Biochemical and Epidemiological Studies of Early-Onset and Late-Onset Pre-Eclampsia

ANNA-KARIN WIKSTRÖM
Dissertation presented at Uppsala University to be publicly examined in Auditorium Minus, Museum Gustavianum, Akademigatan 3, Uppsala, Friday, November 23, 2007 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Biochemical and epidemiological aspects of pre-eclampsia were investigated, with the main focus on possible pathophysiological differences between early-onset and late-onset disease.

In pre-eclamptic women poor correlation was found between albumin-creatinine ratio (ACR) in a random urine sample and total amount of albumin in a 24-hour urine collection. (Paper I)

In a cohort of women giving birth in Sweden in 1973-82 we estimated the adjusted incidence rate ratio (IRR) for ischaemic heart disease (IHD) during the years 1987–2001. The adjusted IRR for development of IHD was 1.6-2.8 in woman exposed to gestational hypertensive disease during her pregnancy compared with unexposed women. The higher risk represents more severe or recurrent hypertensive disease. (Paper II)

Before delivery, in early-onset pre-eclampsia (24-32 weeks) there were pronounced alterations in plasma concentrations of soluble fms-like tyrosine kinase 1 (sFlt1) and placental growth factor (PIGF), and also a higher placental 8-iso-PGF\_2\alpha concentration and an elevated serum ratio of plasminogen-activator inhibitor (PAI)-1 to PAI-2 compared with early controls. In late-onset pre-eclampsia (35-42 weeks) there were only moderate alterations in sFlt1 and PIGF concentrations, and the placental 8-iso-PGF\_2\alpha concentration and PAI-1/PAI-2 ratio were similar to those in late controls. (Papers III, V) There was a rapid postpartum decrease in sFlt1 concentration in all groups. One week postpartum the sFlt1 concentration was persistently higher, however, in women with early-onset pre-eclampsia compared with early controls. (Paper IV)

In conclusion: random ACR cannot replace 24-hour urine collections for quantification of albuminuria in pre-eclamptic women; gestational hypertensive disease, especially severe or recurrent, increases the risk for later IHD; early-onset, but not late-onset pre-eclampsia is associated with pronounced alterations of angiogenesis-related markers and only early-onset pre-eclampsia is associated with placental oxidative stress and an increased PAI-1/PAI-2 ratio, all suggesting a stronger link between early-onset than late-onset pre-eclampsia and a dysfunctional placenta.

Keywords: pre-eclampsia, proteinuria, albumin-creatinine ratio, ischaemic heart disease, angiogenesis, sFlt1, oxidative stress, isoprostane, PAI-1, PAI-2

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“You can’t help respecting anybody who can spell TUESDAY, even if he doesn’t spell it right; but spelling isn’t everything. There are days when spelling Tuesday simply doesn’t count.”

From “The House at Pooh Corner” by A.A. Milne

To: Johan, Carolina, Johanna & Carl
List of papers

This thesis is based on the following papers. They will be referred to in the text by their Roman numerals:


IV Anna-Karin Wikström, Anders Larsson, Ulf J. Eriksson, Peppi Nash, Matts Olovsson. Early postpartum changes in the pro- and anti-angiogenic markers PI GF, VEGF-A and sFlt1 in women with healthy pregnancies and early and late onset preeclampsia. Submitted


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<td>ACR</td>
<td>Albumin-creatinine ratio</td>
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<tr>
<td>AGA</td>
<td>Average for gestational age</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>BP</td>
<td>Blood pressure</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>HELLP</td>
<td>Haemolysis, elevated liver enzymes, low platelets</td>
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<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<td>ICD</td>
<td>International classification of diseases</td>
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<td>IHD</td>
<td>Ischaemic heart disease</td>
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<td>IRR</td>
<td>Incidence rate ratio</td>
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<td>ISSHP</td>
<td>International society for the study of hypertension in pregnancy</td>
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<td>IUGR</td>
<td>Intrauterine growth restriction</td>
</tr>
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<td>LGA</td>
<td>Large for gestational age</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor type 1</td>
</tr>
<tr>
<td>PAI-2</td>
<td>Plasminogen activator inhibitor type 2</td>
</tr>
<tr>
<td>PCR</td>
<td>Protein-creatinine ratio</td>
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<tr>
<td>PE</td>
<td>Pre-eclampsia</td>
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<td>PI GF</td>
<td>Placental growth factor</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>sFlt1</td>
<td>Soluble fms-like tyrosine kinase 1</td>
</tr>
<tr>
<td>SGA</td>
<td>Small for gestational age</td>
</tr>
<tr>
<td>STBM</td>
<td>Syncytiotrophoblast microparticles</td>
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<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor type A</td>
</tr>
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</table>
Introduction

Eclampsia has been recognised since the time of Hippocrates and the name eclampsia has its origin from the Greek word for “lightning”.\textsuperscript{127, 136} For a long time eclampsia was looked upon as a pregnancy-specific seizure disorder. In the beginning of the 20\textsuperscript{th} century new-onset hypertension and proteinuria became accepted as warning signs of eclampsia (pre-eclampsia).\textsuperscript{26} Pre-eclampsia (PE) is now known to be a multi-system disease and can include placental dysfunction, acute renal failure, cerebral oedema, cerebral haemorrhage, seizures (eclampsia), coagulopathy and liver injury.\textsuperscript{1}

The disease is a leading cause of maternal and perinatal morbidity and mortality.\textsuperscript{71, 128} Worldwide, each year, more than four million women develop PE and in developing countries maternal mortality due to this disease is common, accounting for about 50 000 deaths yearly.\textsuperscript{71} The foetus can be affected by intrauterine growth restriction and hypoxia.\textsuperscript{101} PE is also associated with abruptio placentae, with major risks for both the mother and foetus. It has been estimated that 15\% to 25 \% of all preterm births,\textsuperscript{44, 169} with their increased mortality rates and long-range neurological disability, are early deliveries due to PE. The disease can also be mild, usually with onset near to term of pregnancy or intrapartum, with a negligibly increased risk for an adverse pregnancy outcome.\textsuperscript{147, 166} There is no known treatment for PE except for delivering the child (i.e. the placenta), even if it is a long time before term.

The pathophysiology of PE is not completely understood. It has been termed the “disease of theories”,\textsuperscript{17} reflecting the confusion that surrounds its causes and pathophysiology. The understanding of the disease has considerably increased in the past years, but there is also increasing evidence of its heterogeneity, with possible differences in the pathogenesis between subgroups of women.\textsuperscript{97}

Definition of pre-eclampsia

An international agreement was reached a few years ago regarding the definition of PE,\textsuperscript{1, 18} simplifying the comparison between different studies.

According to the International Society for the Study of Hypertension in Pregnancy (ISSHP) the definitions of PE are as follows:\textsuperscript{18}
Research definition

- De novo hypertension after gestational week 20. Hypertension is defined as a systolic blood pressure (BP) ≥140 mmHg and/or diastolic BP≥90 mmHg. Normalisation of blood pressure within 3 months postpartum.

AND

- Proteinuria ≥300 mg/24 hour or a spot urine protein/creatinine ratio ≥30 mg/mmol.

Clinical definition

- De novo hypertension after gestational week 20, AND new onset of one or more of the following:
  - Proteinuria (≥300 mg/24 hour or spot urine protein/creatinine ratio ≥30 mg/mmol)
  - Renal insufficiency: S-creatinine ≥0.09 mmol/L or oligouria
  - Liver involvement: raised S-transaminases and/or severe right upper quadrant or epigastric pain
  - Neurological disorders: convulsions (eclampsia), hyperreflexia with clonus, severe headaches with hyperreflexia, persistent visual disturbances (scotoma)
  - Haematological disorders: thrombocytopenia, disseminated intravascular coagulation, haemolysis
  - Foetal growth restriction

Diagnosis of pre-eclampsia

Diagnostic instruments that are reliable, rapid, and easy to handle are important in screening for and follow-up of PE. Women diagnosed with PE are monitored with frequent examinations, since an initially mild disease may progress rapidly and become severe.

One sign of aggravation of the disease is massive proteinuria. Pre-eclampsia is considered severe if:

- the hypertension is pronounced (systolic blood pressure ≥ 160 mm Hg and/or ≥ 110 diastolic blood pressure )
- and/or the proteinuria is severe (≥ 5 g/24 hours)
- and/or there is multi-organ involvement.

The present “gold standard” for quantification of excreted total protein or albumin is measurement based on 24-hour urine collection. When PE develops, excretion of urinary total protein (or albumin) is monitored with frequent 24-hour samples. This method, however, is time-consuming, inconvenient and not always reliable, because of the difficulty in collecting such a sample correctly.
The degree of proteinuria may fluctuate widely over a 24-hour period and spot urine samples are therefore usually not recommended for quantification of protein excretion. In the presence of a stable glomerular filtration rate, urinary creatinine is excreted at a fairly constant rate, which makes it useful as an internal reference. When calculating the ratio between protein (or albumin) and creatinine, variations in urine volume should be taken into account. In the ISSHP definition of PE, spot urine protein-creatinine ratio (PCR) is accepted as a method for identification of significant proteinuria. However, only a small number of women with significant proteinuria have been included in most previous studies and the reliability of urine PCR as a tool for indirect quantification of proteinuria in women with manifest PE cannot therefore be considered to be fully established.

Prevalence of pre-eclampsia
PE complicates 3–5% of all pregnancies.

Risk factors for pre-eclampsia

Preconceptional and/or chronic risk factors
- Primiparity
- Limited sperm exposure
- Partner who has fathered a PE pregnancy in another woman
- History of previous PE
- Increasing maternal age, long interval between pregnancies
- Family history
- Patient requiring oocyte donation
- Chronic hypertension
- Renal disease
- Obesity, insulin resistance, low maternal weight
- Gestational diabetes, type-1 diabetes mellitus
- Activated protein C resistance, protein S deficiency
- Antiphospholipid antibodies
- Hyperhomocysteinaemia
- Maternal stress

Pregnancy-associated risk factors
- Multiple pregnancy
- Urinary tract infection
- Structural congenital anomalies
- Hydrops fetalis
Pathophysiology of pre-eclampsia

A 2-stage model of PE has been proposed as a means of addressing its pathophysiology.126

The first stage

In 1939 E W Page formulated the concept that placental perfusion was reduced in PE.103 Evidence for this came originally from clinical findings. During the 1970s the association between poor placentation and PE was established.16 During normal placentation, cytotrophoblast cells cross the placental-maternal bridges and invade the maternal decidua and adjacent spiral arteries. They replace the maternal endothelium in the spiral arteries and then invade the media, with resulting destruction of the elastic, muscular and neural tissue. The cytotrophoblast cells become incorporated into the wall of the vessel. These changes create a low resistance to flow and absence of maternal vasomotor control, leading to an enormous increase in blood supply to the growing foetus. By the 20th week of gestation, this process is more or less complete.119 In the preclinical stage of PE, the endovascular cytotrophoblast invasion is restricted, resulting in impaired arterial remodelling ("shallow placentation").16, 119

The aetiology of shallow placentation is unknown, but maternal-foetal immune adaptation could be a main cause.127 In the decidua, trophoblast cells are confronted by natural killer cells.119 During normal pregnancy, these immune cells probably facilitate deep invasion of the trophoblast cells into the myometrial segments and promote extensive spiral artery remodelling. Killer immunoglobulin receptors (KIRs) on natural killer cells interact with specific trophoblast cell markers and this interaction may influence the trophoblast invasion.85 The natural killer cells also have a role in producing several cytokines that are implicated in angiogenesis and vascular stability, including vascular endothelial growth factor (VEGF) and placental growth factor (PIGF).85 Invasive cytotrophoblasts express several angiogenic factors, including VEGF-A, PI GF, and also their soluble receptor VEGFR-1(sFlt1).142
**Figure 1A** During normal placentation endovascular cytotrophoblast cells replace the endothelium of spiral arteries and invade the media, resulting in destruction of the medial elastic, muscular and neural tissue and thus leading to an increased blood supply to the growing foetus. **1B** During abnormal placentation, the first stage of pre-eclampsia, the endovascular cytotrophoblast cell invasion is restricted and the blood supply to the growing foetus is limited. NK= natural killer. From Redman & Sargent SCIENCE 308: 1592-1594 (10 June 2005). Reprinted with permission from AAAS.

