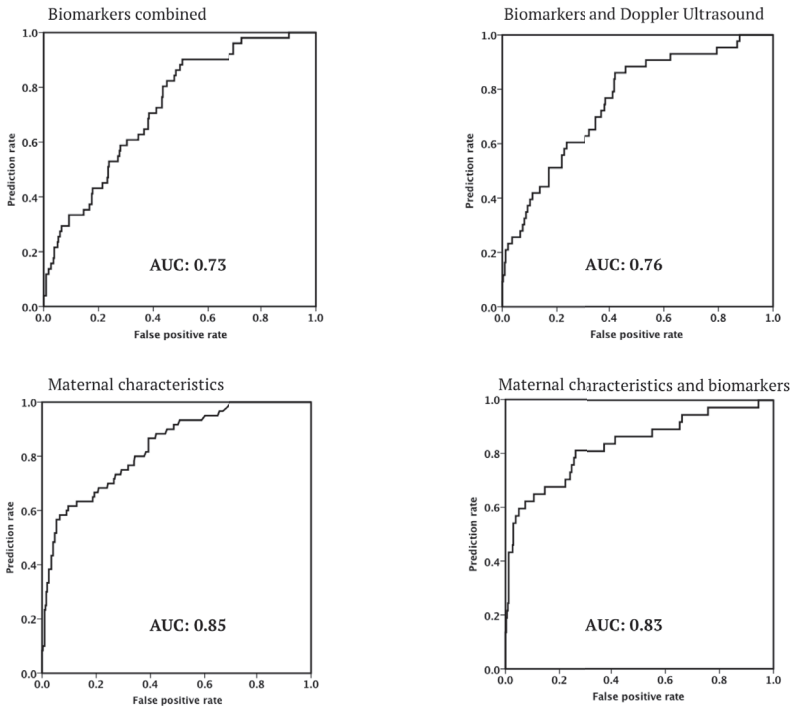

Figure 2: Receiver operation curves of the maternal characteristics, the combination of the biomarkers, biomarkers combined with Doppler ultrasound and maternal characteristics combined with biomarkers. All biomarkers are presented with area under the ROC-curve (AUC). Specific values are found in Table 3.

Paper III

The human endogenous protection system against cell-free hemoglobin and heme is overwhelmed in preeclampsia and provides potential biomarkers and clinical indicators

Magnus Gram[1*¶], Ulrik Dolberg Anderson[2¶], Maria E. Johansson[1], Anneli Edström-Hägerwall[1], Irene Larsson[2], Maya Jälmby[2], Stefan R. Hansson[2&] and Bo Åkerström[1&]

[1]Lund University, Department of Clinical Sciences Lund, Infection Medicine, Lund, Sweden

[2]Lund University, Department of Clinical Sciences Lund, Obstetrics and Gynecology, Lund, Sweden

[*]Corresponding author

Dr. Magnus Gram (MG)

E-mail: magnus.gram@med.lu.se

[¶]These authors contributed equally to this work.

[&]These authors also contributed equally to this work.

Abstract

Preeclampsia (PE) complicates 3-8% of all pregnancies and manifests clinically as hypertension and proteinuria in the second half of gestation. The pathogenesis of PE is not fully understood but recent studies have described the involvement of cell-free fetal hemoglobin (HbF)**.** Hypothesizing that PE is associated with prolonged hemolysis we have studied the response of the cell-free Hb- and heme defense network. Thus, we have investigated the levels of cell-free HbF (both free, denoted HbF, and in complex with Hp, denoted Hp-HbF) as well as the major human endogenous Hb- and heme-scavenging systems: haptoglobin (Hp), hemopexin (Hpx), α_1-microglobulin (A1M) and CD163 in plasma of PE women (n=98) and women with normal pregnancies (n=47) at term. A significant increase of the mean plasma HbF concentration was observed in women with PE. Plasma levels of Hp and Hpx were statistically significantly reduced, whereas the level of the extravascular heme- and radical scavenger A1M was significantly increased in plasma of women with PE. The Hpx levels significantly correlated with maternal blood pressure. Furthermore, HbF and the related scavenger proteins displayed a potential to be used as clinical biomarkers for more precise diagnosis of PE and are candidates as predictors of identifying pregnancies with increased risk of obstetrical complications. The results support that PE pathophysiology is associated with increased HbF-concentrations and an activation of the physiological Hb-heme defense systems.

Introduction

Preeclampsia (PE) complicates 3-8% of all pregnancies and manifests clinically in the second half of gestation [1]. The classical findings that define PE are hypertension and proteinuria appearing after 20 weeks of gestation. PE is a potentially serious condition that in worst case can lead to eclampsia, characterized by general seizures and coma [2-4]. A related disease, the HELLP syndrome, (hemolysis, elevated liver enzymes and low platelets count) develops more rapidly and is accompanied with maternal hemolysis. Uniform classification of the different forms of hypertensive conditions during pregnancy is important in order to optimize patient management. To date several biomarkers have been suggested for screening in the first and second trimester, however none are yet recommended for screening in clinical practice [5]. Several biomarkers have also been suggested to support clinicians in their diagnostics and handling of the patients at term pregnancy [6-8].

The pathogenesis of PE is not fully understood but recent studies have described that extracellular fetal hemoglobin (HbF) is involved [9,10]. Using genomics and proteomics, Centlow et al showed an up-regulated gene expression of HbF and accumulation of cell-free HbF in the vascular lumen of term PE placentas [11]. May et al later showed, in the *ex vivo* human placenta perfusion system, that perfusion with cell-free hemoglobin (Hb) causes tissue damage and leakage of Hb over the placental barrier [12]. It was hypothesized that through the generation of reactive oxygen species (ROS), Hb induces oxidative damage to the placenta and a subsequent leakage over the blood-placental barrier [12]. In fact, Olsson et al [13] demonstrated that pregnant women diagnosed with PE have increased plasma levels of cell-free HbF and adult hemoglobin (HbA) at term and Anderson et al demonstrated that the serum levels of HbF were elevated already in the first trimester of pregnant women that later developed PE [14]. Furthermore, in term pregnancies the plasma concentration of cell-free

total Hb (HbF + HbA) was shown to correlate with blood pressure, *i.e.* the severity of the disease [13].

Hemoglobin is a tetramer consisting of four globin subunits each carrying a heme-group in its active center [15]. In adults the most common Hb isoform is HbA that consists of two α- and two β-subunits ($\alpha_2\beta_2$). In the fetus, the HbF isoform is the predominant type and consists of two α-chains and two γ-chains ($\alpha_2\gamma_2$). Heme consists of an organic ring-structure, protoporphyrin IX, which chelates a ferrous (Fe^{2+}) iron atom with high affinity for free oxygen (O_2). Ferrous Hb binding to O_2 is denoted oxyHb. Autoxidation of oxyHb is a spontaneous intramolecular redox reaction eventually leading to production of ferric (Fe^{3+}) Hb (metHb), ferryl (Fe^{4+}) Hb, free heme and various ROS [16,17]. These compounds are chemically very reactive and have the potential to induce tissue damage and cell destruction by one-electron redox reactions with biomolecules. As described above, it was hypothesized that the increased concentrations of HbF in PE causes oxidative damage to the placenta and a subsequent leakage over the feto-maternal barrier into the maternal circulation [12-14]. As a consequence, the vascular endothelium is damaged and eventually glomerular endotheliosis are developed, a pathognomonic kidney damage that occurs in PE. This damage eventually contributes to development of hypertension and proteinuria, the clinical hallmarks of PE.

Hb is normally found enclosed by the erythrocyte membranes. The autoxidation of intracellular oxyHb and downstream free radical formation is prevented mainly by superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) [18,19]. However, significant amounts of Hb escape from the erythrocytes under healthy conditions and massive amounts can be released during pathological conditions involving hemolysis, causing severe organ

damage. Therefore a number of defense mechanisms have evolved both in plasma and in the extravascular compartments to counteract the damage caused by cell-free Hb.

Haptoglobin (Hp) is perhaps the most well investigated Hb-clearing molecule. It binds cell-free Hb in plasma [20,21] and the resulting Hp-Hb complex is cleared from blood via binding to the macrophage receptor CD163 [22]. The Hp molecule consists of two chains, α and β, and two allelic variants of the α-chains exist, $\alpha 1$ and $\alpha 2$. As a result, three phenotypic variants occur in the human population, Hp 1-1, Hp 2-2, and the allelic mixture, called Hp 1-2. Free heme in blood is sequestered by hemopexin (Hpx) [23,24] and the Hpx-heme complex is cleared from the circulation by the hepatocyte receptor CD91 [25]. In the extravascular compartment, cellular heme oxygenase (HO) is the most essential heme degrading protein, converting heme to free iron, biliverdin and CO [26,27]. Furthermore, the plasma- and extravascular reductase and heme- and radical scavenger α_1-microglobulin (A1M) binds and degrades free heme and can reduce metHb [28-30]. A1M also acts as an antioxidant by reducing and covalently binding the downstream ROS and radicals generated by cell-free Hb [31-34]

In this study we have employed PE as a model disease to study the response of the cell-free Hb-defense network in a pathological situation with prolonged hemolysis. Thus, we have investigated the levels of cell-free HbF (both free, denoted HbF, and in complex with Hp, denoted Hp-HbF) as well as the concentrations of the major human endogenous Hb-scavenging systems: Hp, Hpx, A1M and CD163. The results confirm that PE is associated with increased HbF-concentrations and an activation of the physiological Hb-heme defense systems. The results also suggest that the components of the Hb-Hp-Hpx-A1M network may be employed as diagnostic biomarkers and are potential clinical tools for PE and perinatal pregnancy outcome, individually or in various combinations.

Materials and Methods

Patients and demographics

In an on-going prospective Swedish cohort study, women diagnosed with PE, collected 2003 - 2011, and matched normal pregnancies (controls), collected during the same period, were retrospectively selected from our biobank. In total, 150 pregnant women were included in the study. Exclusion criteria were gestational hypertension, essential hypertension and gestational diabetes. In total 5 cases were excluded and are therefore not included in any of the Tables and Figures. Out of the 145 remaining patients, 98 had PE (cases) and 47 were normal pregnancies (controls). Patient demographics are described in Table 1 and 2.

Sample collection

Patient sampling was performed following written consent and the study was approved by the ethical committee review board for human studies in Malmö/Lund, Sweden. Maternal venous samples were taken prior to delivery (during the last 24 hours of pregnancy) at the Department of Obstetrics and Gynecology, Lund University Hospital, Sweden. Six-ml blood samples were collected into EDTA Vacuette® plasma tubes (Greiner Bio-One GmbH, Kremsmünster, Austria) and centrifuged at 2000 xg for 20 minutes at room temperature (RT). The plasma was then transferred into cryo tubes and stored in -80°C until time of analysis.

Preeclampsia was defined as *de novo* hypertension and proteinuria after 20 weeks of gestation with 2 readings at least 4 hours apart of blood pressure ≥140/90 mmHg and proteinuria ≥300 mg per 24 hours[35]. For quantification of proteinuria dipstick analysis was accepted if no other quantification was made. The PE group was further sub-classified as early-onset PE (diagnosis ≤ 34+0 weeks of gestation, n=22) or late onset PE (diagnosis >34+0 weeks of

gestation, n=74). There were 2 cases of PE with unknown time of diagnosis, and therefore not included in the sub-analyses. The pregnancy outcome was retrospectively obtained from the patient charts.

Reagents and proteins

HbF was purified from whole blood, freshly drawn from umbilical cord blood, as previously described [17]. Human γ-chains were prepared by dissociation of purified HbF with p-mercuribenzoate (Sigma-Aldrich, St-Louis, MO, USA) and acidic precipitation as described by Kajita et al [36] with modifications by Noble [37]. The absolute purity of HbF (from contamination with HbA) and of γ-chains (from contamination with α- and β-chains) was determined as previously described [13]. Human Hp-HbF was prepared by mixing human Hp (1-1; Sigma-Aldrich) with HbF in a 1:1 ratio, and purifying the complex from free Hp and HbF by FPLC size exclusion chromatography. Mouse monoclonal antibodies and rabbit polyclonal antibodies against HbF were prepared by AgriSera AB (Vännäs, Sweden) by immunization with human γ-chains. From the polyclonal antisera, HbF-specific antibodies were purified by immunoglobulin purification (by protein A-Sepharose chromatography, Sigma) followed by HbF-affinity chromatography and removal of unspecific HbA antibodies (by absorbing on an HbA-affinity chromatography). Rabbit anti-Hb IgG were purchased from DAKO (Glostrup, Denmark) and further purified by HbA-affinity chromatography. Antibodies (monoclonal and rabbit polyclonal IgG used as detection antibodies) were conjugated with horseradish peroxidase (Lightning-Link HRP, Innova Biosciences, Cambridge, UK) according to the manufacturer's instruction. Human A1M was purified from urine as described by Åkerström et al [38]. Goat polyclonal antibodies against human A1M and goat anti-rabbit immunoglobulin were prepared as previously described [39].

Fetal hemoglobin (HbF)-concentrations

A sandwich-ELISA was used for quantification of uncomplexed HbF in plasma. Ninety six-well microtiter plates were coated with anti-HbF antibodies (mouse monoclonal (no. 85); 4µg/ml in PBS) overnight at RT. In the second step, wells were blocked for 2 hours using blocking buffer (1% BSA in PBS), followed by an incubation with HbF calibrator or the patient samples for 2 hours at RT. In the third step, HRP-conjugated anti-HbF antibodies (mouse monoclonal (no. 417); diluted 1:5000), were added and incubated for 2 hours at RT. Finally, a ready-to-use 3,3′,5,5′-Tetramethylbenzidine (TMB, Life Technologies, Stockholm, Sweden) substrate solution was added. The reaction was stopped after 20 minutes using 1.0 M HCl and the absorbance was read at 450nm using a Wallac 1420 Multilabel Counter (Perkin Elmer Life Sciences, Waltham, MA, USA).