The second stage

The clinical features of PE appear to arise from a generalised systemic inflammatory response, of which endothelial dysfunction is a prominent component. Several serum markers of endothelial activation are altered in women with PE, including von Willebrand antigen, cellular fibronectin, and endothelin. Another marker of endothelial-cell activation is plasminogen activator inhibitor type 1 (PAI-1). PAI-1 is synthesised mainly by endothelial cells, but also by activated platelets and neutrophils. PAI-1 is an important inhibitor of fibrinolysis which exerts its effect through inhibition of tissue plasminogen activator and urokinase. The concentration of PAI-1 is supposed to be useful as a discriminator between normal and pre-eclamptic pregnancies when it is divided by PAI-2 (PAI-1/PAI-2 ratio), as an increased ratio predates the onset of clinical signs of the disease. PAI-2 is selectively produced by trophoblasts (sometimes called “placental PAI”) and is therefore considered to be a good marker of placental function.
The endothelial dysfunction can give rise to vasospasm due to a decrease in production and activity of vasodilator prostaglandins, especially prostacyclin and nitric oxide, but also by causing an increased sensitivity to pressor agents.\textsuperscript{25, 147}

Endothelial dysfunction can also give rise to activation of the coagulation cascade, with formation of occlusive microthrombi and a loss of fluid from the intravascular space.\textsuperscript{71} All these components contribute to a reduced perfusion, which is seen in virtually any organ (uterus included) examined in women with PE.\textsuperscript{128}

In the kidney, endothelial damage results in proteinuria and produces the characteristic pathological lesion glomerular endotheliosis.\textsuperscript{42} Glomerular endotheliosis is characterised by generalised swelling and vacuolisation of the endothelial cells. Although once considered pathognomonic for PE, recent studies have shown that mild glomerular endotheliosis may also occur in a significant percentage of normal pregnancies at term, but is more severe in PE.\textsuperscript{158}

The linkage between the two stages

The first stage in the 2-stage model is completed before the 20\textsuperscript{th} week and prior to the appearance of clinical signs. In the second and third trimesters of pregnancy, the placenta requires increasing access to the maternal blood supply. As a result of the first stage, leading to a reduced uterine perfusion, the placenta becomes increasingly hypoxic. This hypoxic and dysfunctional placenta is considered to release factors into the maternal circulation that eventually cause the clinical features of PE.\textsuperscript{126} These released factors act as the linkage between the two stages and their identification could hopefully enable the development of therapies for prevention of the clinical stage development.

The identity of the “pre-eclampsia factor” has been elusive, however. Several candidate factors have been studied. Two major candidates are the anti-angiogenic factor sFlt1\textsuperscript{20, 83} and oxidative stress.\textsuperscript{126}

Angiogenesis and pre-eclampsia

There is growing evidence that an alteration of the relation between factors promoting angiogenesis such as VEGF or PI GF and factors antagonising angiogenesis such as sFlt1 have a fundamental role in the pathogenesis of pre-eclampsia.\textsuperscript{3, 83, 156}

VEGF is important both in angiogenesis and in the maintenance of endothelial cell health in the basal state.\textsuperscript{83, 92} PI GF is a VEGF-related molecule and the function of PI GF is still ill-defined, but it appears to act synergistically with VEGF.\textsuperscript{20} VEGF has a family of receptors, the most important of which are Flt1 (VEGFR-1) and Flk1 (VEGFR-2).\textsuperscript{159} SFlt1 is the
soluble form of the Flt1 receptor. It antagonises VEGF and PlGF in the circulation by binding them and preventing their angiogenic effects. SFlt1 is produced in excess quantities by hypoxic trophoblasts. Several studies have shown elevated circulating concentrations of sFlt1 and decreased concentrations of free (unbound) VEGF and free PlGF in the clinical stage of PE. These changes appear to antedate the onset of clinical disease by some weeks.

Maynard et al have reported the occurrence of a syndrome resembling PE, including hypertension, proteinuria, and glomerular endotheliosis, after adenoviral gene transfer of sFlt1 to pregnant rats. The soluble form of Flk1 (which does not antagonise PlGF when given exogenously), did not induce a pre-eclamptic phenotype in pregnant rats. This suggests that antagonism of both VEGF and PlGF is necessary to induce the maternal syndrome.

An association between impaired angiogenesis and PE seems to be evident, but only in a few studies have the altered concentrations of pro- and anti-angiogenic factors accompanying PE been shown to become normalised after delivery.

Oxidative stress and pre-eclampsia

Free radical production occurs continuously in all cells as part of their normal function. Oxidative stress is defined as disequilibrium between the production of free radicals and antioxidant defences in favour of the first. A radical is an atom or group of atoms that have one or more unpaired electrons which give them an extremely high chemical reactivity. In biological systems reactive oxygen species (ROS) are of most concern. Superoxide anion radicals are the most common type of ROS, and hydroxyl radicals and nitric oxide radicals are also rather common examples. Some ROS, e.g. hydroxyl ions and peroxynitrite, do not have unpaired electrons, but nevertheless act as a radical. ROS are able to damage all macromolecules, including lipids, proteins and nucleic acids, and endothelial-cell activation can be evoked through many pathways.

Reactive oxygen radicals (ROS)

\[ \cdot \text{O}_2^+ \text{H} \quad \cdot \text{O}_2^- \quad \cdot \text{OH} \quad \cdot \text{OH}^- \]

Oxygen \[ \text{O}_2 \]
Superoxide anion \[ \text{O}_2^- \]
Hydroxyl radical \[ \cdot \text{OH} \]
Hydroxyl ion \[ \cdot \text{OH}^- \]
Generation of free radicals increases during pregnancy, and placental mitochondria are the major sources of ROS production. In pre-eclamptic pregnancies there is an increase both in the number of mitochondria and in the activity of the mitochondrial enzyme, both of which changes result in increased generation of ROS. Other enzymatic pathways that generate ROS are NADPH oxidase and xanthine oxidase. It is proposed that in women with PE repeated ischaemic/reperfusion insults in the placenta stimulate superoxide generation especially through the xanthine oxidase pathway.

Placental oxidative stress is also probably a cause of the increased amount of syncytiotrophoblast debris (also known as syncytiotrophoblast microparticles (STBM)) found in the maternal circulation of pre-eclamptic women. This STBM has been shown to cause endothelial cell dysfunction in vitro and in isolated vessels.

There are several antioxidant systems that protect against ROS, both enzymatic and non-enzymatic. Examples of enzymatic antioxidants are superoxide dismutase, thioredoxin, thioredoxin reductase, and glutathione peroxidase. The non-enzymatic antioxidants can be lipid-soluble, such as vitamin E or water-soluble antioxidant such as vitamin C. Vitamin E exists in eight forms, of which \( \alpha \)-tocopherol is the most potent antioxidant. It is now well established that there is synergy between vitamins E and C, as vitamin C is able to reduce \( \alpha \)-tocopherol radicals directly or indirectly and thus support the antioxidant activity of vitamin E. A reduction of the antioxidant capacity enhances oxidative stress, and in PE decreased concentrations of several antioxidants have been observed both in the placenta and in the blood. The findings concerning the antioxidant capacity of women with PE are inconclusive, however since other studies have shown unchanged and even increased concentrations of antioxidants in women with PE compared with women with normal pregnancy.

A reliable indicator of oxidative stress and endogenous lipid peroxidation has been shown to be 8-iso-Prostaglandin \( \Delta^{2} \) (PGF\( \Delta^{2} \)), a major isoprostane generated through non-enzymatic peroxidation of arachidonic acid. This isoprostane is a chemically stable product of lipid peroxidation and it has an inherent ability to cause vasoconstriction, endothelium derangement and platelet activation, all of which are major characteristics of the pathophysiology of PE. 8-iso-PGF\( \Delta^{2} \) exists in a free and a phospholipid-bound state in the blood and placental tissue, but only in the free form in the urine. Studies of 8-iso-PGF\( \Delta^{2} \) concentrations in the placenta, blood and urine in women with PE have yielded divergent results.
Maternal factors affecting the second stage

Shallow placentation is not unique for PE. In some pregnancies complicated by intrauterine growth restriction (IUGR), as well as in about one-third of cases of spontaneous preterm births, there is an incomplete process of spiral artery vascular transformation similar to that seen in women with PE.\(^6, 125\) Why some women develop IUGR without PE and others develop PE with or without IUGR after abnormal placentation is unclear.

Shallow placentation is strongly associated with an increased resistance in the maternal uterine arteries, as evaluated by Doppler ultrasound.\(^81\) But studies examining the usefulness of uterine artery Doppler flow velocimetry in the second trimester for prediction of PE have shown limited diagnostic accuracy.\(^27\) Thus, it appears that although shallow placentation leading to a reduced placental perfusion may be a prerequisite for PE, it is by no means sufficient. It has been proposed that the abnormal placentation has to interact with maternal factors to result in the syndrome of PE.\(^125\)

Healthy pregnancy is in itself a state of systemic inflammation, at least in the third trimester.\(^118\) On the basis of this concept, it may be said that PE is not a separate entity, but simply the extreme end of a range of maternal systemic inflammatory responses to pregnancy. Any factor (genetic/behavioural/environmental) that would enhance this response would predispose to PE.\(^126\)

It is likely that the relative contributions of reduced perfusion and maternal factors for the development of PE vary. In some pregnancies, profoundly reduced perfusion could lead to the syndrome in women with a minimal predisposing risk, while in others the maternal constitution could present such a risk that even a minimal (or no?) reduction of placental perfusion would be sufficient for the development of PE.

Many of the maternal factors predisposing to PE are also risk factors for cardiovascular disease (CVD). These include: adiposity,\(^40\) hypertension,\(^122\) diabetes mellitus,\(^149\) lack of physical activity,\(^154\) and a family history of heart disease.\(^96\)

Association between pre-eclampsia and cardiovascular disease

Both PE and CVD are associated with endothelial dysfunction and altered angiogenesis.\(^28, 41, 116\) Both diseases are also associated with the “metabolic syndrome X”.\(^95, 138\) This is a constellation of metabolic disorders that all result from the primary disorder insulin resistance. All the metabolic abnormalities associated with syndrome X can lead to cardiovascular disorders, when present as a group, the risk for CVD and premature death is very high.\(^41\)
The characteristic disorders present in metabolic syndrome X include:  
- insulin resistance
- hypertension
- abnormalities of blood clotting
- low high-density lipoprotein (HDL) and high low-density lipoprotein (LDL) concentrations
- high triglyceride concentrations

Women studied 1.5 to 17 years after an episode of PE have been found to still show higher blood pressures, sFlt1 concentrations, measures of insulin resistance (insulin, glucose, HOMA index of insulin resistance), triglycerides, and uric acid compared with women who had had normal pregnancies. Among 113 women with prior PE studied on average three years after delivery, brachial artery flow-mediated dilation was significantly reduced in women with previous PE as compared to women with previously normal pregnancies, even after adjustment for body mass index (BMI), blood pressure, family history of hypertension, and elevated lipid values. Since flow-mediated vasodilatation is endothelium (nitric oxide)-dependent, that study indicates that endothelial dysfunction appears to be associated with PE but not with normotensive pregnancy.

One hypothesis is that women with a predisposition to endothelial dysfunction and/or with subclinical/clinical features of metabolic syndrome X, are predisposed to develop PE during pregnancy. Pregnancy could act as a vascular and metabolic stress test and women with these underlying features response to pregnancy in an abnormal way. With increasing age the same predisposing factors can develop into a manifest cardiovascular disease.

![Figure 2](image)

*Figure 2. From Sattar & Greer BMJ 325: 157-159 (20 JULY 2002); Reprinted with permission from the BMJ publishing group.*

This is consistent with the higher incidence of CVD that becomes evident many years after an episode of PE.
One possible common underlying factor of PE and CVD is a change in angiogenesis. Early-onset PE, in particular, is strongly associated with both altered angiogenesis and later development of CVD. This theory is supported by a observation of elevated sFlt1 concentrations 18 months postpartum in seemingly healthy women with a history of PE, compared to women with prior normotensive pregnancies. Increased information on this issue could be obtained by determining how the plasma concentrations of pro- and anti-angiogenic factors change in the postpartum period in women with PE, and whether there are any differences between early- and late-onset PE in this respect.

Elucidation of the association between PE and later development of CVD could provide a means of identifying some women at increased risk for CVD. These women could then be advised about lifestyle risk factor modifications in order to reduce the risk for CVD, and be offered follow-up examinations for early diagnosis of CVD.

PE is primarily a disease of first pregnancy. Development of PE in later pregnancies may therefore have a stronger association with the development of CVD later in life than PE in the first pregnancy. According to the two-stage theory discussed above, a more severe form of PE may indicate which women run a greater risk of developing CVD later in life. To address the question whether risk of developing CVD in later life increases with recurrence and/or severity of gestational hypertensive disease therefore seems important.

**Early-onset and late-onset pre-eclampsia**

PE is clinically a heterogeneous disease and major differences are observed between early-onset and late-onset disease.

In a recent study of placental morphology, placentas from women with early-onset (<34 weeks) and late-onset PE (>34 weeks) were studied separately. An abnormal placental morphology was found in early-onset disease, whereas placentas from late-onset disease were morphologically similar to those from gestational age-matched controls. This result is in agreement with studies of findings on second trimester Doppler ultrasound of the uterine arteries, where increased impedance was more associated with early- than with late-onset disease. STBM, which has been shown to cause endothelial cell dysfunction in vitro, has been found in larger amounts in the maternal circulation in women with PE than in normal pregnancy. This excess shedding of STBM may be caused by hypoxia as a result of poor placentation. Recently a study separating women with early-onset PE (<34 weeks), late-onset PE (>34 weeks) and women with normotensive pregnancies complicated with IUGR, showed an increased amount of STBM only in women with early-onset PE. This also indicates a closer association between early-onset PE and poor placental function and thus probably also
poor placentation. Such an association is further supported by the clinical observation that early-onset, but not late-onset PE is accompanied by foetal involvement with intrauterine growth restriction.\textsuperscript{101}

All these findings speak in favour of the existence of two subsets of PE (early-onset and late-onset) and support for the hypothesis that late-onset PE is a maternal, and not a placental disease.

When investigating pathophysiological factors in PE it therefore seems important to separate early-onset from late-onset PE, but this has usually not been done in most previous studies. This is a possible reason for the divergent results in investigations of the association between oxidative stress and PE.\textsuperscript{9, 10, 23, 59, 84, 87, 104, 120, 155, 162} Neither have early- and late-onset PE been separated in studies of the association between impaired angiogenesis and PE.\textsuperscript{20, 21, 74, 75, 83, 130, 144, 160} Since early-onset PE is more strongly associated with impaired placentation than late-onset disease, it is reasonable to assume that the changes in pro-and anti-angiogenic factors also differ between the two groups.
The general aim of the study

The general aim of this study was to increase the knowledge about the pathophysiology of pre-eclampsia, with special emphasis on differences between early-onset and late-onset disease.

The specific aims of the separate studies were

- to assess the value of the urinary albumin-creatinine ratio for quantification of proteinuria in women with manifest pre-eclampsia (study I);

- to address the question whether the risk of developing ischaemic heart disease later in life increases with the severity and/or recurrence of hypertensive disease occurring during pregnancy (study II);

- to evaluate the associations of the anti-angiogenic factor sFlt1 and the pro-angiogenic factors PlGF and VEGF-A with pre-eclampsia and to determine whether there is any difference in this respect between early-onset and late-onset disease (study III);

- to study the longitudinal changes in the anti-angiogenic factor sFlt1 and the pro-angiogenic factors PlGF and VEGF-A in healthy pregnancy and early-onset and late-onset pre-eclampsia during the early postpartum period (study IV);

- and to evaluate the associations of biochemical markers for oxidative stress and placental dysfunction with early- and late-onset pre-eclampsia (study V).
Material and methods

Study populations

Study I

The women participating in the study were admitted to the Department of Obstetrics and Gynaecology at Uppsala University Hospital during the time period February 2002 to September 2004. All women had a new-onset hypertension defined as a blood pressure $\geq 140/90$ at two different measurements performed at intervals of more than 6 hours. Women with a repeated positive urinary test strip (Combur test, Roche) for proteinuria of 2+, corresponding to an albumin concentration of 1000 mg/L, or a single albustix of 3+, corresponding to an albumin concentration of 5000 mg/L, and a planned 24-hour urine collection for albumin measurement, were eligible for inclusion in the study. Only women with significant albuminuria ($\geq 300$ mg) in the 24-hour urine sample were included in the final analysis. Women with a concurrent diagnosis of upper urinary tract infection, chronic hypertension, diabetes mellitus, or pre-existing renal disease were not included.

The study consisted of two parts with different study populations, for which the same inclusion and exclusion criteria were used. The first study population consisted of 35 women and the second study population of five women.

Study II

Two populations were included, one para-1 and one para-2 cohort.

The para-1 cohort comprised all women giving birth to their first child in Sweden during the years 1973-1982. This information was obtained from the Swedish Medical Birth Register. The cohort consisted of 403 550 women.

The para-2 cohort comprised 207 054 women from the para-1 cohort who also gave birth to a second child during the same time period.

Women with multiple births, diabetes mellitus and/or essential hypertension were excluded from both cohorts.
Studies III-V

All women in studies III-V were recruited at the University Hospital in Uppsala during the time period 2001-2005.

Cases: Women with early-onset or late-onset PE were eligible for study inclusion. The group with early-onset PE consisted of pregnant women with PE diagnosed before gestational week 32 and who were delivered prematurely because of PE. The group with late-onset PE consisted of pregnant women with PE diagnosed in gestational week 35 or later. PE was defined as new-onset hypertension (≥ 140/90) observed on at least two separate measurements ≥ 6 hours apart, combined with proteinuria (≥2 on a dipstick or a 24-hour urine sample showing ≥ 300 mg albumin/24 hour).

Controls: We had two early control groups. One consisted of healthy pregnant women in gestational weeks 24-32. These women were recruited during a routine visit to an antenatal clinic during one of these weeks. Only those whose pregnancy continued normally and resulted in a full-term delivery of a healthy child with normal weight were included in the study. In study III this early control group was used and in study V it was used when comparing urine- and serum samples. The other early control group consisted of healthy pregnant women who were delivered in gestational weeks 24-32. These women were admitted because of imminent premature delivery. Women with clinical and/or laboratory signs of infection were excluded. In study IV this early control group was used and in study V it was used when comparing placenta samples. The late control group was the same for all three studies and consisted of healthy pregnant women who delivered in gestational weeks 36-42. In studies III and V a non-pregnant control group was also included. This group consisted of healthy, non-pregnant women visiting a reproduction centre for infertility based on male or tubal factors. They later became pregnant and delivered successfully.

Women with multiple pregnancies or with a concurrent diagnosis of an upper urinary tract infection, chronic hypertension (hypertension before pregnancy and persistently elevated blood pressure before the 20th week of gestation), diabetes mellitus, or pre-existing renal disease were not included in any of the study groups.

Methods

Study I

The first study population was included to estimate the correlation between albumin creatinin ratio (ACR) and the albumin excretion in a 24-hour urine collection. Influence of potential confounders on this correlation was also studied.
Twenty-four hour urine samples were collected by spontaneous voiding and the collection was completed in all cases prior to the onset of labour. The 24-hour urine samples were sent for analysis of albumin and ACR. A single voided urine specimen (5 mL) was obtained randomly during the 24-hour urine collection period. All random samples were collected during the daytime and none was a sample of the first morning void. Twenty of the random samples in the final analysis were marked with the exact time of collection. Eleven of these were collected before noon and nine after noon. The single voided samples were stored at +4°C immediately after collection and within 24 hours they were moved to storage at –18°C. The analyses were performed when all samples had been collected (storage time 1-19 months). To study potential confounders, blood samples were collected during the period of urine collection for estimations of creatinine and albumin concentrations in serum. Other potential confounders studied were BMI, gestational age, systolic or diastolic blood pressure, parity, single versus duplex pregnancy, blood pressure medication, HELLP (haemolysis, elevated liver enzymes, low platelets) syndrome and collecting time of random sample. These data were obtained from each woman’s patient file.

The second study population was included to estimate the intra-individual variability of ACR in single urine samples during a 24-hour period.

One urine sample (2 ml) was collected from each void during a 24-hour collection period. Each urine sample was marked with the time of voiding. The first voided morning sample was marked and urine samples collected after 9 p.m. were regarded as night samples. The urine samples were stored at -18°C (storage time 0-4 months) before analysis.

Analysis of ACR

Urine albumin was measured by nephelometry (Urine albumin, Dade Behring, Deerfield, IL), using a Behring BN ProSpec® analyzer (Dade Behring). The total analytical imprecision of the method was 3.7 % at 9.5 mg/L and 5.0% at 85 mg/L. Urine creatinine was measured with a modified kinetic Jaffe reaction on an Architect Ci8200® analyser (Abbott, Abbot Park, IL) and is expressed as SI units (µmol/L), and creatinine-related urine albumin was calculated from the ProSpec® results. The total analytical imprecision of the creatinine method was 4.8 % at both 94 and 337 µmol/L.