Haptoglobin-fetal hemoglobin (Hp-HbF) concentrations

A sandwich-ELISA used for quantification of Hp-HbF was developed and displayed a high preference for Hp-HbF compared to uncomplexed HbF (>10x higher recovery of a Hp-HbF calibrator series compared to a HbF calibrator series with the same molar content of HbF). No cross-reactivity was observed with Hp or HbA. Ninety six-well microtiter plates were coated with anti-HbF antibodies (HbF-affinity purified rabbit polyclonal ("Bonita"); 4µg/ml in PBS) overnight at RT. In the second step, wells were blocked for 2 hours using blocking buffer (1% BSA in PBS), followed by an incubation with Hp-HbF calibrator or the patient samples for 2 hours at RT. In the third step, HRP-conjugated anti-Hb antibodies (HbA-affinity purified rabbit polyclonal; DAKO; diluted 1:5000), were added and incubated for 2 hours at RT. Finally, a ready-to-use TMB (Life Technologies) substrate solution was added. The reaction was stopped after 30 minutes using 1.0 M HCl and the absorbance was read at 450nm using a Wallac 1420 Multilabel Counter (Perkin Elmer Life Sciences).

Total hemoglobin (Hb-Total)-concentrations

The concentration of total Hb in maternal plasma was determined using a Human Hb ELISA Quantification Kit from Genway Biotech Inc. (San Diego, CA, USA). The analysis was performed according to the manufacturer's instructions and the absorbance was read at 450nm using a Wallac 1420 Multilabel Counter.

α_1-microglobulin (A1M)-concentrations

Radiolabeling of A1M with ^{125}I (Perkin Elmer Life Sciences) was done using the chloramine T method. Protein-bound iodine was separated from free iodide by gel-chromatography on a Sephadex G-25 column (PD10, GE Healthcare, Stockholm, Sweden). A specific activity of around 0.1-0.2 MBq/µg protein was obtained. Radioimmunoassay (RIA) was performed by mixing goat antiserum against human A1M ("Halvan"; diluted 1:6000) with ^{125}I-labelled A1M (appr. 0.05 pg/ml) and unknown patient samples or calibrator A1M-concentrations. After incubating overnight at RT, antibody-bound antigen was precipitated by adding bovine serum and 15% polyethylene glycol, centrifuged at 2500 rpm for 40 minutes, after which the ^{125}I-activity of the pellets was measured in a Wallac Wizard 1470 gamma counter (Perkin Elmer Life Sciences).

Haptoglobin (Hp)-concentrations

The concentration of Hp in maternal plasma was determined using a Human Hp ELISA Quantification Kit from Genway Biotech Inc. The analysis was performed according to the manufacturer's instructions and the absorbance was read at 450nm using a Wallac 1420 Multilabel Counter.

Hemopexin (Hpx)-concentrations

The concentration of Hpx in maternal plasma was determined using a Human Hpx ELISA Kit from Genway Biotech Inc. The analysis was performed according to manufacturer's instructions and the absorbance was read at 450nm using a Wallac 1420 Multilabel Counter.

Cluster of Differentiation 163 (CD163)-concentrations

The concentration of CD163 in maternal plasma was determined using a Human CD163 Duo Set from R&D Systems (Abingdon, UK). The analysis was performed according to the manufacturer's instructions and the absorbance was read at 450nm using a Wallac 1420 Multilabel Counter.

SDS-PAGE and Western blot

SDS-PAGE was performed using precast 4-20% Mini-Protean TGX gels from Bio-Rad (Hercules, CA, USA) and run under reducing conditions using molecular weight standard (precision protein plus dual marker) from Bio-Rad. The separated proteins were transferred to polyvinylidene difluoride (PVDF) or low fluorescence (LF) PVDF membranes (Bio-Rad). The membranes were then incubated with antibodies against Hp (rabbit polyclonal, 12µg/ml, DAKO). Western blot was performed using HRP-conjugated secondary antibodies (DAKO) and the chemiluminescent substrate Clarity Western ECL (Bio-Rad). The bands were detected in a ChemiDoc XRS unit (Bio-Rad).

Statistical analysis

Statistical computer software Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) version 21 for Apple computers (Apple Inc., Cupertino, CA) and Origin 9.0 software

(OriginLab Corporation, Northampton, MA, USA) were used to analyze the data. ANOVA test was used to compare the groups for clinical parameters such as age, BMI, parity, systolic blood pressure, diastolic blood pressure, proteinuria, gestational age at delivery, birth weight, gestational age at time of sampling and APGAR score at10 minutes.

The Chi square test was used to compare the groups for fetal gender, labor induction, mode of delivery (e.g. vacuum extraction, caesarean section or vaginal delivery), need of neonatal intensive care unit (NICU) and preterm delivery.

Mean concentrations of the examined variables (henceforth referred to as biomarkers) were evaluated in women with PE compared to the control group using non-parametric statistics. A univariate logistic regression model was developed for the evaluated biomarkers. The gestational age at sampling was adjusted for in the logistic regression model. The biomarkers displaying a significant difference were further evaluated using Receiver Operational Curve (ROC-curve) by analyzing the area under the ROC-curve (AUC) as well as calculating the detection rates at different false positive levels. Parallel analysis was performed for each of the examined biomarker as well as different combinations of them. Furthermore, sub-group analysis of women with PE, *i.e.* early and late onset PE, compared to the control group was performed. The univariate logistic regression model was also used to further calculate how the biomarkers performed in terms of predicting fetal outcomes (*i.e.* admission to NICU and premature delivery and intrauterine growth restriction (IUGR)) and mode of delivery.

Correlation analysis (Pearson's correlation coefficient) between biomarkers and diastolic- and systolic blood pressure was performed. A p-value of $p \leq 0.05$ was considered statistically significant in all tests.

Results

Patient characteristics

The characteristics of the included patients are shown in Table 1 and 2. There was a significant difference in age, blood pressure, proteinuria, parity, gestational age at sampling, gestational age of delivery and birth weight between women diagnosed with PE and uncomplicated pregnancies (denoted controls). Furthermore, for parameters regarding maternal outcome (e.g. mode of delivery incl. induction and instrumental deliveries) as well as fetal outcome (e.g. admittance to NICU and prematurity) a statistically significant difference was observed between groups. A statistically significant difference in the 10 minutes APGAR score was observed between controls and early onset PE but not late onset PE. There was no statistical significant difference between the groups regarding BMI and fetal gender.

Cell-free Hb

The concentration of cell-free HbF, Hp-HbF and Hb-Total were analyzed in all plasma samples from women with PE and controls (Table 3). A 4-fold increase of the HbF concentration was seen in the PE patients (p-value 0.01) as compared to the controls. When subdividing the PE group into early and late onset PE an almost 5-fold increase in the HbF concentration was observed in the early onset PE group as compared to controls (p-value 0.006). In the late onset PE group, an almost 4-fold increase was observed as compared to controls, but this was not statistically significant (p-value 0.17). A statistically significant increase in the mean Hp-HbF concentration was observed for women with PE as compared to controls (p-value 0.018). This difference was not found when comparing early and late onset

PE separately with the control group, although a clear trend towards an increase could be seen in the early onset PE group (p-value 0.15).

No significant difference in Hb-Total concentration was observed between PE vs. controls (p-value 0.53) or between early (p-value 0.80) and late onset PE (p-value 0.73) vs. controls.

Hp and CD163

Analysis of the Hp concentration in plasma showed a statistically significant decrease in Hp concentration in plasma samples of women with PE as compared to controls (p-value<0.0001). In addition, late onset PE displayed a significant decrease as compared to the controls (p-value 0.001). In contrast, early onset PE showed a slight but not statistically significant increase in Hp concentration as compared to the controls (p-value 0.067).

Soluble, shedded CD163, the macrophage receptor mediating elimination of the Hp-Hb complex, was analyzed in plasma [40-42]. The analysis showed a small but not statistically significant (p-value 0.37) increase in the PE group as compared to the controls (Table 3). Subdividing the PE group into early and late onset PE, a small, not statistically significant, increase was observed in the late onset PE group (p-value 0.07 vs. the controls) whereas a small, not statistically significant, decrease was observed in the early onset PE group (p-value 0.35 vs. the controls).

Hpx

Analysis of the intravascular heme-scavenger protein Hpx showed a statistically significant decrease in plasma Hpx concentration of women with PE (p-value<0.0001) as compared to the controls (Table 3). Subdividing the PE group, displayed a statistically significant decrease

in both the early (p-value<0.0001) and late onset PE (p-value<0.0001) PE groups as compared to the controls.

A1M

Analysis of plasma levels of the heme- and radical scavenger A1M showed a statistically significant increase of plasma A1M concentration in women with PE (p-value 0.035) as compared to controls (Table 3). Subdividing the PE group, a statistically significant increase was observed in the late onset PE group (p-value 0.03) but not in the early onset PE group (p-value 0.26).

Correlation cell-free HbF and Hp

The correlation between plasma cell-free HbF and Hp levels was evaluated. A negative correlation was found, *i.e.* an increased plasma cell-free HbF concentration was associated with a decreased plasma Hp concentration, when including all individuals, controls and women with PE (r = -0.335, p-value<0.0001, n=145)(Fig. 1A). Strikingly, when comparing the correlation in controls (Fig. 1B) and women with PE (Fig. 1C) separately, an increased negative correlation was observed for the PE group (r =-0.437, p-value<0.0001, n=98) whilst in the control group a weak positive correlation was observed (r = 0.142, p-value 0.33, n=47). Similar correlations were observed for Hp vs. Hp-HbF and Hp vs. Hb-Total, but none of them reached statistical significance (Hp vs. Hp-HbF r=-0.05, p-value 0.52; Hp vs. Hb-Total r=0.03, p-value 0.73).

Association between Hp isoform and cell-free HbF, Hpx and A1M

We identified the predominant Hp-isoforms (1-1, 2-2, or both: 1-2) in the patient plasma samples using Western blot (Fig. 2A). As seen in Fig. 2B, a similar distribution of the different phenotypes were observed in both controls and PE, with a predominant presence of Hp 1-2 (C, 45%; PE, 41%) and 2-2 (C, 43%; PE, 44%) as compared to 1-1 (C, 12%; PE, 15%). Subdividing the PE group into early and late onset PE yielded a similar distribution 1-1 (early, 13%; late, 15%), 1-2 (early, 45%; late, 40%) and 2-2 (early, 42%; late, 45%). Furthermore, the association between the Hp-isoforms and the plasma levels of cell-free HbF and Hp-HbF were analyzed (Fig. 2C-D). A striking increase in the concentration of cell-free HbF was observed in the Hp 2-2 group of women with PE (p-value 0.03)(Fig. 2C). A smaller, but similar selective increase in the concentration of Hp-HbF was observed in the Hp 2-2 PE group as compared to controls (p-value 0.05)(Fig. 2D). No significant association was observed between Hb-Total, Hp, CD163, Hpx and A1M to any of the Hp isoform.

Correlation analysis between biomarkers and blood pressure

Correlation analysis using Pearson's correlation coefficient showed statistically significant inverse correlation between Hpx and blood pressure, both systolic (r=-0.511, p-value<0.00001, n=145) and diastolic (r=-0,520, p-value<0.00001, n=145)(Fig. 3). Furthermore, correlation analysis of PE patients only displayed a slight but not statistically significant inverse correlation between Hpx and blood pressure (systolic, r=-0,123, p-value 0.22, n=98; diastolic, r=-0,058, p-value 0.57, n=98). No statistical significant correlation was observed for any of the other biomarkers in relation to the blood pressure.

Evaluation of biomarkers as diagnostic markers of PE

A logistic regression model was used to evaluate the usefulness of the described biomarkers as diagnostic markers of PE. By comparing women with PE vs. controls, a significant difference was detected for HbF (p-value 0.02), A1M (p-value 0.008) and Hpx (p-value<0.0001) but not for Hp (p-value 0.21) and CD163 (p-value 0.42). Each of the significantly altered biomarkers were able to diagnose PE (adjusted for gestational age) but Hpx showed the highest level of significance and a diagnostic detection rate of 64% at a false positive rate of 5% with an AUC of 0.87 (Table 4, Fig. 4C). The combination of Hpx, A1M and HbF was not statistically significant (p-value for HbF 0.08) but displayed a diagnostic detection rate of 69% at a false positive rate of 5% with an AUC of 0.88 (Table 4, Fig. 4A). The combination Hpx and A1M was statistically significant (p-value 0.05 for both biomarkers) and showed a diagnostic detection rate of 66% at a false positive rate of 5% and an AUC of 0.87 (Table 4, Fig. 4B).

Correlation with fetal and maternal outcomes

We further evaluated whether the biomarkers correlated with fetal and maternal outcomes. A logistic regression model was used and the fetal outcome variables were: admission to NICU, presence of IUGR and premature birth. The maternal outcome variables were induction of labor, delivery by cesarean section and instrumental deliveries. The biomarkers HbF (p-value 0.001), Hpx (p-value 0.008) and Hp (p-value 0.03) each showed a association with "admission to NICU". However, in a combined logistic regression model they were not statistically significant. The biomarkers Hpx (p-value 0.0003, AUC=0.71) and CD163 (p-value 0.03, AUC=0.61) showed a association with premature delivery. Furthermore, the combination of Hpx and CD163 also displayed a statistically significant correlation with premature delivery (p-value 0.001 and p-value 0.025, AUC 0.72).

Hpx displayed a statistically significant association with the risk of cesarean section (p-value 0.009, AUC 0.62). No further correlation was found between the evaluated biomarkers and maternal outcomes.

Discussion

In this study cell-free HbF and the endogenous Hb- and heme-scavenger systems were characterized in pregnant women diagnosed with PE and normal pregnancies. Congruent with previous results, a significant increase of HbF was observed in women with PE in term pregnancies [13]. Furthermore, plasma levels of the Hb- and heme scavenger systems Hp and Hpx were statistically significantly reduced, suggesting an increased consumption. In line with previously published studies [13,14] the extravascular heme- and radical scavenger A1M was significantly increased in plasma of women with PE. The diagnostic and clinical utility of the investigated biomarkers was also evaluated and a clear potential in using these as clinical tools for diagnosing women with PE and predicting the obstetrical outcomes was found. The findings of this paper and a possible chain of events involved in the development of PE are discussed in details below and summarized in Fig. 5.

Hemolysis and the subsequent release of cell-free Hb and heme occur in a wide range of clinical conditions and diseases, such as hemorrhage, transfusion reactions, malaria, and sickle cell disease. The release of cell-free Hb and heme causes a range of pathophysiological effects where hemodynamic instability and tissue injury constitutes the major insults [43,44]. Immediate effects include scavenging of the potent vasodilator nitric oxide (NO) that leads to increased arterial blood pressure [45,46]. Furthermore, cell-free Hb and free heme have been described to be accumulated and compartmentalized within the vascular wall causing subsequent organ failure of the kidneys [47,48]. Long-term exposure to cell-free Hb and heme has been described to be associated with NO depletion, inflammation and oxidative stress [44,45]. Thus, inadequate scavenging and protection against extracellular Hb and its metabolites during pregnancy may cause fundamental damage to the vascular bed. In fact, the long-term effects of PE are increased risk of cardiovascular disease and stroke later in life

[49,50]. In a series of recent publications the importance of cell-free HbF and its downstream metabolites free heme and ROS, in the development of PE-related damage and symptoms, have been described [12,47,51,52]. By using the dual placenta perfusion system, May et al [12] described placental damaging effects following exposure to cell-free Hb. Hb caused a significant increase in perfusion pressure and damage to the blood-placenta barrier followed by leakage of extracellular Hb into the maternal circulation. Electron microscopy displayed morphological changes similar to what is seen in placentas of women with PE [53]. In the pregnant ewe PE-model, a starvation-induced hemolysis model, increased amount of extracellular heme, bilirubin and ROS in the blood as well as damage to the placenta and kidneys has been shown [51,54,55].

In order to protect ourselves against extracellular Hb and free heme, humans have evolved several Hb- and heme-detoxification systems. Previous studies have shown that the plasma levels of Hp are decreased in PE pregnancies [13,56]. If Hp becomes depleted, as a consequence of consumption due to high levels of cell-free Hb or prolonged exposure time to cell-free Hb, the uncleared oxyHb will undergo auto-oxidation reactions resulting in the formation of metHb, free heme and ROS. Furthermore, the non-Hp bound Hb will be accumulated within organs, predominantly the kidneys, where it causes damage. In renal tissues, the glomeruli are affected, subsequently leading to leakage of proteins into the urine [57]. Upon release of heme into the blood stream, Hpx, a highly specific and abundant heme-scavenger protein that protects blood and endothelial cells against heme-induced damage, binds the heme and forms an Hpx-heme complex that is cleared by macrophages, hepatocytes, neurons and syncytiotrophoblasts expressing the CD91 receptor. Heme is subsequently internalized by endocytosis of the receptor /Hpx-heme unit and heme is degraded by HO-1 [23,24]. HO-1 is a cytosolic enzyme that participate in heme-detoxification by binding and

degrading the free heme-group [26] and the expression of HO-1 is induced by a variety of conditions of environmental stress [58,59]. It operates in concert with microsomal NADPH-cytochrome P450 reductase to convert heme to biliverdin, CO and Fe^{2+}, utilizing three molecules each of O_2 and NADPH for each molecule of heme [60-62]. The products of HO-activity have important beneficial physiological activities providing further antioxidation effects besides the mere elimination of heme. Biliverdin is reduced to bilirubin by biliverdin reductase [63] and bilirubin is a powerful physiological antioxidant [64]. CO has been reported to have both pro-oxidant and antioxidant effects, mostly as a result of binding to heme-proteins, replacing molecular O_2 (reviewed in [65]). Recently, the importance of HO-1 and CO in sustaining pregnancy was reported. Indeed, CO was suggested as a possible therapy for the treatment of PE [66]. This could be explained by the finding that CO induces vasodilation by binding to the heme-protein guanylyl cyclase [67]. In this paper, we have focused on the extracellular Hb-protection proteins Hp, Hpx and A1M.

Studies of sickle cell anemia patients have reported decreased levels of Hpx following hemolysis [68]. Here we observed a statistically significant decrease of both the Hp and Hpx in maternal plasma of women with PE as compared to normal pregnancies, suggesting a prolonged presence of increased levels of both cell-free Hb and heme. This is in line with the previous study by Anderson et al [14], reporting increased serum levels of cell-free Hb as early as the first trimester in women that later developed PE. Interestingly, the Hp levels in women with late onset PE were considerably lower than in women with early onset PE. In addition, some PE patients displayed a significant increase in cell-free HbF (non Hp-bound), and these high levels were only found in women with the Hp 2-2 isoform (Fig. 2C). Thus, this sub-group of PE patients may have a reduced innate defense system against cell-free Hb and

may constitute a high-risk group. In fact, a majority developed a severe condition of PE (3 out of 7) or had early onset PE (4 out of 7).

Due to scavenging of HbF by Hp it is expected that the maternal plasma samples contain two major forms of HbF: cell-free, non Hp-bound HbF and Hp-HbF. In order to target these, two different ELISA assays for separate quantification of the two molecular species were developed. The results showed significantly increased concentrations of both free HbF and Hp-HbF complex in women with PE but the magnitude of the increase was much less for Hp-HbF than free HbF. As described above, once cell-free HbF appears in blood it is rapidly scavenged by Hp and the Hp-HbF complex is quickly cleared from the blood by internalization in CD163-bearing cells. Therefore, a possible explanation for the small differences in Hp-HbF complex concentrations between the PE and control groups may be that the high turnover-rate of the Hp-HbF complex tends to counteract accumulation of the complex in PE and thus obscures the differences.

Hpx concentration was shown to have a significant negative correlation to the blood pressure (Fig. 3). It could be speculated that this is also correlated to the severity of the disease, although no statistical significance was seen between Hpx and blood pressure when separating PE patients from controls. Previous studies have shown that enzymatically active Hpx can affect the renin-angiotensin system (RAS) in *in vitro* by downregulating the vascular angiotensin II receptor (AT(1)) and promoting an expanded vascular bed [69,70]. It could be speculated that increased heme levels, resulting from elevated levels of cell-free HbF, in women with PE leads to a consumption of Hpx and consequently a reduced Hpx activity, resulting in an enhanced AT(1) receptor expression and a contracted vascular bed. In fact, Bakker et al [71] showed that plasma from women with PE had an increased AT(1) receptor

expression on monocytes as compared with plasma from normal pregnancies. This, together with NO consumption, may be important blood pressure regulating effects caused by elevated extracellular HbF observed in PE.

We have previously shown that the radical scavenger A1M binds and degrades heme [28,29,72]. Addition of the protein protects cells and tissues against oxidative insult, structural- and functional damage and prevents cell death [12,32,73,74]. In line with previous studies [13,14] the A1M plasma concentration was shown to be significantly increased in women with PE. This increase was statistically significant in women with late onset PE, but not in women with early onset PE.

Why are the A1M-levels increased while the Hp- and Hpx-levels are decreased in the PE patients? Several reports describe that the A1M gene expression is rapidly upregulated in the liver, skin, placenta and other organs as a response to increased levels of Hb, heme and ROS [12,13,74]. This will lead to increased secretion of the protein resulting in increased plasma concentrations in pathological situations with increased Hb and ROS loads [57]. Furthermore, no specific receptor-mediated clearance system of A1M has been shown to be triggered during hemolysis or oxidative stress, whereas Hp and Hpx are cleared from plasma upon binding to Hb and heme [24,25]. As a result, the concentrations of A1M in plasma and extravascular fluids will increase, while Hp and Hpx will be exhausted and hence their plasma concentrations will decrease.

There is an increased attention towards the use of biomarkers in clinical prediction and diagnosis of PE [35,75,76]. Several biomarkers have been suggested but so far, but no available guidelines recommend the use of biomarkers in clinical screening programs [6-8]. Recently, the American College of Obstetricians and Gynecologists (ACOG) suggested that

the definition of severe PE should replace proteinuria by the use of biomarkers; thrombocytes (<100,000/microliter), serum creatinine (>1.1 mg/dl) and liver transaminases (twice the normal concentration) [76]. In this study, we present data suggesting that HbF, Hpx and A1M can be used as clinical biomarkers in supporting the diagnosis of PE. The combination of HbF, Hpx and A1M displayed the highest correlation to diagnosis (detection rate of 69% at 5% false positives, AUC = 0.88, Fig. 4A) and the combination of Hpx and A1M also displayed a high detection rate (66% at 5% false positive, AUC=0.87, Fig. 4B). Thus, HbF, Hpx and A1M constitute possible future markers that could support the diagnosis of PE.

Being able to predict fetal and maternal outcomes is of great clinical value as it can help clinicians in the difficult task to optimize timing of delivery and mobilize neonatal resources. In this study the correlation between investigated biomarkers and a range of maternal and fetal outcomes were evaluated. The results indicated that HbF, Hp and Hpx correlated with admission to NICU. Furthermore, Hpx was strongly associated to premature birth. However, since all prematurity in this cohort was associated with PE this strong association could be as result of the strong correlation between Hpx and PE rather than prematurity itself.
It is of importance to note that the cohort used in this case-control study contains an over - representation of women with PE. Consequently, detection and prediction rates reported in this study will most likely be different than in a normally distributed cohort, containing 3-8% of PE cases. Studies on such normally distributed, and larger, cohorts have been initiated.

In summary, we have characterized cell-free HbF and the endogenous Hb- and heme-scavenger systems in pregnancies complicated by PE. Plasma levels of HbF were significantly elevated whereas Hp and Hpx were significantly decreased in women with PE. The extravascular heme- and radical scavenger, and marker of oxidative stress, A1M was

significantly increased in plasma of women with PE. Furthermore, HbF and the related scavenger proteins displayed a potential to be used as clinical biomarkers for more precise diagnosis of PE and as predictors that help identifying pregnancies with increased risk of obstetrical complications.

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Competing Interests

I have read the journal's policy and the authors of this manuscript have the following competing interests: The authors MG, BÅ, and SRH are co-founders of the company A1M Pharma AB and holds patents regarding diagnosis and treatment of preeclampsia. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

List of Abbreviations

A1M, α_1-microglobulin; ACOG, American College of Obstetricians and Gynecologists; AT(1), Angiotensin II Receptor; AUC, Area Under the ROC-curve; CD163, Cluster of Differentiation 163; Fe^{2+}, Ferrous Iron; Fe^{3+}, Ferric Iron; Fe^{4+}, Ferryl; GPx, Glutathione Peroxidase; Hb, Hemoglobin; HbA, Adult Hemoglobin; HbF, Fetal Hemoglobin; HELLP, Hemolysis, Elevated Liver Enzymes and Low Platelets Count; HO, Heme Oxygenase; Hp, Haptoglobin; Hp-Hb, Haptoglobin-Hemoglobin Complex; Hp-HbF, Haptoglobin-Fetal Hemoglobin Complex; Hpx, Hemopexin; HRP, Horseradish Peroxidase; IUGR, Intrauterine Growth Restriction; LF, Low Fluorescence; MetHb, Ferric (Fe^{3+}) Hemoglobin; NICU, Neonatal Intensive Care Unit; NO, Nitric Oxide; O_2, Oxygen; PE, Preeclampsia; PVDF,

Polyvinylidene Difluoride; RAS, Renin-Angiotensin System; ROC, Receiver Operational Curve; ROS, Reactive Oxygen Species; RT, Room Temperature; SOD, Superoxide Dismutase; SPSS, Statistical Package for the Social Sciences; TMB, 3,3',5,5'-Tetramethylbenzidine

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Figure Legends

Fig. 1. Correlation between cell-free HbF- and Hp concentrations. Samples were from normal pregnancies (Control) and women diagnosed with PE. The cell-free HbF plasma concentration of each patient sample (Control and PE) was plotted against the Hp plasma concentration (**A**). The cell-free HbF plasma concentration of Controls was plotted against the Hp plasma concentration (**B**). The cell-free HbF plasma concentration of women diagnosed with PE was plotted against the Hp plasma concentration (**C**). Associations between variables were assessed by linear regression analysis (Pearson's).

Fig. 2. Correlation between Hp phenotype, cell-free HbF- and Hp-HbF concentration. Hp-phenotypes (1-1, 1-2 or 2-2) were investigated in plasma using SDS-PAGE and Western blot with anti-Hp antibodies as shown in the three patient examples (**A**) as described in Materials and Methods and the distribution of the different isoforms are presented as mean percentage of women with Hp 1-1, 1-2 and 2-2 for respective group (**B**). The plasma concentration of cell-free HbF (**C**) and Hp-HbF (**D**) are shown separately in patient samples with each Hp phenotype (Hp 1-1, 1-2 and 2-2). Results are presented as mean percentage of respective Hp phenotype (Hp 1-1, 1-2 and 2-2) in **B**. Results are presented as mean ± SEM plasma concentration of cell-free HbF and Hp-HbF in **C** and **D**.

Fig. 3. Correlation between Hpx concentration and systolic/diastolic blood pressure. Highest systolic (**A**) and diastolic (**B**) blood pressure (BP) measured within the last two weeks before delivery were plotted against the plasma concentration of Hpx. Correlation analysis of PE patients and controls using Pearson's correlation coefficient between Hpx and blood pressure, systolic (**A**, **solid line**; r=-0.511, p-value<0.00001, n=145) and diastolic (**B**, **solid**

line; r=-0,520, p-value<0.00001, n=145). Correlation analysis of PE patients only using Pearson's correlation coefficient between Hpx and blood pressure, systolic (**A**, **dashed line**; r=-0,123, p-value 0.22, n=98) and diastolic (**B**, **dashed line**; r=-0,058, p-value 0.57, n=98).

Fig. 4. Receiver operating characteristic (ROC) curves. ROC curves showing sensitivity and specificity for the combination of HbF, A1M and Hpx (**A**), Hpx and A1M (**B**) and Hpx (**C**). Area under curve (AUC) is 0.88 for the combination of HbF, A1M and Hpx, 0.92 for the combination of A1M and Hpx and 0.87 for Hpx.

Fig. 5. Schematic representation of the tentative chain of events involving HbF, Hp, Hpx, A1M and ROS and leading to PE. The figure shows a schematic placenta with impaired feto-maternal barrier function causing leakage of placenta factors. 1: Early events in the placenta induce an upregulation of the placenta HbF genes and protein and ROS. 2: Oxidative damage and leakage of the feto-maternal barrier results in 3: increased maternal plasma concentrations of HbF. Excess oxyHb undergoes auto-oxidation reactions resulting in free heme-groups and formation of ROS. 4: A complex network of scavenger proteins, composed of Hp, Hpx and A1M, binds, inhibits and eliminate HbF, heme and ROS. Cell-free HbF is bound by Hp and cleared by CD163 receptor-mediated uptake in monocytes and macrophage-cells. Free heme-groups are bound by Hpx and heme is cleared via the Hpx receptor CD91, preferably expressed on macrophages and hepatocytes. In this study, a highly significant decrease of both the Hp and Hpx was observed in maternal plasma of women with PE as compare to normal pregnancies. This indicated a prolonged presence of increased levels of both extracellular Hb and heme. Analysis of the plasma A1M levels in the present study displayed a significantly increase in women with PE as compared to normal pregnancies, most likely as a result of oxidative stress-induced up-regulation of the A1M gene expression.