Study II

The Swedish Medical Birth Register contains data on more than 99% of all births in Sweden.29 It includes demographic and administrative data and information on the reproductive history and on complications during pregnancy, delivery and the neonatal period. Complications during pregnancy and delivery are classified according to the Swedish version of
the International Classification of Diseases (ICD), as noted by the responsible physician at the time of discharge from the hospital. As regards the reliability of the register information, a previous validation study has shown a positive predictive value of 95% for a classification of normotension during 1973–1978. For PE, the positive predictive value (during 1973–1986) was lower (68%), mostly because of a missing second record of proteinuria that is needed for classification (personal communication).

*The para-1 cohort* was divided into one group exposed to gestational hypertensive disease and one unexposed group.

Exposed women were women who were classified as having gestational hypertensive disease during her pregnancy. This group was divided into subgroups based on the severity of the hypertensive disease. Two different classifications of the severity were used.

The first classification was based on the ICD codes for gestational hypertensive disease of different degrees of severity:

a) gestational hypertension  
b) mild pre-eclampsia  
c) severe pre-eclampsia

The second classification was based on the hypothesis that preterm delivery and giving birth to a small for gestational age (SGA) infant can be an effect and a marker of a more severe hypertensive disorder. The cohort was thus divided into two subgroups:

a) women who were exposed to gestational hypertensive disease, but who did not have a premature delivery or gave birth to an SGA infant.

b) women who were exposed to gestational hypertensive disease, had a premature delivery and gave birth to an SGA infant.

Gestational age (GA) was defined as completed weeks of gestation calculated from the estimated date of delivery, and preterm delivery was defined as occurring before the 37th week of gestation. SGA was defined as a birthweight <2 SDs below the mean birthweight for the gestational age. Using the birthweights of all children born in Sweden between 1973 and 1982, and their respective gestational ages at delivery, we calculated mean birthweights for all gestational ages. These were used for the identification of SGA children.

Unexposed women were women who were not exposed to gestational hypertensive disease during their pregnancy.

*The para-2 cohort* was divided into one exposed and one unexposed group of women.
The *exposed group* was divided into three subgroups:

a) women with hypertensive disease (of any severity) in their first pregnancy but not in the second.

b) women with hypertensive disease in the second pregnancy but not in the first.

c) women with two consecutive hypertensive pregnancies.

*Unexposed women* were women who were not exposed to gestational hypertensive disease during either of these two pregnancies.

*The endpoint for follow-up* in both the para-1 and para-2 cohorts was fatal or non-fatal ischaemic heart disease (using ICD codes associated with this disease). Using national identification numbers, we linked the records of the studied women in the Medical Birth Register to the Cause of Death Register and the Hospital Discharge Register. Both of the latter registers are held by the National Board of Health and Welfare. The Hospital Discharge Register contains data on individual hospital discharges and the coverage is approximately 99%. This register has existed nationwide since 1987 and owing to limited coverage of the register before 1987, the follow up was restricted to the period 1987–2001. The first admission to hospital with ischaemic heart disease (IHD) as the main diagnosis, or death with IHD as the underlying cause, was designated the event.

We considered age, category of hospital at delivery and socio-economic status to be possible confounders. Information about the socio-economic status of the women’s household was collected from the Swedish Population and Housing Censuses of 1990, 1985, 1980 and 1970. We used the most recent information other than codes indicating a retired person or a non-classified person.

Studies III-IV

*Collection of plasma samples*

In study III, plasma samples for measurements of sFlt1, PlGF and VEGF-A were collected from each woman on entry into the study.

In study IV, the first plasma sample for measurements of sFlt1, PlGF and VEGF-A was collected from each woman 1 hour to 4 days before delivery (82% of the samples within one day before delivery). All samples were collected before the start of active labour. After delivery, plasma samples were also collected on days 1, 3 and 7 postpartum. For reasons of discharge from the hospital, transfer to other hospitals, lost/inadequate marking of blood samples or already consumed blood samples, we did not have samples available for analysis from all women on all three occasions (Table 1). The
late controls on day 7 postpartum were excluded from the final analysis, since samples from only two women were collected at that time point.

*Table 1.* Number (n) of samples collected before delivery/day one/day three/day seven postpartum in each study group.

<table>
<thead>
<tr>
<th></th>
<th>Early control</th>
<th>Early pre-clampsia</th>
<th>Late control</th>
<th>Late pre-clampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-Flt1 samples (n)</td>
<td>9/8/8/6</td>
<td>18/18/16/14</td>
<td>17/16/11/2</td>
<td>20/18/18/11</td>
</tr>
<tr>
<td>PlGF samples (n)</td>
<td>9/8/7/6</td>
<td>16/17/16/14</td>
<td>17/16/11/2</td>
<td>18/17/18/11</td>
</tr>
<tr>
<td>VEGF-A samples (n)</td>
<td>8/8/8/4</td>
<td>16/17/16/13</td>
<td>14/15/11/2</td>
<td>18/14/17/10</td>
</tr>
</tbody>
</table>

After collection, the plasma samples in both studies were immediately put into a refrigerator, where they were kept from 20 minutes to 2 (in a few cases up to 4) hours before being centrifuged for 10 minutes at 1500 g. The samples were then stored at -70°C until analysed.

*Measurements of sFlt1, PlGF and VEGF-A*

Samples were analysed for sFlt1, PlGF, and VEGF-A, using commercially available ELISA kits (DVR100B, DPG00 and DVE00, R&D Systems, Minneapolis, MN). Briefly, the microtitre plates had been coated with monoclonal antibodies specific for the antigen in question and the first step was to add standards and samples to the wells. During the following incubation period the antigen present in the standards and samples became bound to the immobilised antibody. After a thorough wash an enzyme-linked polyclonal antibody specific for each protein was pipetted into the wells and after a second incubation and wash step a substrate solution was added and colour developed in proportion to the amount of antigen bound. The colour development was subsequently stopped and the intensity of the colour was measured by photospectrometry. The results were calculated according to the manufacturer’s recommendations. The inter-assay CV was approximately 7%.

**Study V**

*Collection of placental, urine and serum samples*

Placental samples were collected immediately after delivery. From each placenta, 4-6 pieces (about 1x1x1 cm) were cut. These samples were repeatedly rinsed in saline, excess saline was reduced by putting the sample on a piece of paper for a few seconds, and the sample was then snap-frozen in liquid nitrogen. The frozen samples were then stored at -70°C.

Urine and serum samples were collected from each woman upon entry into the study. All samples were collected before the start of active labour. After collection, the samples were immediately put into a refrigerator, where
they were kept from 20 minutes to 2 (in a few cases up to 4) hours before being centrifuged for 10 minutes at 1500 g. The samples were then stored at -70ºC until analysed.

Some of the samples were lost as a result of human errors (delay in obtaining the placenta, missed / damaged blood or urine samples), and thus the actual number of samples analysed differed slightly from the number of patients in each group.

Measurement of isoprostane
The concentrations of total isoprostane 8-isopGF2α in placenta, urine and serum were measured using a commercial kit (Cayman Chemical Company, Ann Arbor, MI), following the manufacturer’s instructions. The assay is based on the competition between 8-isoprostane and 8-isoprostane-acetyl-cholinesterase conjugate for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. One frozen placental sample from each patient was selected randomly and a small piece was cut and homogenised with 500 μl of water. The homogenised placential tissue was purified according to the protocol in the assay kit, based on the methods of Bligh and Dyer, and Morrow and collaborators. The amount of isoprostanes was determined as a ratio to protein in the sample, which was determined by the method of Lowry and collaborators. The urine and serum samples were purified and analysed in a similar manner.

Measurements of vitamins C and E
Vitamin C concentrations were determined in serum using the method of Jagota and Dani. 200 μl of serum was precipitated on ice with 800 μl of trichloroacetic acid for 5 minutes and then centrifuged at 3000 rpm for 5 minutes. A total of 500 μl of the supernatant was diluted with distilled water to the volume of 2 ml. 200 μl of Folin-Ciocalteau’s solution, diluted 1:10 in distilled water, was added to the samples, which were immediately mixed. After 10 minutes the absorbance at 760 nm was measured using a Beckman DU-65 spectrophotometer (Beckman Instruments, Fullerton, CA). The sample values were compared with values of standard samples prepared in distilled water from ascorbic acid (Merck, Darmstadt, Germany).

The concentrations of vitamin E in serum were estimated as α-tocopherol concentrations through a method described by Simán and Eriksson. 500 μl of serum was mixed with an equal amount of methanol, and 2 ml of hexane was added. The samples were mixed for 3 minutes, and centrifuged at 3000 rpm for 10 minutes, and the hexane phase was then collected. The samples were separated with HPLC using a Spheri-sob amnio-column (4.6 x 250 mm; Phase Separation, Deeside, UK). The system was isocratic with iso-octane / tert-butyl-methyl-ether / methanol (75 / 25 / 5) as mobile phase and a flow of 1 ml / min. The effluent was analysed fluorometrically with Shimadzu RF-10A (Shimadzu, Kyoto, Japan), using an excitation
wavelength of 295 nm and an emission wavelength of 327 nm. The sample values were compared with values of standard samples prepared from \( \alpha \)-tocopherol (Merck, Darmstadt, Germany). Because of the metabolic relationship between \( \alpha \)-tocopherol and lipoprotein,\(^6\) we also expressed concentrations as a ratio of \( \alpha \)-tocopherol/ cholesterol.

**Measurements of PAI-1 and PAI-2**

PAI-1 and PAI-2 were measured in serum with commercial ELISA kits nos 822 and 823 (American Diagnostica Inc. Stamford, CT) in accordance with the manufacturer’s instructions. The samples were diluted 10-40 times prior to analysis.

**Statistical analysis**

**Study I**

The correlation between ACR and albumin measurements in the 24-hour urine samples was assessed with Pearson’s correlation coefficient. Multiple linear regression was used to detect possible confounders. A \( p \) value of 0.05 was used as the threshold for statistical significance. The variability of the ACR during a 24-hour collection was assessed by dividing the highest by the lowest ACR during the collection period.

**Study II**

Poisson regression was used to estimate the effect of hypertensive disease during pregnancy on the likelihood of developing IHD later in life. Incidence rate ratios (IRRs) (i.e. in this case the ratio between the estimated number of IHD events per 1000 person-years among those with gestational hypertensive disease and the corresponding number among the non-hypertensive women) and 95% confidence intervals were calculated. We considered age, category of hospital at delivery and socio-economic status to be possible confounders. Age was treated as a continuous variable and the other variables as categorical.

**Studies III-IV**

The Kolmogorov-Smirnov test was used to test the data for a normal distribution. Mann-Whitney U tests were thereafter used to estimate whether there were any differences in median concentrations of sFlt1, PlGF and VEGF-A between the study groups. Changes in sFlt1, PlGF and VEGF-A concentrations between the different time points within one study group in
study IV were analysed with the Wilcoxon signed rank test. In cases where the concentration of VEGF-A was below detection limit (15 pg/mL) the concentration was recorded as 15 pg/mL in the protocol. For continuous variables in the tables describing the clinical characteristics of the study population, we used ANOVA for overall comparisons of means and Tukey’s test for multiple pair-wise comparisons. When only one comparison was made, Student’s t-test was used. Chi-square and Fisher’s exact tests were used for comparisons between categorical variables.