Tables

Table 1. Description of pregnancies

Outcome	Normal pregnancy (Control; n=47)	Preeclampsia (n=98)	Early onset PE[1] (n=22)	Late onset PE[2] (n=74)
Age	29 (28-30)	31** (30-32)	32 **NS** (30-34)	30 **NS** (29-32)
BMI (kg/m^2)	25.0 (23.7-26.3)	26.1 **NS** (25.1-27.0)	27.1 **NS** (24.3-29.9)	25.9 **NS** (24.9-26.9)
Parity (n)	0.2 (0.02-0.32)	0.5* (0.28-0.64)	0.82* (0.23-1.41)	0.37* (0.20-0.54)
Systolic BP[3] (mmHg)	123 (120-126)	161** (157-165)	176** (167-185)	157** (153-160)
Diastolic BP[4] (mmHg)	77 (75-79)	101** (99-103)	108** (103-112)	99** (97-101)
Proteinuria (g/L)	0.02 (0.00-0.04)	2.32** (2.02-2.61)	3.35** (2.68-4.02)	2.08** (1.77-2.39)
Gestational age at delivery (days)	282 (279-285)	256** (250-262)	212** (199-225)	269** (265-273)
Twin pregnancies (n)	0	8 (8%)	2 (9%)	6 (8%)
Gestational age at sampling (days)	281 (278-284)	253** (247-260)	208** (196-220)	266** (262-270)
IVF (n)	1 (2%)	8 (8%)	1 (5%)	7 (10%)
ICSI (=n)	1 (2%)	1 (1%)	1 (5%)	0
Egg donor recipient (n)	0	1 (1%)	0	1 (1%)
Medication to stimulate ovulation[5] (n)	0	2 (2%)	0	2 (3%)

[1] Early onset PE was defined as diagnosis before 34+0 weeks of gestation.
[2] Late onset PE was defined as diagnosis before gestational week > 34+0.
[3] Highest systolic blood pressure recorded within two weeks prior to delivery.
[4] Highest diastolic blood pressure recorded within two weeks prior to delivery.
[5] In one case not known, the other patient medicated with Pergotime.

Table 1. Patient demographics of PE cases and normal pregnancies (controls). Time of PE diagnosis was not known for 2 PE cases and therefore not included in the sub-classification of early and late onset PE. Values are shown as mean (95% confidence interval) or number (%).

Statistical comparison vs. controls. p-value <0.05 is considered significant. **NS:** Not significant; *:p=<0.05; **:p=<0.001.

Table 2. Outcome of pregnancies.

Outcome	Normal pregnancy (Control; n=47)	Preeclampsia (n=98)	Early onset PE[1] (n=22)	Late onset PE[2] (n=74)
Birth weight (gram)	3602 (3477-3726)	2834** (2621-3047)	1434** (1105-1764)	3213** (3045-3381)
Fetal gender (M:F)	23:24	46:49 **NS**	7:15 **NS**	37:34 **NS**
HELLP[3]	0	7 (7%)	3 (14%)	4 (5%)
Eclampsia[4]	0	5 (5%)	2 (9%)	3 (4%)
Induction (n)	10 (21%)	58** (59%)	2** (9%)	55** (75%)
Vaginal delivery (n)	35 (75%)	46* (47%)	3* (14%)	43* (59%)
Vacuum extraction (n)	8 (17%)	8* (8%)	0**	8** (11%)
Cesarean section (n)	12 (26%)	47** (48%)	18** (82%)	27** (37%)
SGA[5]	0	1 (1%)[5a]	0	1 (1%)
IUGR[6]	0	8 (8 %)	5 (23%)	3 (4%)
Admitted to NICU[7] (n)	2 (4%)	32** (36%)	14** (82%)	18*** (25%)
Neonatal death	0	1 (1%)	1 (5%)	0
Preterm[8] (=n)	0	34** (35%)	20** (95%)	12** (16%)
APGAR10[9]	9.80 (9.64-9.96)	9.75 **NS** (9.62-9.89)	9.30* (8.80-9.70)	9.90 **NS** (9.70-10.0)

[1] Early onset PE was defined as diagnosis before 34+0 weeks of gestation.
[2] Late onset PE was defined as diagnosis before gestational week > 34+0.
[3] HELLP syndrome (Hemolysis, Elevated Liver enzymes, Low Platelets) diagnosed according to Mississippi classification.
[4] Eclampsia was defined as seizures occurring during pregnancy and after delivery in the presence of PE.
[5] SGA (Small for Gestational Age) defined as growth curve on Ultrasonography constant below curve.
[5a] Patient defined as both SGA and IUGR.
[6] IUGR (Intra Uterine Growth Restriction) was defined as growth below -2 standard deviations (-22%) on Ultrasonography (equivalent to growth below 3rd percentile).
[7] NICU (Neonatal Intensive Care Unit).
[8] Preterm was defined as delivery before 36+6 weeks of gestation (258 days).
[9] APGAR (Appearance, Pulse, Grimace, Activity, Respiration) score at 10 minutes.

Table 2. Patient demographics of PE cases and normal pregnancies (controls). Values are shown as mean (95% confidence interval) or number (%). Statistical comparison vs. controls. p-value <0.05 is considered significant. **NS:** Not significant; *:p=<0.05; **:p=<0.001.

Table 3. Biomarker results

Biomarker	Normal pregnancy (Control; n=47)	Preeclampsia (n=98)	Early onset PE[1] (n=22)	Late onset PE[2] (n=74)
HbF (ng/ml)	3.85 (2.51-5.20)	15.26 (7.0-23.6) p=0.01	18.72 (1.6-39.05) p=0.006	14.60 (5.10-24.0) p=0.17
Hp-HbF (µg/ml)	0.59 (0.003-1.18)	0.61 (0.31-0.90) p=0.018	1.07 (-0.10-2.24) p=0.15	0.48 (0.29-0.66) p=0.02
Total-Hb (µg/ml)	277 (232-321)	285 (238-331) p=0.53	290 (152-430) p=0.80	284 (237-331) p=0.73
Hp (mg/ml)	1.17 (1.04-1.30)	0.97 (0.75-1.19) p=<0.0001	1.34 (0.39-2.30) p=0.067	0.89 (0.77-1.02) p=0.001
CD 163 (µg/ml)	461 (408-512)	485 (445-527) p=0.37	433 (324-543) p=0.35	508 (465-551) p=0.07
Hpx (mg/ml)	0.93 (0.88-0.98)	0.69 (0.66-0.73) p=<0.0001	0.69 (0.61-0.77) p<0.0001	0.69 (0.65-0.73) p<0.0001
A1M (µg/ml)	29.93 (27.89-31.97)	33.50 (31.90-35.10) p=0.035	34.07 (30.31-37.83) p=0.26	33.70 (31.90-35.50) p=0.03

[1] Early onset PE was defined as diagnosis before 34+0 weeks of gestation.
[2] Late onset PE was defined as diagnosis before gestational week > 34+0.

Table 3. The mean concentrations of the biomarkers in the PE group and normal pregnancies (controls). Statistical comparison vs. controls. Significance was calculated with non-

parametric statistics (Mann-Whitney). Values are mean values with (95% confidence interval). A p-value <0.05 was considered significant.

Table 4. Biomarker detection rates

False positive rate	HbF combined with A1M and Hpx[1]	A1M combined with Hpx[2]	Hpx
5%	69%	66%	64%
10%	69%	67%	70%
20%	81%	81%	75%
30%	83%	85%	79%
AUC	0.88	0.87	0.87

[1] Based on logistic regression including all three parameters.
[2] Based on logistic regression including both parameters.

Table 4. Detection rates at fixed positive values for the combination of 1) HbF, A1M and Hpx, 2) A1M and Hpx and 3) Hpx alone. Detection rates for PE at different false positive rates and AUC for the ROC curve. Calculations are for all PE vs. controls.

Table 5. Prediction of fetal and maternal outcomes

Admittance to NICU	Significance	AUC
HbF	0.001	0.69
Hp	0.03	0.62
Hpx	0.008	0.66
Prematurity		
Hpx	0.001	0.70
CD 163	0.04	0.61
Combination Hpx + CD 163	0.001 0.025	0.72
Cesarean section		
Hpx	0.009	0.62

Table 5. Area Under the ROC-curves (AUC) for fetal outcomes (admittance to Neonatal Intensive Care Unit (NICU) and prematurity) and maternal outcomes (risk of cesarean section). The fetal outcome IUGR and the maternal outcomes induction of labor and vacuum extraction were not significantly related to any of the biomarkers. All calculations were based on univariable logistic regression analysis.

Paper IV

Fetal Hemoglobin and the hemoglobin degradation pathways in patients with preeclampsia - heme oxygenase 1 and Hemopexin activity as diagnostic biomarkers

Ulrik Dolberg Anderson[1a,b*], Maya Jälmby[1a,b], Marijke M Faas[2] and Stefan R Hansson[1a,b]

1a: Section of Obstetrics and Gynecology, Department of Clinical Sciences Lund, Lund University, Sweden

1b: Skåne's University Hospital, Malmö/Lund, Sweden

2: Department of Pathology and Medical biology and Department of Obstetrics and Gynecology, University of Groningen and University Medical Center Groningen, the Netherlands

*Corresponding author.

Ulrik Dolberg Anderson

Ulrik.dolberg_anderson@med.lu.se

Abstract:

Objective: The aim of this study was to investigate how maternal cell-free fetal hemoglobin and heme impacts the scavenger enzyme systems hemopexin and heme oxygenase 1 in patients with preeclampsia (PE). The secondary aims were to correlate the severity of PE, i.e. blood pressure, to the Hx activity in early- and late onset PE and evaluate their potential as biochemical markers for preeclampsia

Material and methods: Plasma samples from 135 patients were analyzed, 89 with PE and 46 normal controls. All samples were analyzed for cell-free fetal hemoglobin (HbF), heme, hemopexin enzymatic activity (Hx activity), hemopexin concentration (Hx), and heme oxygenase 1 concentration (HO-1).

Results: There were significantly higher levels of HbF ($p=0.01$) and heme (0.01) but significantly lower Hx activity ($p=0.02$), Hx ($p<0.0001$) and HO-1 ($p=0.03$) in PE plasma. The Hx activity was significantly correlated ($p=0.04$) to the diastolic blood pressure and HO-1 concentration was inversely correlated to both the systolic ($p=0.01$) and diastolic blood pressure ($p=0.003$). ROC-curve analysis showed 84% detection rate at 10% false positive rate by combining these biomarkers.

Conclusions: Hemopexin exhibits decreased enzymatic activity in PE plasma. Hemopexin and HO-1 concentrations were reduced in PE and the heme levels increased. Components of the heme degradation systems may be used as potential predictive and diagnostic biomarkers for PE in combination with HbF concentration.

Introduction:

Preeclampsia (PE) is a pregnancy related syndrome that causes major maternal and fetal morbidity and mortality worldwide [1]. It is estimated that PE causes up to 75.000 maternal and 500.000 fetal deaths each year, especially in developing countries [2].

The pathogenesis underlying PE is still not fully understood but the two-stage model is up to date, the most accepted way to describe how the disease progresses [3, 4]. The first stage is characterized by impaired remodeling of the maternal spiral arteries, which induce oxidative stress in the placenta due to uneven blood flow [5]. A number of placental factors have been suggested to leak over to the maternal blood circulation where inflammation causes vascular damage. General endothelial damage/ endotheliosis are typical findings in the second stage of PE, which affect all organs and eventually give rise to the clinical manifestations seen in PE. The second stage of PE is characterized by the maternal clinical manifestations; hypertension and proteinuria [6]. The International Society for the Study of Hypertension in Pregnancy (ISSHP) define PE by its clinical findings: *de novo* hypertension and proteinuria [7, 8].

Increased synthesis and accumulation of cell free fetal hemoglobin (HbF) has been shown in PE placentas [9]. Furthermore, increased concentrations of HbF have been shown in maternal plasma/serum in both early- [10] and late pregnancy complicated by PE suggesting it to be an important factor linking stage one and two in the etiology [11]. Free HbF has been shown to cause placental tissue damage and oxidative stress, which consequently leads to leakage over the blood-placenta barrier into the maternal circulation [12]. To prevent toxicity of hemoglobin and its degradation metabolites heme and free iron, several scavenger systems protects the human body. Haptoglobin (Hp) is the most well described hemoglobin scavenging system that binds free hemoglobin and transports it to macrophages and hepatocytes where the uptake is facilitated by the CD 163 receptor-mediated endocytosis [13]. In the intracellular compartment of primarily macrophages, hemoglobin is degraded to heme by lysosomes, and furthermore catabolized by heme oxygenase 1 (HO-1) to biliverdin, carbon monoxide (CO), and free iron [14]. Biliverdin is further reduced to bilirubin, which is excreted via the bile system. Carbon monoxide has dilating effects on the vascular bed as it relaxes the smooth muscle layer of the vessels and consequently lowers blood pressure. Hemopexin (Hx) is a circulating plasma glycane, mainly synthesized in the liver. It acts as an acute phase reactant that binds free heme with high affinity [15, 16]. The heme affinity to Hx

is affected by several factors, such as decreased pH, reducted state of the heme iron, binding of nitric oxide (NO) to the heme iron, or presence of chloride anions and other divalent metal ions [17]. Sodium cations increase heme affinity to Hx [17]. The Hx-heme complex is transported to macrophages and hepatocytes expressing the LDL receptor-related protein 1 (LRP1), which facilitates uptake of the Hx-heme complex [18]. In this way Hx serve a back up system to Hp. When the Hp-system is overwhelmed, Hx clears the blood from heme [17]. Hemopexin has indeed been shown to prevent endothelial damage in a mouse model [19]. Like other circulating plasma proteins Hx also present with enzymatic activity (Hx activity). These enzymatic processes affects functions such as neutrophil necrosis [20], inhibition of cellular adhesion[21] and attenuation of inflammation [22]. On top of this, serine protease activity has been observed, i.e. that Hx has protease properties that break down the amino acid Serine [23]. The Hx enzymatic activity can be measured by a method described by Bakker et al [23, 24]. Hx activity is described to increase from 10 weeks of gestation and onwards [25]. *In vitro* results show that the Hx activity can affect the renin-angiotensin system (RAS). Hemopexin activity has been shown to down-regulate the angiotensin II receptor in monocytes, endothelial cells, and in the rat aorta [25]. Furthermore the Hx activity has been suggested to regulate vascular responsiveness to angiotensin II [26].