All significance tests were two-tailed. A p value of 0.05 or less was considered to denote a statistically significant difference. Bonferroni corrections of the significance levels were made for multiple Mann-Whitney U tests. In study III a p value of 0.017 or less and in study IV a p value of 0.025 or less, was considered to denote a statistically significant difference for pair-wise comparisons of sFlt1, PlGF and VEGF-A between study groups. All statistical analyses were performed with the SPSS 12.0 (Chicago, IL) for Windows software package.

Study V

Results are presented as mean ± standard error of the mean (SEM). ANOVA was used for overall comparisons and Fisher’s protected least significant difference test (PLSD) was used for pair-wise comparisons between means of the study groups. Chi-square and Fisher’s exact tests were used for comparisons between proportions. A p value of less than 0.05 was considered significant. The statistical analyses were performed with Statview (SAS institute Inc. Cary, NC) and figures were created with SPSS 12.0 for Windows software package (Chicago, IL).
Results

The correlation between the albumin-creatinine ratio and 24-hour albumin excretion in urine in women with manifest pre-eclampsia (study I)

**ACR vs. albumin measurement in 24-hour urine samples**

Thirty-one of 35 recruited women were included in the final analysis. Two were excluded because of incorrect sampling of urine for ACR measurement. Two additional women were excluded because of insignificant proteinuria despite +2 on dipstick.

The correlation between ACR in the 24-hour samples and the 24-hour urine albumin excretion had a Pearson coefficient of 0.98 ($R^2 = 0.94$) (Fig.3).

![Figure 3](image)

_Figure 3._ The ACR in the 24-hour urine sample is almost perfectly correlated to the total urine albumin content, illustrating the consistency of the daily creatinine excretion between the subjects.

The correlation between random ACR and 24-hour urine albumin was also significant ($p < 0.01$), but was rather poor, with a Pearson coefficient of 0.65 ($R^2 = 0.42$) (Fig.4).
In randomly voided urine samples, the ACR is only weakly correlated with the corresponding 24 h urine albumin content. There was no significant confounding effect of BMI, gestational age, systolic or diastolic blood pressure, parity, single versus duplex pregnancy, labetolol medication, HELLP syndrome, collecting time of random sample, or serum concentration of albumin or creatinine on the correlation between random ACR and 24-hour urine albumin excretion. Adjustment for maternal age and nifedipine medication significantly (p= 0.04 and p= 0.02, respectively) improved the correlation between random ACR and albumin in 24-hour urine collection (R²= 0.60).

*Intra-individual variability of ACR in single urine samples during a 24-hour period*

The mean variability (highest divided by lowest) value of ACR during a 24-hour urine collection period was 222% (range 178-269%). On exclusion of samples collected during the night and from the first morning voiding, the variability was 202% (range 161-243%).

The association between hypertensive disease occurring during pregnancy and ischaemic heart disease later in life (study II)

*The para-1 cohort*

In the para-1 cohort 20 469 women suffered from hypertensive disease during their first pregnancy (Table 2). Of the total cohort 0.6% were hospitalised or died of IHD during the period 1987–2001, compared with
1.3% of the women who had hypertensive disease during their first pregnancy. After severe PE the risk for IHD was even higher (1.9%). The socio-economic level had a low impact on the risk of developing IHD, with an exception for unskilled manual labour, where the risk was higher (Table 2).

Table 2. Baseline characteristics of para-1 cohort.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No.</th>
<th>%</th>
<th>IHD (n)</th>
<th>Person-years (PY)</th>
<th>IHD/10 000 PY</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age 1987 (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-24</td>
<td>4 328</td>
<td>1</td>
<td>3</td>
<td>63 928</td>
<td>0.5</td>
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<tr>
<td>25-34</td>
<td>199 474</td>
<td>49</td>
<td>698</td>
<td>2 950 428</td>
<td>2.4</td>
</tr>
<tr>
<td>35-44</td>
<td>186 045</td>
<td>46</td>
<td>1 523</td>
<td>2 745 936</td>
<td>5.5</td>
</tr>
<tr>
<td>45-54</td>
<td>13 472</td>
<td>3</td>
<td>335</td>
<td>195 885</td>
<td>17.1</td>
</tr>
<tr>
<td>55-64</td>
<td>231</td>
<td>0.1</td>
<td>20</td>
<td>3 247</td>
<td>61.6</td>
</tr>
<tr>
<td><strong>Hypertensive disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No hypertension</td>
<td>383 081</td>
<td>95</td>
<td>2 306</td>
<td>5 658 019</td>
<td>4.1</td>
</tr>
<tr>
<td>Gestational hypertension</td>
<td>7 936</td>
<td>2</td>
<td>97</td>
<td>116 723</td>
<td>8.3</td>
</tr>
<tr>
<td>Mild pre-eclampsia</td>
<td>9 718</td>
<td>2</td>
<td>123</td>
<td>143 336</td>
<td>8.6</td>
</tr>
<tr>
<td>Severe pre-eclampsia</td>
<td>2 815</td>
<td>0.7</td>
<td>53</td>
<td>41 345</td>
<td>12.8</td>
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<tr>
<td><strong>SGA</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>383 579</td>
<td>95 2 321</td>
<td>5 666 532</td>
<td>4.1</td>
<td></td>
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<tr>
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<td>17 031</td>
<td>4</td>
<td>233</td>
<td>249 905</td>
<td>9.3</td>
</tr>
<tr>
<td>Missing</td>
<td>2 940</td>
<td>0.7</td>
<td>25</td>
<td>42 987</td>
<td>5.8</td>
</tr>
<tr>
<td><strong>Gestational age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22-32 weeks</td>
<td>4 133</td>
<td>1</td>
<td>53</td>
<td>60 712</td>
<td>8.7</td>
</tr>
<tr>
<td>33-36 weeks</td>
<td>18 164</td>
<td>4</td>
<td>176</td>
<td>267 265</td>
<td>6.6</td>
</tr>
<tr>
<td>37-41 weeks</td>
<td>315 639</td>
<td>78 2 010</td>
<td>4 661 817</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>42-45 weeks</td>
<td>63 252</td>
<td>16</td>
<td>321</td>
<td>935 089</td>
<td>3.4</td>
</tr>
<tr>
<td>missing</td>
<td>2 362</td>
<td>0.6</td>
<td>19</td>
<td>34 540</td>
<td>5.5</td>
</tr>
<tr>
<td><strong>Socio-economic level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unskilled manual labour</td>
<td>63 980</td>
<td>16</td>
<td>573</td>
<td>939 523</td>
<td>6.1</td>
</tr>
<tr>
<td>Skilled labour</td>
<td>61 659</td>
<td>15</td>
<td>415</td>
<td>911 618</td>
<td>4.6</td>
</tr>
<tr>
<td>Non-manual, low</td>
<td>62 784</td>
<td>16</td>
<td>395</td>
<td>930 002</td>
<td>4.2</td>
</tr>
<tr>
<td>Non-manual, intermediate</td>
<td>95 591</td>
<td>24</td>
<td>518</td>
<td>1 417 272</td>
<td>3.7</td>
</tr>
<tr>
<td>Non-manual, high</td>
<td>78 304</td>
<td>19</td>
<td>393</td>
<td>1 157 724</td>
<td>3.4</td>
</tr>
<tr>
<td>Farmers and self-employed</td>
<td>36 160</td>
<td>9</td>
<td>201</td>
<td>535 994</td>
<td>3.8</td>
</tr>
<tr>
<td>Others</td>
<td>5 072</td>
<td>1</td>
<td>84</td>
<td>67 289</td>
<td>12.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>403 550</td>
<td>100 2 579</td>
<td>5 959 425</td>
<td>4.3</td>
<td></td>
</tr>
</tbody>
</table>

IHD, ischaemic heart disease; SGA, small for gestational age

After adjusting for maternal age, socio-economic level and category of hospital in which the woman gave birth, we found an increased risk of being hospitalised for or dying of IHD if the first pregnancy was complicated with hypertensive disease (Table 3). The difference in this respect between
gestational hypertension and mild PE was modest and not significant, while the difference in IRR between mild and severe PE was significant.

*Table 3.* Adjusted IRR (95% CI) of being hospitalised for, or dying from, IHD in women with hypertensive disease during their first pregnancy compared with non-hypertensive women.

<table>
<thead>
<tr>
<th>Pregnancy complication</th>
<th>Adjusted IRR^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational hypertension</td>
<td>1.6 (1.3-2.0)</td>
</tr>
<tr>
<td>Mild pre-eclampsia</td>
<td>1.9 (1.6-2.2)</td>
</tr>
<tr>
<td>Severe pre-eclampsia</td>
<td>2.8 (2.2-3.7)</td>
</tr>
</tbody>
</table>

^a Adjusted for maternal age, socio-economic level and hospital in which the woman gave birth.

In more than 99% of the cohort the birthweight and gestational age were recorded. This made it possible to assess the relative risk of IHD separately for gestational hypertension, preterm delivery and delivery of an SGA child, and for combinations of these complications. All complications had an impact on the risk for later development of IHD (Table 4).

*Table 4.* Adjusted IRR (95% CI) of being hospitalised for, or dying from, IHD in women with a first pregnancy complicated by hypertensive disease, preterm delivery, delivery of an SGA child, or with a pregnancy with all three complications.

<table>
<thead>
<tr>
<th>Pregnancy complication</th>
<th>Adjusted IRR^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertensive disease</td>
<td>1.7 (1.5-2.0)</td>
</tr>
<tr>
<td>Preterm delivery</td>
<td>1.3 (1.1-1.5)</td>
</tr>
<tr>
<td>SGA</td>
<td>1.8 (1.6-2.3)</td>
</tr>
<tr>
<td>All three complications</td>
<td>2.6 (1.8-4.7)</td>
</tr>
</tbody>
</table>

^a Adjusted for maternal age, socio-economic level and hospital in which the woman gave birth.

*The para-2 cohort*

During the years of follow-up, 0.6% of the para-2 cohort developed IHD. Of the women who had hypertensive disease in either or both of the pregnancies, 1.3% developed IHD. There was a significant increase in adjusted IRR for IHD in women with hypertension in both pregnancies compared with women who were hypertensive in only the first pregnancy (Table 5).
Table 5. Adjusted IRR (95% CI) of being hospitalised for, or dying from, IHD in women with a first, second or recurrent hypertensive disease during pregnancy, compared with women with two non-hypertensive pregnancies.

<table>
<thead>
<tr>
<th></th>
<th>Adjusted IRR^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>First hypertensive pregnancy</td>
<td>1.9 (1.5-2.4)</td>
</tr>
<tr>
<td>Second hypertensive pregnancy</td>
<td>2.4 (1.8-3.2)</td>
</tr>
<tr>
<td>Both pregnancies hypertensive</td>
<td>2.8 (2.0-3.9)</td>
</tr>
</tbody>
</table>

^a Adjusted for maternal age, socio-economic level and category of hospital in which the woman gave birth.