The physiological heme and radical scavenger α_1-microglobulin (A1M) has been shown to be up regulated in early [10] and late PE [11]. Circulating maternal plasma concentrations of Hp and Hx are decreased in PE [27].

The aim of this study was to investigate the role of the hemoglobin/heme degrading pathways in PE, i.e. to further understand how HbF and heme impact the scavenger systems Hx and HO-1 levels in PE. The secondary aims were to correlate the severity of PE, i.e. blood pressure, to the Hx activity in early- and late onset PE, and to evaluate these proteins as potential biomarkers for PE diagnosis.

Materials and Methods

Patients and demographics

A random sample of 98 women with preeclampsia and 47 women with normotensive pregnancies (recruited between 2006 and 2011) was initially identified for a study of hemoglobin-related metabolites as potential biomarkers of preeclampsia [27]. The current

study included 135 of these 145 study participants for whom plasma samples were collected up to 24-hours prior to delivery. The patients were randomly selected from an on-going prospective cohort study. Exclusion criteria were gestational hypertension, essential hypertension and gestational diabetes. In total 5 cases were excluded due to pre-gestational diabetes or pregnancy related diabetes. In total, 89 of the included patients had PE and 46 were normal pregnancies used as controls. A detailed patient demographics were previously described by Gram et al [27].

Sample collection

The study was approved by the ethical committee review board for studies on human subjects at Lund University, Sweden. The patients signed informed consent after orally and written information. Maternal venous samples were taken within the last 24 hours prior to delivery from patients admitted to the Department of Obstetrics and Gynecology, Lund University Hospital, Sweden. The blood samples were collected in 6 ml EDTA Vacuette® plasma tubes (Greiner Bio-One GmbH, Kremsmünster, Austria) and centrifuged at 2000 x g for 20 minutes. The plasma was then transferred into cryo tubes and stored at -80°C until time of analysis. Pregnancy outcome were retrospectively obtained from the patients charts after delivery. The samples were shipped on dry ice from Lund, Sweden to Groningen, the Netherlands for blinded Hx activity analysis. The samples were still frozen upon arrival.

Preeclampsia was defined as *de novo* hypertension after 20 weeks of gestation with 2 readings at least 4 hours apart of blood pressure ≥140/90 mmHg and proteinuria ≥300 mg per 24 hours according to the ISSHP definition [7]. Dipstick analysis was accepted if there was no quantification of proteinuria. Furthermore, the PE group was further sub-classified as early-onset PE (diagnosis ≤ 34+0 weeks of gestation) or late onset PE (diagnosis >34+0 weeks of gestation).

Hx activity

Plasma Hx activity was measured in EDTA plasma samples using the Hx-MCA substrate (synthesized by Pepscan, Lelystad, the Netherlands). The plasma samples (40 µl) were diluted 1:4 with the substrate solution (0.2M Tris + 0.9% NaCl pH 7.6 (substrate concentration 80 µM/L) to a final volume of 200 µl. The emission was measured at 460 nm on a Varioskan spectrophotometer (Thermo Fisher) at 37°C. The Hx activity was measured after 0 min, 30

min (Hx30), 60 min (Hx60) and 24 hours. The measured value represented the total amount of Hx-MCA substrate catabolized by Hx at the given time point. The scale of the value is arbitrary. If the value was <5 after 24 hours of incubation, the activity was considered too low to be included. Reasons for low levels are either technical problems with the assay or the sample quality.. The area under the curve analysis was based on Hx30 and Hx60 measurements (HxAUC). The measures Hx30, Hx60 and HxAUC were similar and therefore only Hx30 was used for the analysis, mentioned Hx activity in the following.

Hx concentration

The Hx concentration was measured with a Human Hemopexin ELISA Kit (Genway Biotech Inc). The analysis was performed according to manufacturer's instructions and the absorbance read at 450nm with a Wallac 1420 Multilabel Counter.

Cell free HbF was measured with a monoclonal Sandwich ELISA as previously described by Gram et al. [27]. Heme was measured with the QuantiChrom Heme Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's instructions. The HO-1 concentration was measured with ELISA (Enzo Lifesciences Inc., Farmingdale, New York) according to the manufacturer's instructions.

The concentration of total cell-free Hb was measured with a Human Hb ELISA Quantification Kit (Genway Biotech Inc., San Diego, CA, USA). The analysis was performed according to the manufacturer's instructions and the absorbance was read at 450nm using a Wallac 1420 Multilabel Counter.

Correlation to previous findings

The same cohort has previously been used for HbF, A1M, Hp, Hx and CD163 measurements [27]. Data from this study was used as complement to build the algorithms presented in this study

Statistical analysis

All statistical analysis was performed with the software Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) version 21 for Apple computers (Apple Inc., Cupertino, CA).

Mann-Whitney test was used to compare Hx activities, Hx, HO-1, heme, HbF and total Hb concentrations between PE and controls (table 2). Subgroup-analyses were performed for early- and late onset PE.

Correlation analysis

Correlation between Hx activity and the Hx concentration was calculated using the non-parametric Kendall's correlation coefficient. Furthermore, correlation analysis was performed between Hx activity and maternal blood pressure (defined as the highest measured blood pressure within 24 hours prior delivery).

Correlation analyses were also done between cell-free Hb (HbF and Total Hb), heme, HO-1 and hemopexin concentrations. Furthermore, heme and HO-1 were correlated to both systolic and diastolic blood pressure.

Logistic regression analysis

The detection rate was determined by ROC-curve analysis for each of the potential biomarkers. The detection rates were obtained at 10% and 20% false positive rates. The combined detection potential for the biomarkers was obtained by stepwise logistic regression analysis of the biomarkers and ROC-curve analysis.

Results.

The cohort

Description of the patient cohort is displayed in Table 1. In total 89 patients with PE were included in the study matched with 39 uncomplicated pregnancies as controls. Of the 89 PE cases, 17 were diagnosed with PE before 34+0 weeks of gestation and classified as early-onset PE. The groups were comparable concerning maternal age, BMI and parity.

The systolic and diastolic blood pressures along with proteinuria were by definition significantly higher in the PE group. Due to increased incidence of preterm labor in the PE group the blood sampling was consequently performed earlier in the PE group.

Hx activity

Eleven of the samples (8 controls and 3 PE) showed “very low value” after 24 hours of incubation and therefore excluded from further analysis.
The Hx activity was significantly lower in the PE groups compared to controls after 30 min (p=0.02, Table 2). However, when subdividing the PE patients into early- and late-onset, the early-onset group (Hx activity = 0.81) showed identical values to the control group (Hx activity = 0.80, Table 2). In contrast, the late onset group showed a more marked decrease in the Hx activity compared to all PE (Hx activity = 0.54, p = 0.007, Table 2).

Heme.

The heme concentration was significantly higher in patients with preeclampsia compared to controls (75.03 µM vs. 59.86 µM, p=0.01). The concentrations were significantly higher in late onset PE (77.55 µM, p=0.02, table 2).

HO-1.

The heme oxygenase 1 concentration was significantly lower in the PE group compared to controls (4.48 ng/ml vs. 5.29 ng/ml p=0.03). Both early- and late onset PE showed significantly lower HO-1 concentrations (4.67 ng/ml, p=0.02) and (4.42 ng/ml, p=0.01) respectively (table 2).

HbF and Hemopexin protein concentration

There was a statistically higher HbF-concentration in all PE groups [27]. The hemopexin concentration was significant lower in the PE groups as described in Gram et al [27]. Total Hb was not significantly different between PE and controls (p=0.53).

Correlation analysis

The Hx activity was not correlated to the corresponding plasma concentration in the study group (p=0.90) or for the PE subgroups early- (p=0.17) and late onset PE (p=0.24) respectively.

Hx activity was significantly inversely correlated to the diastolic blood pressure in all patients (p=0.04). When the early onset patients were excluded from the analysis there was a clear correlation between the diastolic blood pressures and Hx activity (p=0.009).

The heme concentration was not correlated to the HbF level (p=0.31) but did significantly correlate to the total Hb concentration (Correlation coefficient=0.18, p= 0.002). Furthermore,

there were no correlations between heme and Hx activity (p=0.82). Heme and HO-1 were not statistically significantly related (p=0.08).

The HO-1 level was significantly inversely correlated to both the systolic (p=0.01, correlation coefficient=-0.15) and the diastolic blood pressure (p=0.003, correlation coefficient=-0.25). The HO-1 concentration did not correlate to the Hx activity (p=0.92).

Logistic regression analysis

The results from the logistic regression and ROC-curve analyses are presented in table 3. The Hx activity had a detection rate (DR) of 30% at a 10% false positive rate (FPR) with 0.66 AUC. The HO-1 concentration showed 21% DR at 10% FPR, AUC=0.64 and the heme concentration showed 22% DR at 10% FPR and AUC=0.63.

The combination of HbF, Hx activity, Hx concentration, heme and HO-1 together showed a DR of 84% at 10% FPR, AUC=0.93 (table 3, figure 1).

Discussion

The main findings in this study were decreased plasma Hx activity and HO-1 concentrations in patients with PE in combination with increased plasma heme concentration. Furthermore, the Hx activity, and in particular, HO-1 levels were significantly correlated to the maternal blood pressure, i.e. the severity of PE. In combination, HbF, Hx activity, Hx concentration, heme and HO-1 are potential biomarkers for PE as they are able to detect 84% of the PE patients at 10% FPR.

The role of HbF in the development of PE has been investigated in several studies [9-12, 27-32]. Here the role of the hemoglobin/heme degrading pathways in PE was specifically evaluated. Furthermore, to investigate the impact of circulating cell-free fetal hemoglobin on Hx enzymatic activity in plasma in women with PE and to study the correlations of Hx activity to free heme and HO-1.

In concordance to previously published results, a decreased Hx activity was shown in patients with manifest PE [24]. Interestingly, the results showed that Hx activity only decreased in patients with late-onset PE although previous studies have shown the plasma Hx activity to be significantly decreased in both early and late onset PE [24]. A reason for this discrepancy in Hx activity regarding the sub groups could be the study design. In this study, early onset patients were compared with term controls. If instead gestational age matched controls had

been used there might have been lower Hx activity in the early onset group, as it has been shown in previous studies [24].
The current data showed that the Hx activity not correlated to the Hx concentration, which indicate that the activity is not solely dependent on its concentration. Elevated heme levels and low hx levels suggest a consumption of Hx due to increased circulating heme load in the PE group [11, 27]. Several factors influence Hx activity in the maternal circulation but *in vitro* studies indicate that extra-cellular adenosine triphosphate (ATP) to be an important inhibitor of Hx activity [23]. *In vitro* studies suggest that the increase in Hx activity seen in normal pregnancy may down-regulate the endothelial expression of the Angiotensin II receptor 1 (AT1) and thereby promote a relaxed and dilated maternal vascular bed [25]. It has further been suggested that the decreased Hx activity seen in PE may increases the maternal blood pressure via the RAS [24].

Decreased Hx activity has been shown to induce inflammation [21]. Increased inflammation has been described in both stages of PE development [5]. Endothelial damage/ endotheliosis are cardinal mechanism behind the development of hypertension in PE [6]. The reduced Hx activity shown in PE patients might therefore add to the inflammation and endotheliosis.

Reduced HO-1 levels have previously been described in PE placentas [33]. Furthermore, HO-1 have also been shown to be protective against inflammation, apoptosis, and to be involved in regulation of angiogenesis [33]. A HO-1 knock out mouse model suggest that HO-1 is critically involved in placentation, spiral artery remodeling and placental blood pressure regulation [34]. In the present study the HO-1 concentration was significantly reduced, particularly in the late onset PE group. The low concentration of HO-1 could be due to continuous strain on this system due to the elevated heme and HbF levels described in PE. The HO-1 enzyme is being gradually depleted throughout pregnancy and is therefore only lower in late onset PE.
The plasma heme concentration was elevated both in early and late onset PE, however only significantly elevated in late onset PE. The heme concentration obviously correlated well with total Hb concentration. Previously published studies have indicated that the increased levels of HbF throughout a PE pregnancy, slowly put a strain on and deplete the maternal Hb and heme scavenging systems including A1M, haptoglobin and hemopexin concentration [10, 11, 27]. A constant over-production of HbF in the placenta induces damage to the placenta and the maternal endothelium. The strength of the maternal scavenger and enzyme systems may

be important constitutional factors that determine how and when the clinical symptoms present in stage two. The more the systems are strained and/or depleted, the more severe are the clinical symptoms.

Correlation analysis showed a significantly inverse correlation between Hx activity and diastolic blood pressure in all the patients. This is in accordance with previously published data that described that Hx active promote down regulation of the angiotensin II receptor (AT1 receptor), which consequently lead to an expanded vascular bed and lower blood pressure [25, 26]. Diminished Hx activity, seen in PE patients may therefore lead to up-regulation of the AT1 receptor and consequently to a contracted vascular bed and hypertension.
Heme oxygenase 1 was also inversely correlated to both systolic and diastolic blood pressure. The higher heme load might explain why HO- 1 was lower in PE patients. Depletion of HO-1 diminishes the anti-inflammatory properties, which in turn may aggravate maternal endotheliosis. Furthermore the degradation of heme by HO-1 produces CO, which is a potent vaso-dilator. Diminished levels of HO-1 consequently lead to decreased degradation of heme and less production of CO that may add to the increased contractility of the vascular bed seen in PE.

In this present study we present a range of potential biomarkers based on HbF and hemoglobin- and heme scavenger proteins and -enzymes. Used as individual biomarkers, none of the evaluated biomarkers reach a sufficient detection level acceptable for clinical use. However, when analyzed together, Hx activity, Hx, HO-1, Heme and HbF concentrations were able to detect 84% of the PE cases at 10% FPR, which is similar to other suggested biomarkers such as PlGF and sFlt-1 [35]. An important advantage of the presented biomarkers is the correlation to blood pressure and hence with the severity of the disease.

Conclusions

Hemopexin exhibits decreased enzymatic activity in PE plasma. In addition, both Hx and the HO-1 concentrations were reduced in PE whereas heme levels were increased suggesting a depletion of the protective heme degradation systems. By measuring components of the Hb degradation system as potential diagnostic biomarkers, a more precise PE diagnosis can be

made. Future studies will evaluate the value of these biomarkers as predictive biomarkers in the first trimester.

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Table 1. Description of pregnancies

Outcome	Normal pregnancy – controls (n=39)	Preeclampsia (n=89)	Early-onset PE[1] (n=17)	Late-onset PE[2] (n=72)
Age	29 (28-30)	31** (30-32)	32 **NS** (30-34)	30 **NS** (29-32)
BMI (kg/m^2)	25.0 (23.7-26.3)	26.1 **NS** (25.1-27.0)	27.1 **NS** (24.3-29.9)	25.9 **NS** (24.9-26.9)
Parity (n)	0.2 (0.02-0.32)	0.5* (0.28-0.64)	0.82* (0.23-1.41)	0.37* (0.20-0.54)
Systolic BP[3] (mmHg)	123 (120-126)	161** (157-165)	176** (167-185)	157** (153-160)
Diastolic BP[4] (mmHg)	77 (75-79)	101** (99-103)	108** (103-112)	99** (97-101)
Proteinuria (g/L)	0.02 (0.00-0.04)	2.32** (2.02-2.61)	3.35** (2.68-4.02)	2.08** (1.77-2.39)
Gestational age at delivery (days)	282 (279-285)	256** (250-262)	212** (199-225)	269** (265-273)
Gestational age at sampling (days)	281 (278-284)	253** (247-260)	208** (196-220)	266** (262-270)

[1] Early-onset PE was defined as diagnosis before 34+0 weeks of gestation.
[2] Late-onset PE was defined as diagnosis after 34+0 weeks of gestation.
[3] Highest systolic blood pressure recorded within two weeks prior to delivery.
[4] Highest diastolic blood pressure recorded within two weeks prior to delivery.

Patient demographics of PE cases and normal pregnancies (controls). Values are shown as mean (95% confidence interval) or number (%). Statistical comparison of the groups was performed with ANOVA. A p-value <0.05 was considered significant.

NS: Not significant; *:p=<0.05; **:p=<0.001.

Table 2

	Controls N=39	**Preeclampsia n= 89**	**Early onset preeclampsia n=17**	**Late onset preeclampsia n=72**
Hemopexin activity (mean)	0.80 (0.66-0.93)	0.59 (0.49-0.69) p=0.019	0.81 (0.54-1.07) p=0.96	0.54 (0.44-0.65) p=0.004
Hemopexin plasma concentration[1] (mean)	0.93 (0.88-0.98)	0.69 (0.66-0.73) p<0.0001	0.69 (0.61-0.77) p<0.0001	0.69 (0.56-0.73) p<0.0001
HbF[1] (mean)	3.85 (2.51-5.20)	15.26 (7.0-23.6) p=0.01	18.72 (1.6-39.05) p=0.006	14.60 (5.10-24.0) p=0.17
Heme µM (mean)	59.86 (52.34- 67.38)	75.03 (67.43-82.62) p=0.01	69.54 (55.07-84.02) p=0.26	77.55 (68.37-86.74) p=0.02
HO -1 ng/ml (mean)	5.29 (4.69-5.9)	4.48 (4.04-4.93) p=0.03	4.67 (3.37-5.97) p=0.02	4.42 (4.69-5.89) p=0.01

Plasma concentrations of the biomarkers for the control group, the preeclampsia group and for early- and late onset preeclampsia. Early onset preeclampsia was defined as preeclampsia diagnosed before 34+0 weeks of gestation.

[1]: Previously published by Gram et al [27].

Table 3

	10%	20%	Area under the ROC-curve
Hx activity	30%	44%	0.66
HO-1	21%	31%	0.64
Heme	22%	31%	0.63
Combination of biomarkers*	84%	87%	0.93

The results of the logistic regression / ROC-analysis for PE vs. controls. Detection rates are displayed at fixed false positive rates (10% and 20%). *=Combination of HbF, Hx activity, Hx concentration, Heme and HO-1.

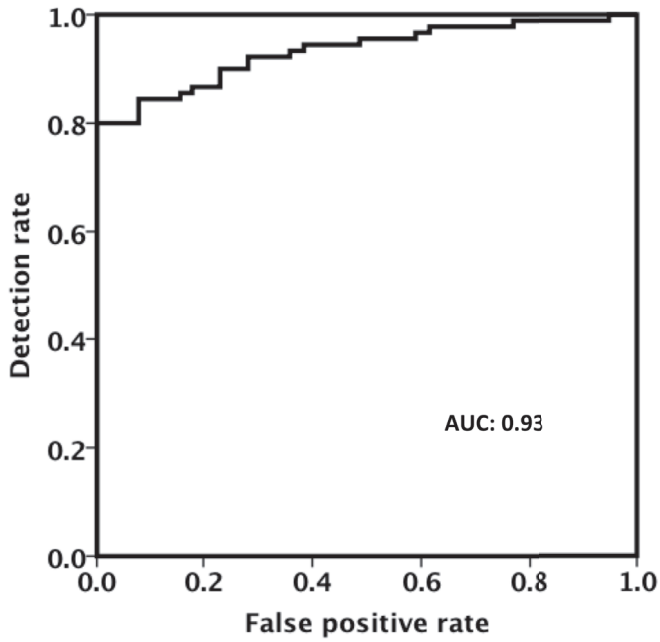

Figure 1 ROC-curve based on the logistic regression analysis for the combination of the biomarkers HbF, Hx activity, Hx concentration, Heme and HO-1

Paper V

Urinary Extracellular Vesicles Positive for Podocyte-specific Proteins are Associated with Renal Injury in Preeclampsia

Sarwat I. Gilani,[*, 1] Ulrik Dolberg Anderson,[†, 1] Muthuvel Jayachandran[‡] Tracey L. Weissgerber,[*] Ladan Zand,[*] Wendy M. White,[§] Natasa Milic,[*, l] Joseph P. Grande,[*] Karl A. Nath,[*] Magnus Gram,[¶] Bo Åkerström,[¶] Stefan R. Hansson,[†, 2] Vesna D. Garovic,[*, §, 2]

[1] First co-authors (SIG and UDA)

[2] Senior co-authors (SRH and VDG)

[*] Division of Nephrology and Hypertension, Mayo Clinic, Rochester, MN USA

[†]Department of Obstetrics and Gynecology, Institute of Clinical Sciences Lund, Lund University, University Hospital Skåne, Malmö, Sweden

[‡]Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN USA

[§]Department of Obstetrics and Gynecology, Mayo Clinic, Rochester, MN USA

[l] Department of Biostatistics, Medical Faculty, University of Belgrade, Serbia

[¶]Department of Clinical Sciences, Lund, Infection Medicine, Lund University, Sweden

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Address for Correspondence: Vesna D. Garovic, MD

Division of Nephrology and Hypertension
Mayo Clinic
200 First Street SW
Rochester, MN 55905 USA
Phone: 507-266-1963
Fax: 507-266-7891
Email: Garovic.vesna@mayo.edu

ABSTRACT

Proteinuria in preeclampsia may result from podocyte injury, the latter representing the principal determinant of glomerular permselectivity. We hypothesized that renal injury in preeclampsia is associated/correlated with the presence of urinary extracellular vesicles (EV) of podocyte origin, and that maternal cell-free fetal hemoglobin (HbF) in plasma may represent the proximate cause for podocyte injury.

We studied 49 preeclamptic and 43 normotensive pregnant women recruited at the time of delivery in the Lund University Hospital, Malmö, Sweden. Plasma measurements included clinical renal function tests (creatinine and cystatin C), concentrations of HbF and endogenous chelators of HbF itself (haptoglobin) or its potential toxic heme moiety (hemopexin and α_1 microglobulin). Proteinuria was measured by dipstick. Urine samples were analyzed for urinary extracellular vesicles (EVs) in a blinded fashion at Mayo Clinic, Rochester, MN USA. Podocyte injury was assessed by the concentrations of urinary EVs that stain for podocyte-specific proteins by digital flow cytometry.

Preeclamptic, compared to normotensive pregnancies, demonstrated decreased kidney function, elevated cell-free HbF and α_1-microglobulin levels, decreased levels of haptoglobin and hemopexin, elevated annexin, nephrin , podocin positive urinary EVs, and nephrin positive over podocin positive EVs (nephrin$^+$ EVs/ podocin$^+$ EVs) ratios: 0.86 ± 0.15 versus 0.42 ± 0.19 ($p<0.001$).There was a positive correlation between cell-free HbF and both the degree of proteinuria and the nephrin$^+$ EVs/ podocin$^+$ EVs ratio.

Renal injury in preeclampsia is thus associated with an elevated urinary nephrin^{+} EVs/ podocin^{+} EVs ratio, which may, in part, be mediated by HbF-induced podocyte injury.

INTRODUCTION

Preeclampsia is a multi-system pregnancy disorder characterized by hypertension and proteinuria,[1] and in which endothelial dysfunction is a central pathogenetic process.[2] Glomerular endotheliosis, which is the characteristic (but not pathognomonic) renal lesion in preeclampsia, is characterized by occlusion of capillary lumens, glomerular endothelial swelling and loss of endothelial fenestrations.[3] Over the last decade, evidence has increasingly spotlighted injury to podocytes, the principal determinant of glomerular permselectivity, as a critical contributor to proteinuria in preeclampsia.[4] Podocytes are terminally differentiated cells located on the side of the glomerular basement membrane that faces the urinary space.[5] They interdigitate via their foot processes, which connect via specialized cell-to-cell junctions to form glomerular slit diaphragms. The slit diaphragm appears to be a modified adherens junction that provides the main size selective filtration barrier in the kidney.

Several lines of evidence suggest that both podocytes and podocyte-specific proteins are present in urine samples obtained from women at the time of their preeclampsia diagnosis, supporting podocyte loss and injury as the mechanism of proteinuria in preeclampsia. [6-8] The clinical use and mechanistic studies of podocyte injury in preeclampsia are limited by the performance characteristics of the currently available podocyturia assays. These are largely cytology-based, time-consuming, lengthy procedures, and require special expertise (a trained pathologist) for the interpretation of cytologic findings. Furthermore, podocytes recovered from urine and cultured for 24 hours offer little to no opportunity to perform mechanistic studies.

We hypothesized that renal injury in preeclampsia - as demonstrated by the presence of

proteinuria and renal dysfunction - is associated with and correlates to the presence of urinary extracellular vesicles (EVs) of podocyte origin. The EVs are small (0.03 to 1 μm) membrane-enclosed sacs that are shed from activated or injured cells, and have been identified in different body fluids, including urine.[9] Podocyte injury was measured by the concentrations of urinary EVs that stain for podocyte-specific proteins by digital flow cytometry, a methodology that was adopted from studies detecting EVs in blood,[10] and was applied recently to urine studies.[11]

In our effort to delineate a cause for podocyte injury in preeclampsia, our attention was drawn to both clinical and experimental evidence pertaining to fetal hemoglobin (HbF). Clinical studies[12, 13] demonstrate that cell-free HbF levels are elevated in preeclamptic vs. normotensive pregnancies as early as in the first trimester. The pathophysiologic significance of such observations was raised by experimental studies demonstrating that renal injury in preeclamptic models in sheep and rabbits may be instigated by starvation-induced hemolysis and species-specific cell-free HbF infusions, respectively.[14, 15] We thus linked these observations back to podocyte injury in preeclampsia, postulating that levels of cell-free fetal hemoglobin (HbF) in maternal plasma, along with derangements in mechanisms that protect against hemoglobin/heme toxicity (haptoglobin, hemopexin, a1-microglobulin) would correlate with maternal renal dysfunction and podocyte injury, as reflected by the presence of urinary EVs of podocyte origin.

Results

Demographic and Clinical Variables

A total of 92 pregnant women were included in this study, 49 with preeclampsia and 43 with normotensive pregnancies (Table 1). Women with preeclampsia did not differ significantly from those with normotensive pregnancies with respect to age, BMI, parity or nulliparous status, but delivered at an earlier gestational age and were more likely to undergo induction of delivery. Their infants were more likely to have lower birth weights.

Table 2 summarizes the biochemical characteristics of the women with preeclampsia versus those with normotensive pregnancies at the time of delivery. Kidney function, as evaluated by both cystatin C and serum creatinine, was decreased in women with preeclampsia. Uric acid levels were elevated as expected based on previous reports.[16] The concentrations of cell-free HbF and α_1-microglobulin were elevated in preeclampsia, while haptoglobin and hemopexin were decreased as previously described.[17]

Characterization and quantification of urinary EVs

Urinary EVs that stain for nephrin, podocin, and annexin were significantly increased in preeclampsia compared to normotensive pregnancy; a trend towards an increase in EVs staining for synaptopodin was noted (Table 3). A significant overlap in values between preeclamptic and normotensive pregnancies was noted (Figure 1). This ultimately resulted in the suboptimal diagnostic performance of each of these markers when analyzed separately (Table 4).

In the next step (and as justified in Methods), we calculated the ratios of podocyte-specific proteins - positive EVs (podocin, synaptopodin and nephrin) over annexin, a common marker of microvesicles that identifies the surface phosphatidyl serine, and nephrin$^+$ EVs/ podocin$^+$ EVs

ratio (Table 5, Figure 2). The ratios of podocyte- specific proteins - positive EVs over annexin were significantly decreased in preeclampsia compared to normotensive pregnancy, while the nephrin$^+$ EVs/ podocin$^+$ EVs ratio was significantly increased in women with preeclampsia at the time of delivery (Table 5, Figure 2).

Nephrin$^+$ EVs/ podocin$^+$ EVs ratio as novel biomarker of acute renal injury in preeclampsia

The nephrin$^+$ EVs/ podocin$^+$ EVs ratio was higher in preeclamptic than in normotensive pregnancies: 0.86 ± 0.15 versus 0.42 ± 0.19 ($p<0.001$). ROC curve was drawn (Figure 3) which revealed an AUC of 0.952 (SE=0.022, $p<0.001$). The cut off level of >0.6 for the nephrin$^+$ EVs/ podocin$^+$ EVs ratio reflected the optimal cut off (Table 6) and demonstrated 91.8% sensitivity and 86.0% specificity in distinguishing a clinical diagnosis of preeclampsia from normotensive pregnancy. Positive and negative predictive values with respective confidence intervals are presented in Table 6.