The associations of the anti-angiogenic factor sFlt1 and the pro-angiogenic factors PlGF and VEGF-A and early-onset and late-onset pre-eclampsia (study III)

Characteristics of the women

The study groups showed only minor differences in their baseline characteristics (Table 6). Length of gestation at delivery was on average 13 days shorter in the late-onset PE group than in their controls, and BMI was higher in the group with early-onset PE than in the early controls. All women had normal blood pressure in the first trimester, but women who later developed early-onset PE had a significantly higher mean diastolic blood pressure compared with their controls.

sFlt1

Non-pregnant women had a median plasma concentration of sFlt1 of 48 pg/mL (Fig.5A). Late controls had a higher concentration (7827 pg/mL) than early controls (886 pg/mL) (p< 0.001). Women with early-onset PE had a higher concentration (37 700 pg/mL) than early controls (p< 0.001). Women with late-onset PE had a higher concentration (26 106 pg/mL) than late controls (p< 0.001). The relative increase, compared with the respective control group, was greater in women with early-onset (43 fold) than in those with late-onset (3 fold) PE.

The plasma concentration of sFlt1 in women with early-onset PE and giving birth to an SGA infant (41 818 pg/mL) did not differ from that in women with early-onset PE and an appropriate-for-gestational age (AGA) infant (34 414 pg/mL).
Table 6. Clinical characteristics and outcomes of the study population

<table>
<thead>
<tr>
<th></th>
<th>Non-pregnant</th>
<th>Early control</th>
<th>Early PE</th>
<th>Late control</th>
<th>Late PE</th>
<th>Significance level (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>22</td>
<td>18</td>
<td>18</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23± 2.5</td>
<td>23± 3</td>
<td>27± 5</td>
<td>25± 4</td>
<td>27± 6</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.55&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>7</td>
<td>9</td>
<td>22</td>
<td>6</td>
<td>20</td>
<td>0.38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BP (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 1&lt;sup&gt;st&lt;/sup&gt; trimester, systolic</td>
<td>114± 12</td>
<td>123± 14</td>
<td>113± 10</td>
<td>121± 14</td>
<td>0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>- 1&lt;sup&gt;st&lt;/sup&gt; trimester, diastolic</td>
<td>69± 7</td>
<td>77± 8</td>
<td>71± 7</td>
<td>76± 8</td>
<td>0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>- at delivery, systolic</td>
<td>125± 18</td>
<td>151± 18</td>
<td>114± 29</td>
<td>131± 30</td>
<td>0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>- at delivery, diastolic</td>
<td>81± 12</td>
<td>95± 12</td>
<td>76± 10</td>
<td>91± 7</td>
<td>0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Any BP medication (%)</td>
<td>82</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
<td>0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>≥ two drugs for BP (%)</td>
<td>71</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td>0.001&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C-reactive protein (mg/L)</td>
<td>0.9± 0.7</td>
<td>4.7± 3.5</td>
<td>15± 21</td>
<td>4.1± 4.4</td>
<td>5.6± 4.2</td>
<td>0.008&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.99&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine (mg/L)</td>
<td>82± 8.0</td>
<td>67± 5.6</td>
<td>76± 12</td>
<td>71± 6.4</td>
<td>76± 7.4</td>
<td>0.007&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.42&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gestational length (weeks)</td>
<td>28± 2</td>
<td>29± 3</td>
<td>40± 1</td>
<td>38± 2</td>
<td></td>
<td>0.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SGA/ AGA/ LGA (n)</td>
<td>0/22/0</td>
<td>10/8/0</td>
<td>0/16/2</td>
<td>1/19/1</td>
<td></td>
<td>0.045&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

plus-minus values represent mean ± standard deviation; PE, pre-eclampsia; n, number of women; BMI, body mass index in first trimester; BP, blood pressure; C-reactive protein and creatinine values represents data from analyses of plasma collected from nonpregnant women at sampling time, from early controls between 24 and 32 weeks of pregnancy, and for the others before the start of active labor; gestational length represents length of gestation at sampling for early controls and at delivery for the others; SGA, small for gestational age (GA); AGA, average for GA, LGA, large for gestational age; <sup>a</sup> early PE compared to early controls; <sup>b</sup> late PE compared to late controls; <sup>c</sup> early PE compared to late PE.
**PlGF**

Non-pregnant women had a median plasma PlGF concentration of 110 pg/mL (Fig.5B). The median plasma concentration in late controls (221 pg/mL) was lower than that in early controls (577 pg/mL) (p< 0.001). Women with early-onset PE had a lower concentration (27 pg/mL) than early controls (577 pg/mL) (p< 0.001). Women with late-onset PE had a lower PlGF concentration (48 pg/mL) than late controls (221 pg/mL) (p= 0.01).

The median plasma concentration of PlGF in women with early-onset PE and a SGA infant (8 pg/mL) was lower than that in women with early-onset PE and an AGA infant (61 pg/mL) (p= 0.007).

**VEGF-A**

Non-pregnant women had a median plasma VEGF-A concentration of 35 pg/mL (Fig.5C). In 67% of the early controls, 71% of the late controls, 69% of the women with early-onset PE, and 65% of the women with late-onset PE the plasma concentrations were under 15 pg/mL (the detection limit of the ELISA test) and therefore all pregnant groups had a median plasma concentration of 15 pg/mL.

There were no significant differences between the study groups with respect to the median plasma concentration of VEGF-A (p= 0.27).
Figure 5A-C. Boxplots of sFlt1 (A), PlGF (B) and VEGF-A (C) concentrations in plasma in each study group. PE, pre-eclampsia. The top and bottom of the box represent the third and first quartiles (the box length is the interquartile range). The horizontal line within the box represents the median value. The bars on the sides of the box represent the highest and lowest value. Circles indicate outliers (cases with values more than 1½ box lengths from the upper or lower edge of the box). Asterisks indicate extremes (cases with values more than 3 box lengths from the upper or lower edge of the box).

The longitudinal changes in the anti-angiogenic factor sFlt1 and the pro-angiogenic factors PlGF and VEGF-A in healthy pregnancy and early-onset and late-onset pre-eclampsia during the early postpartum period (study IV)
Characteristics of the women

Maternal age and BMI in the first trimester were similar between the study groups, and smoking habits did not differ between the early-onset PE group and early controls, or between the late-onset PE group and late controls. All women had normal blood pressure in the first trimester, but women who later developed early-onset PE had a higher mean diastolic BP and a trend for a higher mean systolic BP than early controls (123/77 vs. 110/69 mmHg). Women developing late-onset PE did not differ significantly in their first-trimester BP compared to late controls (121/76 vs. 113/71 mmHg).

At delivery women with early-onset PE had higher systolic BP than women with late-onset PE (151 vs. 131 mmHg; p = 0.02), even though more women with early-onset PE were treated with BP medication (p = 0.03). The mean gestational age at delivery was 29 weeks in the early-onset PE group and 38 weeks in the late-onset PE group. Neither the early- nor the late-onset PE group differed in gestational length at delivery from their control group. Early- but not late-onset PE was associated with giving birth to an SGA infant (10 of 18 infants were estimated to be born SGA in the group of women with early-onset PE but none in the group of women with late-onset PE).

SFlt1

Before delivery, women with early-onset PE had a median plasma concentration of sFlt1 (38 021 pg/mL) that was 17 times higher than that in the early controls (2 182 pg/mL) (p < 0.001). The late-onset PE group had a three times higher median concentration (24 883 pg/mL) than the late controls (7 827 pg/mL) (p = 0.001) (Fig.6A).

All groups of pregnant women showed a rapid decrease in sFlt1 after delivery. The decreases between the different time points during the postpartum period were significant in all study groups.

The decrease in sFlt1, expressed as the percent change in the median plasma sFlt1 concentration, from before delivery to three days postpartum, was 96 % in women with early-onset PE and 84 % in early controls. Women with late-onset PE had a 97 % decrease, compared to 93 % in late controls.

Women with early-onset PE showed a persistently elevated median sFlt1 concentration on day seven postpartum compared with early controls (p = 0.025), whereas the median concentration of sFlt1 did not differ between women with late-onset PE and their controls at any analysed time point postpartum (Fig. 6A).

PlGF

Before delivery, women with early-onset PE (32 pg/mL) had a lower median concentration of PlGF than early controls (213 pg/mL) (p = 0.003). Women with late-onset PE (62 pg/mL) also had a lower median concentration than late controls (221 pg/mL) (p = 0.023) (Fig.6B).
On day one postpartum both the early and late controls showed a rapid decrease in the medium PlGF concentrations compared with the concentrations before delivery (p= 0.012 and p= 0.013, respectively). After day one postpartum there was no further decrease in the PlGF concentrations, either in early and late controls.

There was no change in the median PlGF concentration in women with early- or late-onset PE between the pre-delivery measurement and any of the measurements postpartum.

The PlGF concentrations did not differ between early-onset PE and early controls or between late-onset PE and late controls at any time point during the first week of the postpartum period.

**VEGF-A**

Before delivery, all groups of pregnant women had median VEGF-A concentrations of 15 pg/mL; since 71% of the early controls, 69% of the women with early-onset PE, 71% of the late controls and 61% of the women with late-onset PE had VEGF-A concentrations of ≤ 15 pg/mL (the detection limit of the ELISA test) (Fig.6C).

On day seven, all study groups had slightly higher median concentrations of VEGF-A than before delivery, but only in women with late-onset PE (median: 22 pg/mL) was the increase statistically significant (p= 0.04).

There were no significant differences in VEGF-A concentrations between women with early- or late-onset PE and their respective control group, either before delivery or at any measurement postpartum.
Figure 6 A-C. Median concentrations of sFlt1 (A), PI GF (B) and VEGF-A (C) in plasma before delivery and at days one, three and seven postpartum. EC= early controls, EPE= early-onset pre-eclampsia, LC= late controls, LPE= late-onset pre-eclampsia.

The associations of biochemical markers for oxidative stress and placental dysfunction with early- and late-onset pre-eclampsia (study V)

Characteristics of the women
The women of all groups were of similar age. Parity was similar between the groups of pregnant women. Smoking habits in first trimester did not differ between women with early-onset PE and any of the early control groups, or between women with late-onset PE and the late control group. The women
with early-onset PE had higher BMI in the first trimester compared with the early control group/term delivery. All women had a normal BP in the first trimester, but women who later developed PE had higher BP compared with their controls (Table 7).

The length of gestation at delivery was on average 12 days shorter in the group of women with late-onset PE than in the late control group. Before delivery, the systolic BP in women with early-onset PE was higher than in women with late-onset PE, even though more women with early-onset PE were treated with antihypertensive medication. Early- but not late-onset PE was clearly associated with giving birth to an infant small for gestational age (Table 7).