Correlations between the nephrin$^+$ EVs/ podocin$^+$ EVs ratio and biochemical parameters at the time of delivery are presented in Table 7. Most notably, there was a positive correlation with renal functional indices (cystatin C and creatinine) and markers of preeclampsia severity (uric acid). The nephrin$^+$ EVs/ podocin$^+$ EVs ratio showed a positive correlation with cell-free HbF, and negative correlations with haptoglobin and hemopexin (Table 7). There was a positive correlation between cell-free HbF and the degree of proteinuria (rho=0.244; $p=0.021$).

Discussion

Our data demonstrate significant differences in the number of urinary EVs of podocyte origin between preeclamptic and normotensive pregnancies at the time of delivery. The nephrin^{+} EVs/ podocin^{+} EVs ratio was higher in preeclamptic than in normotensive pregnancies and demonstrated 92 % sensitivity and 86 % specificity in distinguishing preeclamptic from normotensive pregnancies at delivery. Women with preeclampsia delivered at an earlier gestational age, as they were more likely to undergo induction of delivery for termination of pregnancy, the latter representing the only cure for preeclampsia. Concentrations of cell-free HbF were elevated in preeclampsia as reported previously for this cohort, [17] and were positively correlated with the degree of proteinuria, indicating a possible role of cell-free HbF in renal injury in preeclampsia. Furthermore, a positive correlation was discerned between the and HbF, leading us to speculate that renal injury in preeclampsia may be, in part, mediated by direct HbF-induced podocyte injury. This speculation is further supported by the negative correlations observed between the nephrin^{+} EVs / podocin^{+} EVs ratio and plasma levels of both haptoglobin and hemopexin; specifically, the greater consumption of these hemoglobin- and heme-binding proteins may reflect the higher levels of HbF to which the podocyte is exposed, and the severity of podocyte injury that then ensues.

Podocyte injury and loss are recognized increasingly as one of the mechanisms underlying renal injury in preeclampsia. Differential expressions of the podocyte-specific proteins that contribute to the structural and functional integrity of the glomerular slit diaphragm have been reported by studies of renal tissue in preeclamptic versus normotensive pregnancies.[18, 19] The expressions of nephrin and synaptopodin were decreased in preeclampsia, while the expression of podocin was comparable. These findings in renal tissue may affect podocyte urinary markers in two ways. First, podocyte detachment and shedding in the urine (i.e., podocyturia) may be best identified by

podocin, which is least affected in preeclampsia with respect to its expression in renal tissue sections.[18] Our initial study of podocyturia in preeclampsia indicated that staining for podocin, compared to other podocyte-specific proteins, was superior in identifying urinary podocytes in preeclampsia.[6] Secondly, the down-regulation of nephrin in renal tissue can be, at least in part, due to urinary nephrin losses, i.e., nephrinuria. Studies of urinary supernatants in preeclampsia using a nephrin ELISA showed that urine nephrin levels were elevated compared to levels in normotensive pregnancies[8, 20] and that urine nephrin levels correlated with proteinuria, diastolic blood pressure, and renal dysfunction.[20]

Urinary EVs originate from the cells facing the urinary space and contain cargo (protein, lipids and micro RNA) representative of their cells of origin.[21] Based on size, content, and biogenesis, EVs can be classified further into exosomes, microvesicles, and apoptotic bodies. The term, EVs, is preferred, however, due to their overlapping physical and biological properties, and the lack of scientific accord regarding how these sub-classes are best defined.[22] One of the significant challenges in the field relates to the best way of expressing and normalizing changes in the EVs number and their content that would facilitate not only adequate comparisons between the study groups, but comparisons across studies.[9, 23] In the current study, we normalized the number of podocyte protein-specific positive EVs to annexin$^+$ EVs and calculated the ratio between nephrin$^+$ EVs / podocin$^+$ EVs, which may serve as a marker of disease activity.[24] Our results show that the ratios between EVs staining for podocyte-specific proteins and annexin$^+$ EVs were consistently lower in preeclampsia compared to normotensive pregnancies, likely due to an increase in denominator, i.e., the absolute number of annexin$^+$ EVs in preeclampsia. This suggests that

nephron structures other than podocytes may be affected in preeclampsia in a way that results in EVs generation.

A recent comprehensive study of urinary EVs demonstrated a variety of EVs that were positive for cell-specific markers from different nephron segments, including podocytes, parietal cells, proximal tubule, thin and thick loop of Henle, distal tubule, and collecting duct.[11] Urinary EVs to date have been studied as markers of renal disease in several disease entities, including glomerular diseases such as focal segmental sclerosis,[25] and diabetic nephropathy.[26] An increase in podocyte-specific EVs has been viewed as a marker of direct glomerular injury, while a reduction may herald podocyte loss and related chronic disease. Our study extends previous findings to acute renal injury in preeclampsia by demonstrating that elevation of podocyte-specific EVs is a marker of direct podocyte damage, and by implicating HbF as the proximate cause for such injury.

An acute increase in glomerular permeability through oxidative stress was reported in an HbF rat kidney perfusion model.[27] Additional evidence for the injurious effects of HbF was provided in a model of preeclampsia in rabbits, induced by species-specific cell-free HbF infusions:[15] podocytes exhibited ultrastructural evidence of mitochondrial and endoplasmic reticulum swelling along with apoptosis. Constituents of the hemoglobin molecule such as its tetrapyrrole heme prosthetic group and the iron it contains are cytotoxic because of their oxidant, inflammatory, and pro-apoptotic effects.[14, 15, 28] The potential of HbF to inflict cell injury needs to be viewed within the context of the endogenous elimination pathways that bind hemoglobin (haptoglobin), sequester its free heme moiety (hemopexin and α_1-microglobulin) and those that degrade heme, heme oxygenase (HO)-1. Interestingly, aspects of these pathways are receiving increasing interest with respect to pregnancy.

For example, elevated heme levels, achieved by either the administration of exogenous heme in wild-type mice, or by using heme oxygenase (HO)-1 knockout mice, were shown to recapitulate several of the critical findings in preeclampsia, including suboptimal placentation followed by IUGR and fetal lethality.[29] The classical preeclamptic renal lesion of endotheliosis was present in the pregnant ewe preeclampsia model whereby starvation leads to the signs and symptoms of preeclampsia via hemolysis.[14] Renal endotheliosis was absent in rescue experiments where starved animals were treated with the heme scavenger, α_1-microglobulin, further supporting the role of free heme in inducing renal injury in preeclampsia,[14] and raising the exciting possibility of α_1-microglobulin serving as a therapeutic agent in preeclampsia. α_1-microglobulin binds and degrades heme, and originates from placental tissue in response to oxidative stress.[13]

An important limitation of our study is that we did not include adult hemoglobin. Elevated free adult hemoglobin may contribute to the overall heme burden, particularly in HELLP syndrome, where intravascular hemolysis occurs. We have compared women with HELLP (n=5) to those with preeclampsia without HELLP (n=44) and have found no differences in plasma analytes and urinary EVs or their ratios (data not shown). We performed, in addition, a sensitivity analysis by excluding five HELLP cases and by comparing 44 cases of preeclampsia to 42 normotensive controls. Again, the results were similar (data not shown).

Our study has several important strengths. It utilized well-defined clinical samples, and all analyses were performed in a blind manner. Our results provide preliminary evidence that heme-induced renal toxicity may be a mechanism of renal injury in preeclampsia. It sets the stage for future studies that will aim to characterize the content of EVs in preeclampsia; to determine

whether urinary EVs expressing podocyte-proteins predate proteinuria in preeclampsia; and to study the dynamics of EVs after preeclamptic pregnancies as a marker of ongoing renal injury that may potentially lead to permanent renal damage.

CONCISE METHODS

Patient recruitment strategy and study sample collection

Our participants were retrospectively identified from an on-going prospective Swedish study of women diagnosed with preeclampsia and normotensive pregnancies. The study started in 2006 and has recruited close to 900 participants to date. A random sample of 98 women with preeclampsia and 47 women with normotensive pregnancies (recruited between 2006 and 2011) was initially identified for a study of heme-related metabolites as potential biomarkers of preeclampsia.[17] The current study included 92 of these 145 study participants for whom urine samples were collected up to 24-hours prior to delivery and were available for EVs analyses: 43 women with normotensive pregnancies and 49 with preeclampsia. The study was approved by the Lund University Hospital institutional review board and all participants gave written informed consent. Urine samples were sent to the research laboratory at Mayo Clinic for the analysis of urinary EVs positive for podocyte proteins in a blind fashion.

Normotensive, preeclamptic, eclamptic and HELLP (**H**emolysis, **E**levated **L**iver enzymes, and **L**ow **P**latelet count) syndrome pregnancies were defined based on published criteria.{, #890;Jones, 1998 #305} Exclusion criteria included having gestational hypertension, essential hypertension and/or gestational diabetes. Maternal venous blood and urinary samples were collected prior to delivery. The 2-mL aliquots of unspun urine samples were stored at -80°C until the time of analysis. All clinical identifiers were removed to secure sample processing in a blind fashion prior to sending the samples to Mayo Clinic.

Characterization and quantification of urinary EVs

Urine sample preparation for EVs analysis by digital flow cytometry

Frozen urine samples were thawed in 37°C water bath for 5 min. Each sample was thawed twice: the first thaw to perform a concentration check, and the second thaw to stain with a pre-determined set of antibodies for flow cytometric analysis using our standardized methodology.[11] Each urine sample was stained with annexin-V, nephrin, podocin, and synaptopodin.

Digital flow cytometry analysis of urinary EVs

A digital flow cytometer (FACSCanto ™) was used to perform the analysis of urinary EVs. The flow cytometer settings and gates for recording events and analysis were recently published.[11] The absolute counts of single and double fluorescently labeled urinary EVs were expressed as urinary EV/µL of urine using the standardized method previously described.[10, 11, 30] The number of podocyte protein-specific positive EVs was normalized to annexin$^+$ EVs. The number of EVs was not expressed as a ratio to urine creatinine concentration because podocytes are situated on the outer aspect of the glomerular basement membrane, and, notably, EVs originating from podocytes are not subject to glomerular filtration. In contrast, urine metabolites that commonly are normalized by the creatinine concentration in the respective urine sample originate in the blood and are filtered in the urine, thereby requiring to be factored for urine creatinine concentration so as to control for the concentration/dilution of urine. In addition, we calculated the ratio between nephrin$^+$ EVs/ podocin$^+$ EVs ratio as previous studies have indicated that the nephrin mRNA/ podocin mRNA ratio may serve as a marker of disease activity/progression.[24]

Plasma analytes

Plasma uric acid, creatinine, and cystatin C concentrations were measured by standard methods on a Cobas 6000 (Roche Diagnostics Limited, Rotkreuz, Switzerland) in the Clinical Chemistry Laboratory at the Skåne University Hospital in Lund, Sweden. Cell-free HbF, haptoglobin, α_1-microglobulin, and hemopexin plasma concentrations were determined as described previously.[17]

For a more detailed description of the methods, see the Supplemental Material.

1. Statistical Analysis

Quantitative variables are expressed as mean values with standard deviations, or as medians with interquartile ranges (for data with no Gaussian distribution). Categorical data are presented as absolute numbers with percentages. The normal distribution of each variable was tested by the Kolmogorov-Smirnov's test. The Student's t test and Mann Whitney U test were used to assess differences in quantitative variables between the pregnancy groups (preeclamptic vs. normotensive pregnancies) for independent samples. Categorical variables were analyzed using the chi-square test. The accuracy of the specific EV markers and nephrin^{+} EVs/ podocin^{+} EVs ratio in distinguishing a clinical diagnosis of preeclampsia from a normotensive pregnancy was assessed by means of the Receiver Operating Characteristic (ROC) curve. As the ROC curve is a graph of sensitivity versus 1-specificity for various cut-off points of a positive diagnostic test result, the optimal cut off level was established by the model. Correlations among various parameters in the studied population were analyzed using Pearson correlation or the Spearman correlation coefficient according to data distribution. Statistical analysis was performed using SPSS (SPSS for Windows, version 21.0, SPSS, Chicago, IL). $P < 0.05$ was considered to be statistically significant.

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DISCLOSURES

Dr. Garovic is the inventor of technology referenced in this article. That technology has been patented by Mayo Clinic, but is currently not licensed.

All other authors report no conflict of interest.