Table 7. Clinical characteristics and outcomes of the study population

<table>
<thead>
<tr>
<th></th>
<th>Non-pregnant</th>
<th>Early control/term delivery</th>
<th>Early control/preterm delivery</th>
<th>Early pre-eclampsia</th>
<th>Late control</th>
<th>Late pre-eclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>22</td>
<td>6</td>
<td>18</td>
<td>18</td>
<td>21</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>30 ± 1</td>
<td>31 ± 1</td>
<td>31 ± 3</td>
<td>31 ± 1</td>
<td>32 ± 1</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>Primipara (%)</td>
<td>67</td>
<td>45</td>
<td>67</td>
<td>39</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>7</td>
<td>9</td>
<td>50</td>
<td>22</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23 ± 1</td>
<td>23 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 ± 2</td>
<td>27 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25 ± 1</td>
<td>27 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BP (mmHg) - 1st trimester, systolic</td>
<td>114 ± 2</td>
<td>104 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>123 ± 3</td>
<td>113 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>121 ± 3</td>
<td></td>
</tr>
<tr>
<td>BP (mmHg) - 1st trimester, diastolic</td>
<td>69 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77 ± 2</td>
<td>70 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>77 ± 2</td>
<td></td>
</tr>
<tr>
<td>BP (mmHg) - at delivery, systolic</td>
<td>125 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>151 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>114 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>131 ± 7</td>
<td></td>
</tr>
<tr>
<td>BP (mmHg) - at delivery, diastolic</td>
<td>81 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95 ± 3</td>
<td>76 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91 ± 2</td>
<td></td>
</tr>
<tr>
<td>BP medication (%)</td>
<td>83&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>≥ two drugs (%)</td>
<td>71&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Gestational length (weeks)</td>
<td>28 ± 0.4</td>
<td>30 ± 0.4</td>
<td>29 ± 1</td>
<td>40 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>SGA/ AGA/ LGA (n)</td>
<td>0/ 22/ 0</td>
<td>0/ 5/ 1</td>
<td>10/ 8/ 0</td>
<td>0/ 16/ 2</td>
<td>1/ 19/ 1</td>
<td></td>
</tr>
</tbody>
</table>
Values with plus-minus are means ± SEM; Early control group/term delivery served as early controls when comparing data from urine- and serum samples; Early control group/preterm delivery served as early controls when comparing data from placenta samples; BMI, body mass index (first trimester); BP, blood pressure; Gestational length refers to weeks of gestation at delivery except for the early control group/term delivery where length of gestation at sampling time is denoted; SGA, small for gestational age (GA); AGA, appropriate for GA; LGA, large for GA; \(^a\)p < 0.05 when compared to early-onset or late-onset preeclampsia; \(^b\)p < 0.05 when early-onset preeclampsia is compared to late-onset preeclampsia; \(^c\)p < 0.05 when compared to non-pregnant women.

Isoprostanes (8-iso-PGF\(_{2\alpha}\))

Placental tissue concentrations of isoprostanes were higher in women with early-onset PE than in early controls (Fig. 7A). Further, placental isoprostanes was higher in placentas from women with early-onset PE than those from women with late-onset PE and late controls. Placental tissue concentrations of isoprostanes did not differ significantly between women with late-onset PE and late controls.

Non-pregnant women had lower urine concentrations of isoprostanes than women with early-onset and late-onset PE. Early and late controls displayed numerically intermediary values, but their urine concentration was not significantly different from the other study groups (Fig. 7B). However, urine concentrations of 8-iso-PGF\(_2\) tended to be higher in pregnant than in nonpregnant women, with the highest values in women with preeclampsia. The outcome did not change when the isoprostane concentrations were adjusted to the urine-creatinine concentration.

Serum concentrations of isoprostanes did not differ between pregnant and non-pregnant women (Fig. 7C). Neither did the concentrations differ between women with early- or late-onset PE and their respective control group.
Vitamins C and E

The serum concentrations of vitamin C did not differ between the study groups (Table 8).

Nonpregnant women and early controls had a lower vitamin E concentration than women with late-onset PE and late controls. The vitamin E concentrations, either nonadjusted or adjusted to cholesterol levels, did not differ between women with PE and their respective control (Table 8). When the vitamin E concentrations were adjusted to serum-cholesterol there were still no differences between the groups.
Table 8. Serum concentrations (mean ± standard error of mean) of vitamins C and E.

<table>
<thead>
<tr>
<th></th>
<th>Non-pregnant</th>
<th>Early control</th>
<th>Early preeclampsia</th>
<th>Late control</th>
<th>Late preeclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C (µM)</td>
<td>35 ± 2</td>
<td>33 ± 3</td>
<td>33 ± 2</td>
<td>33 ± 3</td>
<td>34 ± 5</td>
</tr>
<tr>
<td>Vitamin E (µM)</td>
<td>20 ± 3a</td>
<td>22 ± 3a</td>
<td>28 ± 3</td>
<td>32 ± 3</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>Vitamin E/ cholesterol (µM/ mM)</td>
<td>4.6 ± 0.7</td>
<td>3.7 ± 0.4a</td>
<td>4.7 ± 0.4</td>
<td>5.0 ± 0.4</td>
<td>5.5 ± 0.4</td>
</tr>
</tbody>
</table>

* p < 0.05 compared to late controls and late-onset pre-eclampsia

**PAI-1 / PAI-2 ratio**

The serum level of PAI-1 was higher in early than in late controls (Table 9). Neither early- nor late-onset PE had significantly increased levels of PAI-1 compared to their corresponding control group.

Early controls had a lower mean PAI-2 concentration in serum than late controls (Table 9). Women with early-onset PE had lower PAI-2 levels than early controls, while women with late-onset PE had similar PAI-2 levels as their controls.

Table 9. Serum concentrations (mean ± standard error of mean) of plasminogen activator inhibitor (PAI)-1 and PAI-2.

<table>
<thead>
<tr>
<th></th>
<th>Early control</th>
<th>Early preeclampsia</th>
<th>Late control</th>
<th>Late preeclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1 (ng/mL)</td>
<td>569 ± 41a</td>
<td>541± 67a</td>
<td>344± 31</td>
<td>404±44</td>
</tr>
<tr>
<td>PAI-2 (ng/mL)</td>
<td>790 ± 47b</td>
<td>317± 41b</td>
<td>1092± 83</td>
<td>1035± 90</td>
</tr>
</tbody>
</table>

*a p< 0.05 compared to late controls; *p< 0.05 compared to all other groups.

The PAI-1/ PAI-2 ratio was higher in women with early-onset PE compared with early controls (Fig.8). Women with early-onset PE also had a higher ratio than late-onset PE and late controls. The ratio did not differ significantly between women with late-onset PE and late controls.
Figure 8. The mean PAI-1/PAI-2 ratio in early controls (EC), early-onset pre-eclampsia (EPE), late controls (LC) and late-onset pre-eclampsia (LPE). The error bars demonstrate standard error of mean (SEM).
Discussion

Study I

In study I we showed that random ACR was poorly correlated with 24-hour urine albumin estimates and that ACR fluctuated widely during the day in women with significant proteinuria. The ACR from a 24-hour urine collection, however, showed a high correlation with the total 24-hour albumin measurement.

We have found six earlier studies of the correlation between random ACR or PCR and 24-hour samples where the women all had significant albuminuria/proteinuria and the number of women included was 30 or more. The correlation varied widely in these studies. The authors of the largest study (including 220 women of whom 168 had significant proteinuria) found a correlation similar to that in our study ($r^2 = 0.41$). Complete urine collections were ensured in that study by the use of indwelling Foley catheters in the majority of women. In most earlier studies the association between PCR and 24-hour urine protein excretion has been evaluated. In most Scandinavian centres urine albumin excretion is measured instead of total protein excretion. The reason for this is that albuminuria is considered to give a more accurate reflection of the glomerular dysfunction associated with the glomerular endotheliosis of PE, compared with total proteinuria. In one previous study, by Nisell et al, the association between random ACR and 24-hour urine albumin excretion was assessed and a fairly good correlation was found between these two parameters in women with significant albuminuria ($r = 0.88$). One difference between the study by Nisell et al and our study concerns the timing of the random urine sample collection. We chose to collect the random ACR sample during any time of the day, avoiding the first-voided sample in the morning. This choice was made because these samples showed the best correlation with the 24-hour protein excretion in the novel study by Ginsberg et al. Ginsberg’s study did not, however, include pregnant women. In the study by Nisell et al the random urine sample was collected at eight o’clock in the morning (immediately prior to the start of the 24-hour collection). In our study we found that the mean variability of ACR was more than 200% during the day; this variability might be explained by the previously established variation in protein excretion with posture, with an increase in the erect position. Standardisation of the random urine sampling to sampling in the morning...
might decrease the ACR variability and increase the association with the 24-hour urine albumin excretion.

We have only found one study addressing the effect of possible confounding factors on the association between PCR and 24-hour urine protein in pregnant women. That investigation showed no significant confounding effect of maternal age, gestational age, or parity. In our study, on the other hand, we found that adjustment for maternal age and treatment with nifedipine increased the correlation significantly, although only to an intermediate level ($R^2 = 0.60$). The effect of age could at least partly be explained by the decline in creatinine clearance with increasing age, which in turn affects the ACR. The confounding effect of nifedipine medication is more difficult to explain. We have found no evidence supporting an effect of nifedipine medication on the creatinine excretion. A possible explanation for the confounding effect could be that nifedipine usually was given as a supplement to another antihypertensive drug, which indicates a more serious hypertensive disease, with the possibility of an abnormal creatinine excretion rate.

The ACR from the 24-hour urine collection had a very high correlation with the total 24-hour albumin measurement. Our interpretation of this is that the urine excretion rate of creatinine was stable enough to be useful as an internal reference, compensating for differences in urine osmolality. One possible use of ACR could therefore be for testing of the 24-hour urine collection instead of measuring the total protein/albumin. By use of a quick-test for ACR, this could save time and possibly also be less expensive than conventional 24-hour albumin measurement. In addition, ACR measurement of the 24-hour collection is likely to be less sensitive to errors from incomplete collections.

In the ISSHP statement concerning classification and diagnosis of hypertensive disorders of pregnancy, random PCR is claimed to be equivalent to total protein excretion in a 24-hour sample when it comes to diagnosing significant proteinuria. In our study all women had significant albuminuria and we were thus unable to study the ability of the ACR to diagnose significant albuminuria. We aimed rather to investigate the correlation between ACR in a random urine sample and the albumin excretion in a 24-hour urine specimen in patients with significant albuminuria.

Study II

In study II we found that gestational hypertensive disease entailed an increased risk of developing IHD later in life, and that the risk was further increased with increasing severity and recurrence of the hypertensive disease.
Our finding of an increased risk of later development of IHD after a gestational hypertensive disease is in agreement with earlier studies. Previous studies, however, have had limitations in terms of relatively short follow-up times,\textsuperscript{58, 67, 153} large drop-out fractions at follow-up,\textsuperscript{67, 153, 170} or small materials.\textsuperscript{63} Three earlier studies,\textsuperscript{58, 67, 153} all with shorter follow-up periods than in our study, showed a similar or even higher risk of developing IHD or CVD after a first pregnancy with hypertensive complications. In a study from Iceland with a smaller material but a longer follow-up,\textsuperscript{63} a risk for IHD that was slightly lower than in our study was found. This suggests that hypertensive disease during pregnancy is primarily related to the risk of early-onset IHD. This hypothesis is supported by another study from Iceland;\textsuperscript{7} there the follow-up time was at least 50 years and a larger increase in the risk of dying of IHD was found before the age of 65 years (RR: 2.4) than after that age (RR: 1.5).