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Table 1. Baseline characteristics of the women with preeclampsia and normotensive pregnancies

Characteristic	**Normotensive pregnancies (n=43)**	**Preeclamptic pregnancies (n=49)**	**p value**
Maternal age, y; mean (±SD)	29.16±3.48	30.4 (5.49)	0.190
BMI at booking mean (±SD)	25.17±4.56	25.14 (4.01)	0.980
Parity >1, n (%)	7 (16.3%)	14 (28.6%)	0.234
Singleton pregnancy, n (%)	43 (100%)	47 (95.9%)	0.497
GW delivery median (IQR)	40 (39-41)	38 (35-39)	<0.001
Birth weight, g median (IQR)	3700 (3320-3925)	2940 (1981-3490)	<0.001
Induction, n (%)	10 (23.3%)	29 (59.2%)	0.001
Cesarean section, n (%)	13 (30.2%)	24 (49.0%)	0.067
Systolic BP at delivery, mmHg mean (±SD)	123.00 (10.42)	162.6 (18.39)	<0.001
Diastolic BP at delivery, mmHg mean (±SD)	77.29 (7.97)	103.14 (8.76)	<0.001
Urine protein dipstick, n (%)			
<1	41(95.4%)	9 (18.7%)	<0.001
1	1 (2.3%)	14 (29.1%)	
>1	1 (2.3%)	25 (52.2%)	
Late preeclampsia	NA	39 (79.6%)	
Eclampsia, n (%)	NA	3 (6.1%)	
HELLP, n (%)	NA	5 (10.2%)	
Abruption, n (%)	0	2 (4.1%)	
IUGR, n (%)	0	5 (10.2%)	

BMI, body mass index; GW, gestational weeks; HELLP, Hemolysis, Elevated Liver enzymes, and Low Platelet count syndrome; IQR, interquartile range; IUGR, intrauterine growth restriction defined as fetal growth 2 standard deviations below the average for gestational age; SD, standard deviation

Table 2. Biochemical characteristics of the women with preeclampsia and normotensive pregnancies at the time of delivery

Characteristic	**Normotensive pregnancies (n=43)**	**Preeclamptic pregnancies (n=49)**	**P value**
Cystatin C, mg/l; mean (±SD)	1.19±0.27	1.44±0.35	0.001
Uric acid, mg/dl; mean (±SD)	4.50±1.07	6.68±1.77	<0.001
Creatinine, mg/dl; mean (±SD)	0.62±0.13	0.77±0.20	<0.001
Fetal hemoglobin, ng/ml; median (IQR)	2.14 (1.58-3.43)	2.98 (1.85-5.86)	0.015
α_1-microglobulin, μg/ml; mean (±SD)	30.13±6.98	33.54±8.19	0.036
Haptoglobin, mg/ml; median (IQR)	1.15 (0.95-1.55)	0.73 (0.47-1.15)	<0.001
Hemopexin, mg/ml; mean (±SD)	0.94±0.15	0.68±0.16	<0.001

IQR, interquartile range; SD, standard deviation

Table 3. Extracellular vesicles that stain for podocyte-specific proteins and annexin-V in preeclampsia versus normotensive pregnancies at the time of delivery

Number of EVs/μl urine	**Normotensive pregnancies (n=43)**	**Preeclamptic pregnacies (n=49)**	**p value**
Annexin^{+}; median (IQR)	260 (133-1302)	1052 (394-3189)	0.008
Podocin^{+}; median (IQR)	1155 (308-2551)	3120 (1334-7382)	0.037
Nephrin^{+}; median (IQR)	337 (147-1035)	2248 (917-4707)	<0.001
Synaptopodin^{+}; median (IQR)	1361 (455-2923)	2680 (1208-5990)	0.095

IQR, interquartile range

Table 4. Performance characteristics of extracellular vesicles that stain for podocyte-specific proteins and annexin as diagnostic tools in distinguishing preeclamptic versus normotensive pregnancies

Diagnostic test statistic	**Annexin**	**Podocin**	**Nephrin**	**Synaptopodin**
Cut-off value	500	1400	550	2000
Sensitivity	71.4	73.5	87.8	59.2
Specificity	61.9	60.5	72.1	60.5
Positive predictive value	68.6	67.9	78.2	63.0
Negative predictive value	65.0	66.7	83.8	56.5

Table 5. Ratios of extracellular vesicles that stain for podocyte-specific proteins and annexin-V in preeclampsia versus normotensive pregnancies at the time of delivery

Characteristic	**Normotensive pregnancies (n=43)**	**Preeclamptic pregnancies (n=49)**	**P value**
Podocin/annexin-V; median (IQR)	4.04 (1.10-6.04)	1.23 (0.34-2.50)	0.001
Synaptopodin/annexin-V; median (IQR)	5.19 (1.48-9.22)	2.03 (0.74-5.41)	0.036
Nephrin/annexin-V; median (IQR)	1.10 (0.60-1.36)	0.33 (0.10-0.79)	<0.001
Nephrin/podocin; mean (±SD)	0.42±0.19	0.86±0.15	<0.001

IQR, interquartile range; SD, standard deviation

Table 6. Performance of urinary nephrin^{+} EVs/ podocin^{+} EVs ratio>0.6 as a diagnostic test for preeclampsia

Diagnostic test statistic	Performance	95% CI
Sensitivity	91.8% (45/49)	80.4-97.7%
Specificity	86.0% (37/43)	72.1-94.7%
Positive predictive value	88.2% (45/51)	76.1-95.6%
Negative predictive value	90.2% (37/41)	76.9-97.3%
Accuracy	89.1% (82/92)	82.8-95.5%

CI, Confidence interval

Table 7. Correlation between urinary nephrin^{+} EVs/ podocin^{+} EVs ratio and biochemical parameters

Variable	**r**	**ρ**	**p value**
Cystatin C, mg/l	0.283		0.008
Uric acid, mg/dl	0.531		<0.001
Creatinine, mg/dl	0.343		0.001
Fetal hemoglobin, ng/ml		0.268	0.011
α_1-microglobulin (μg/ml)	0.162		0.122
Haptoglobin (mg/ml)		-0.293	<0.001
Hemopexin (mg/ml)	-0.496		<0.001

r, Pearson correlation coefficient; ρ, Spearman correlation coefficient

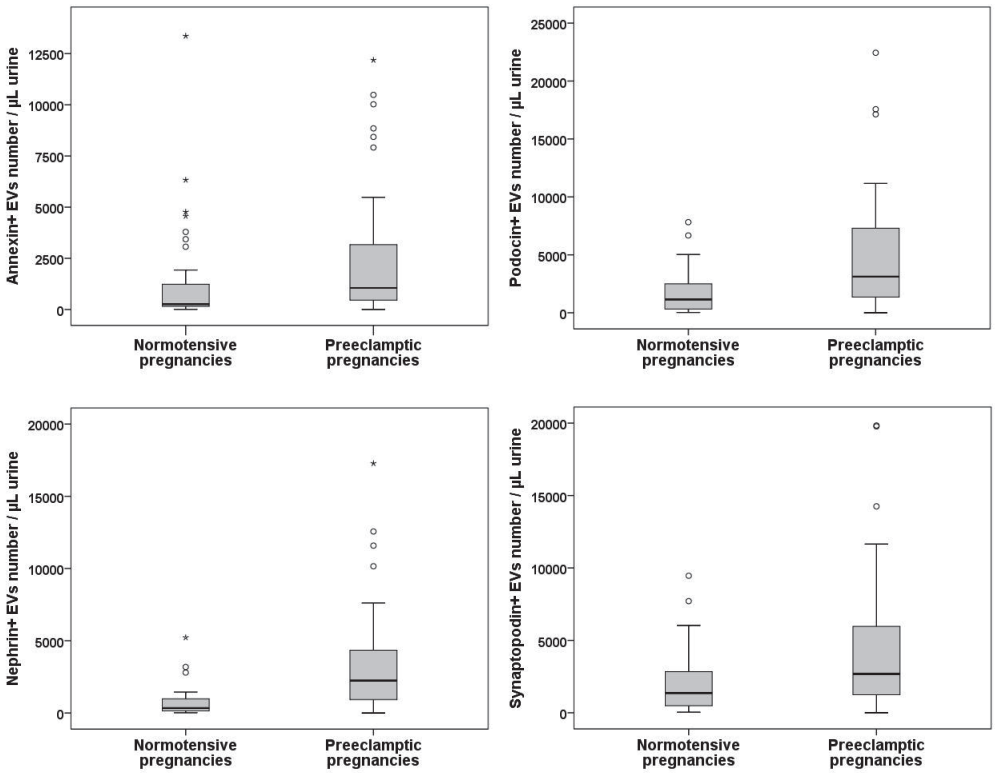

Figure 1. Extracellular vesicles that stain for podocyte-specific proteins and annexin in preeclamptic versus normotensive pregnancies

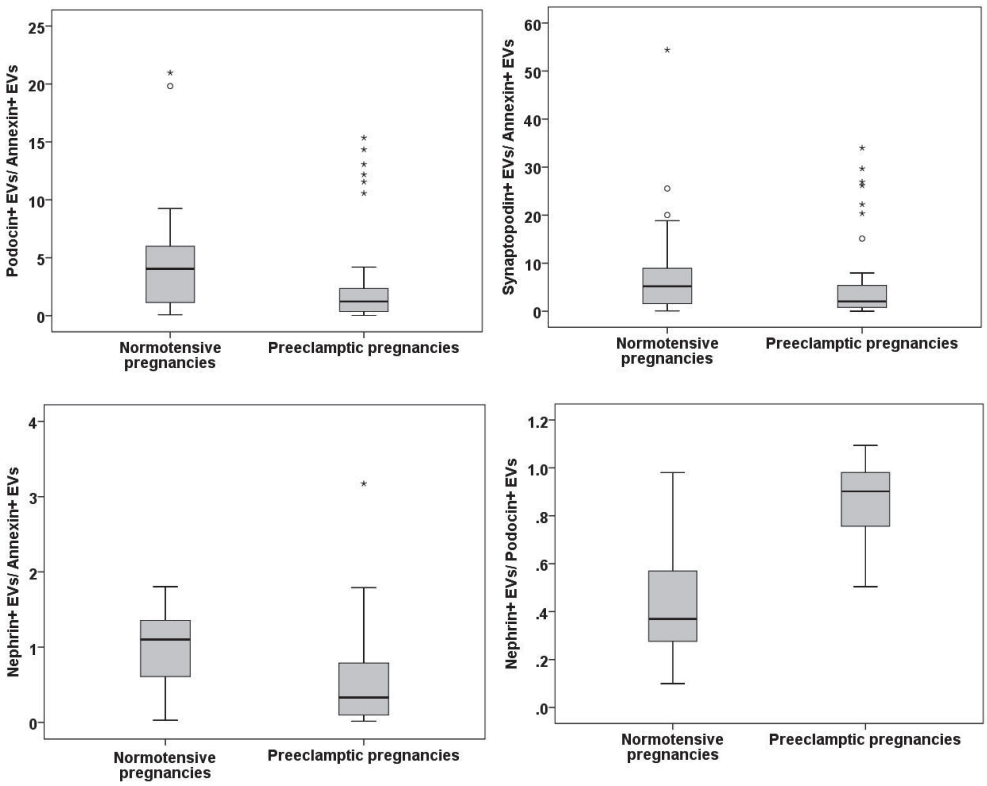

Figure 2. Ratios of extracellular vesicles that stain for podocyte-specific proteins and annexin in preeclampsia versus normotensive pregnancies

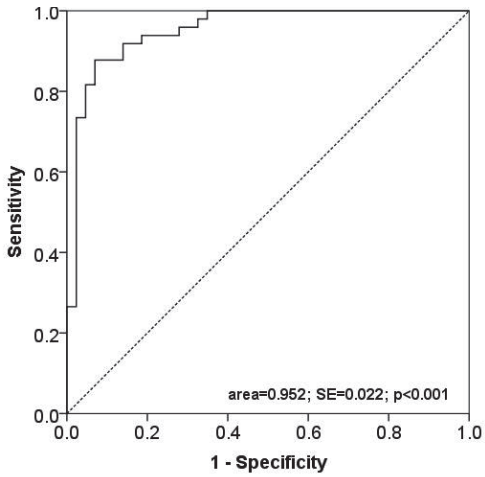

Figure 3. Receiver operating curve for nephrin^{+} EVs/ podocin^{+} EVs ratio as a novel biomarker of acute renal injury

Urinary Extracellular Vesicles Positive for Podocyte-specific Proteins are Associated with Renal Injury in Preeclampsia

Supplemental Material

Patient recruitment strategy and study sample collection

A normotensive pregnancy was defined as a previously healthy woman with normal blood pressures throughout pregnancy, who gave birth at a gestational age between 37 and 42 weeks of gestation. Preeclampsia was defined as *de novo* hypertension and proteinuria after 20 weeks of gestation. Hypertension was defined as having a blood pressure ≥140/90 mmHg on 2 occasions at least 4 hours apart, and proteinuria as 1+ by dipstick.[1] ENREF 34 The diagnosis of HELLP syndrome (**H**emolysis, **E**levated **L**iver enzymes, and **L**ow **P**latelet count), considered to be a severe form of preeclampsia, was confirmed by the presence of microangiopathic hemolytic anemia, elevated liver enzymes, and thrombocytopenia.[2] Progression of preeclampsia to its convulsive form, eclampsia, was diagnosed in the presence of new-onset seizures. Intrauterine growth restriction (IUGR) was defined as fetal growth 2 standard deviations below the average for gestational age. Exclusion criteria included having gestational hypertension, essential hypertension and/or gestational diabetes.

Maternal venous blood and urinary samples were collected prior to delivery at the Department of Obstetrics and Gynecology, Lund University Hospital, Sweden. Six-ml blood samples were collected in EDTA Vacuette® plasma tubes (Greiner Bio-One GmbH, Kremsmünster, Austria) and centrifuged at 2000 x g for 20 minutes at room temperature (RT). The plasma was then transferred into cryo tubes and stored at -80°C for later analysis. Urine was collected in a 10 mL Sarstedt tube containing 300uL of a protease inhibitor cocktail as previously described.[3] The 2-mL aliquots of unspun urine samples were stored at-80°C until the time of analysis.

Characterization and quantification of urinary EVs

Concentration checks for all samples were performed using a fixed urine volume (20uL) diluted in HEPES/Hanks' (H/H) buffer (80uL) and incubated with annexin-V FITC (3uL) and CD9 PE (3uL) for 30 min. The purpose of the concentration check was to determine the optimal urine volume required to yield total urinary EVs of 10,000 or more, and annexin-V FITC positive urinary EVs of 2500 or more. Each urine sample was stained with a pre-determined panel of antibodies which consisted of the following: annexin-V FITC (3uL; undiluted), Nephrin FITC (3uL; dilution, 1:10), Podocin PE (3uL; dilution, 1:10), Synaptopodin PE (3uL; dilution, 1:10) and annexin-V PE (3uL; undiluted) after determining the urine volume needed. The urine samples were diluted in twice filtered H/H buffer and mixed with fluorophore conjugated antibodies/recombinant proteins, and then ncubated for 30 min in the dark; samples containing EVs were analyzed following the addition of 800uL of H/H buffer and 100uL of TruCOUNT beads prepared in H/H buffer.[4]

Antibodies and Reagents

Phycoerythrin (PE) and/or fluorescein isothiocyanate (FITC) conjugated recombinant Annexin-V, mouse anti-human CD9, and TruCOUNT™ beads were purchased from BD Biosciences, San Jose, CA USA. Rabbit anti-synaptopodin and anti-podocin polyclonal antibodies conjugated with PE, and rabbit anti-nephrin polyclonal antibodies conjugated with FITC were purchased from Bioss, Woburn, MA. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and Hanks' balanced salts were purchased from Sigma Chemicals Co., St. Louis, MO USA. All reagents and antibodies were filtered twice through 0.2 μm membrane filters to decrease chemical particles of similar size to urinary EVs.

Plasma analytes

HbF was determined by an in-house developed enzyme-linked immunosorbent assay (ELISA), α_1-microglobulin by an in-house developed radioimmunoassay, and haptoglobin and hemopexin were measured by commercially-available ELISA Quantification Kits (Genway Biotech Inc, San Diego, CA USA).

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