One of the studies from Iceland addressed the question of whether the severity of the hypertensive disease had any impact on the risk of developing IHD later in life.\textsuperscript{63} It was found that the risk of death from IHD was 1.5 times higher if the woman had a hypertensive pregnancy compared with a non-hypertensive pregnancy. A pregnancy complicated by PE further increases the risk for IHD to 1.9 and for eclampsia to 2.6. Our study and the one from Iceland\textsuperscript{63} are thus in general agreement with each other, although the latter study showed a more modest increase in the risk for IHD. It must be kept in mind that the set-up of these studies differs, since we included both hospitalisation and death due to IHD events, whereas the study from Iceland was based only on death due to IHD events.

In our study we also classified the severity of the hypertensive disease in the hypertensive women of the para-1 cohort on the basis of the co-occurrence of preterm delivery and/or SGA. All three complications (hypertensive disease, preterm delivery and SGA) were found to have an independent impact on the risk of developing IHD later in life (IRR 1.7, 1.3 and 1.8, respectively). These figures are slightly lower than those in a study of a Scottish population,\textsuperscript{153} with a shorter follow-up from the index pregnancy. In that study a sevenfold increase in the risk of developing IHD was found in women who had all three complications, which is considerably higher than our finding of an IRR of 2.6 in this group.

Very few studies have addressed the risk of developing IHD after recurrent hypertensive disease during pregnancy. In one above mentioned Icelandic study the risk of death from IHD was analysed in women whose index pregnancy was parous, and the risk was found to be twice as high as that in primigravidae women.\textsuperscript{63} There was no information on whether the parous women had recurrent hypertensive disease. In our study we made separate analyses depending on whether the hypertensive disease was recurrent or only present in the first or second pregnancy. We found that the highest risk of developing IHD was in the women with recurrent
hypertensive disease (with a risk ratio of 2.8, compared with 1.9 when the hypertensive disease only occurred in the first pregnancy).

One strength of this population-based study is that the population was nationwide and losses to follow-up were almost non-existent. Another strength is that we were able to include both hospitalisation and death due to ischaemic heart events. At the end of the follow-up period the women were older than in several previous studies, 58, 67, 153 but the results still apply to relatively young women (mean age 48 years at the end of follow-up).

Limitations of our study are that we were not able to adjust for the women’s BMI or smoking habits, as neither of these possible confounders was reported to the Swedish Birth Register during the study years. Earlier studies have shown that a high BMI is a risk factor both for hypertensive disease during pregnancy and IHD.36, 56 Smoking reduces the incidence of PE but increases the risk of developing IHD.30, 48, 54 In our study we have adjusted the results for socio-economic status. In lower socio-economic classes, higher BMI and smoking are more common.39, 62 This adjustment should therefore partly compensate for the lack of adjustment for the possible confounders BMI and smoking.

Study III

In study III we found support for the suggestion that PE is associated with a disturbed relationship between pro- and anti-angiogenic factors. We also found this disturbance to be much more pronounced in early-onset than in late-onset PE.

We found increased plasma concentrations of sFlt1 in both early- and late-onset PE compared with their controls. Women with early-onset PE had an approximately 43 times higher median concentration of sFlt1 compared with early controls. In late-onset PE the corresponding value was 3 times higher than that in the controls. Our findings are consistent with data presented by Chaiworapongsas et al.,20 who found a 10-fold increase in median plasma sFlt1 in early-onset PE (diagnosed at ≤ 34 weeks of gestation) compared with controls, and a 2-fold increase in late-onset PE (diagnosed at > 37 weeks of gestation) compared with controls. In our study the mean length of gestation at delivery in women with early-onset PE was less than 29 weeks. The mean length of gestation at parturition in the group of women with early-onset PE was not reported in the study by Chaiworapongsas et al.20 In our study the early-onset PE group may have been delivered more prematurely and may therefore have included more severe cases of PE, which seem to lead to more pronounced changes in sFlt1.

In addition, our results are consistent with those in a longitudinal study by Levine et al.,75 who found elevated concentrations of sFlt1 five weeks before
the onset of PE and a larger change in the sFlt1 concentration in women with earlier onset of the disorder.

In a study by Robinson including women with early-onset (delivered in gestational week 31-36, described as “severe PE”) and late-onset (delivered in gestational week 36-39, described as “mild PE”) PE they did not find any difference in sFlt1 concentrations in women with early- compared with late-onset PE. Levine et al found that the serum concentrations of sFlt1 increased during normal pregnancy, and we therefore consider that comparisons between different groups should be made in women at corresponding weeks of gestation.

In the present study there was a difference in the median concentration of PlGF between early and late controls, with a higher concentration observed in early than in late controls. This is consistent with findings in previous longitudinal studies where increasing concentrations have been noted during normal pregnancy up to the end of the second trimester, followed by a decrease during the third trimester. The relative decreases in the median plasma PlGF concentrations in the PE groups, compared with their respective control groups, were larger in early-onset PE (21-fold) than in late-onset PE (5-fold). Women with early-onset PE giving birth to SGA infants had a lower median PlGF concentration than those with early-onset PE giving birth to AGA infants. Our study thus indicates a progressively greater change in PlGF from late-onset PE to early-onset PE with AGA infants and finally the most pronounced changes in women with early-onset PE and SGA infants. These findings are consistent with those in earlier investigations of the PlGF concentrations in women with PE and in women giving birth to SGA infants, where the lowest concentrations were found in pregnancies complicated by both.

In earlier studies extremely low concentrations of VEGF-A have been found during pregnancy. This is in accordance with our finding that 69% of the pregnant women (both women with PE and controls) had VEGF-A concentrations below the detection limit (15 pg/mL). We were therefore unable to investigate possible differences in VEGF-A concentrations between women with PE and controls. To address this question there is a need for more sensitive test systems.

There were some differences between women with PE and their controls regarding some baseline characteristics. The early control group had a lower BMI in the first trimester compared with women who later developed early-onset PE. This was expected, since obesity is a risk factor for PE. Earlier studies have not shown any correlation between BMI and sFlt1 or PlGF concentrations, and therefore the higher BMI in women with early-onset PE in the present study should not have influenced our findings concerning
sFlt1 or PlGF. The length of gestation at delivery was lower in women developing late-onset PE (mean: 38 weeks) than in their controls (mean: 40 weeks). In normal pregnancy the sFlt1 concentration increases by 145 pg/mL per week after gestational weeks 33-36 and the concentrations of PlGF have been found to decrease after 30 weeks of gestation. This means that we may have slightly underestimated the differences in plasma concentrations of sFlt1 and PlGF between women with late-onset PE and their controls.

In the current study we have presented data on measurements at only one time point for each woman. The results of the study awaken the question if the alterations of sFlt and PlGF can be used as a screening procedure for PE. In a very recent published study this question has been addressed. The authors of the study showed that the ratio in serum between sFlt1 and PlGF at 22-26 weeks’ gestation was highly predictive of early-onset PE (less than 34 weeks) in a high-risk population. They also showed that a rapid rise in the sFlt1 to PlGF ratio with advancing gestation may be predictive of PE occurring any time during gestation. The sample size of the study was small (only 12 women developed PE) and larger studies are encouraged.

Study IV

In study IV we found a rapid decrease in sFlt1 in all groups during the first week after delivery, indicating that the large fraction of sFlt1 produced during pregnancy, both in women with PE and in healthy pregnancies, is derived from the placenta. We also found that women with late-onset PE had sFlt1 concentrations that were similar to those in late controls as early as one day after delivery, while women with early-onset PE continued to have increased concentrations of sFlt1 one week postpartum compared with early controls. The PlGF concentrations decreased rapidly in healthy pregnancies after delivery, while the concentrations in women with PE did not change from the pre-delivery measurement to any time point postpartum.

Our findings concerning the sFlt1 concentrations postpartum are in agreement with those in one previous study and partly in agreement with data presented in two other previous studies. Maynard et al found a rapid decrease in sFlt1 48 hours postpartum in both normal and pre-eclamptic pregnancies, without reporting any further analysis of the data.

The study by Koga et al showed that the sFlt1 concentrations were higher one week postpartum in women who had suffered from PE compared with women with a previous normotensive pregnancy. This is in confirmity with our data concerning women with early-onset PE, but not with the data on
women with late-onset PE. Obvious limitations of the study by Koga et al are that the sample size was small (only six women with PE and six controls) and that there is a lack of information about the severity of the disease.

Powers et al reported on concentrations of sFlt1 48 hours after delivery in 33 women with mild PE (mean length of gestation at delivery was 39 weeks) and in 11 women with severe PE (mean length of gestation at delivery was 36 weeks).\textsuperscript{109} They had one control group with samples from women who delivered at term. A decrease in the sFlt1 concentration within 48 hours after delivery was found in all groups, but this concentration was at a higher level both in women with mild and in those with severe PE compared with controls. When the decrease in plasma sFlt1 from delivery to 48 hours postpartum was expressed as percent, it was found to be significantly lower in the women with PE than in those with healthy pregnancies. Further, women with severe PE showed a slower decrease in sFlt1 than women with mild PE. We did not find a slower decrease in sFlt1 in women with PE compared with those with healthy pregnancies from before delivery to three days postpartum. One possible explanation for the different results could be differences in inclusion criteria. We studied women with early- and late-onset PE, while Powers et al studied women with either mild or severe PE and the mean length of gestation at delivery also differed significantly between our study and theirs. These differences may also explain the less pronounced difference in sFlt1 before delivery between women with healthy pregnancies and those with PE found by Powers et al compared with the difference found in our study and other previous studies.\textsuperscript{20, 83, 130}

Before delivery, the plasma concentrations of PIGF were lower in women with both early- and late-onset PE compared with their controls, which is in agreement with earlier studies.\textsuperscript{130, 160, 161} Both early and late controls showed a rapid decrease in PIGF during the first day after delivery, which is also in agreement with earlier findings.\textsuperscript{172} In women with PE the PIGF concentrations in plasma during the first week of the postpartum period were similar to those before delivery. The reason for this might be that PIGF binds to excess sFlt1 and keeps the PIGF concentrations so suppressed in women with PE that the concentrations do not decrease further after delivery of the placenta, which is the major source of PIGF during pregnancy.\textsuperscript{8} This means that all PIGF produced by the placenta in women with pre-eclampsia might be neutralised by sFlt1.

Previous studies have shown extremely low plasma and serum concentrations of VEGF-A during pregnancy.\textsuperscript{106, 172} This is in agreement with our finding that 68% of the pregnant women (both women with PE and healthy controls) had pre-delivery VEGF-A concentrations below the detection limit (15 pg/mL) of our test system. There is thus a need for more sensitive methods of analysing VEGF.
There was a numerical increase in VEGF-A in all study groups by day seven after delivery, but not earlier. In a previous study, however, a rapid increase in VEGF within three days post-partum was seen in normal pregnancies.\textsuperscript{172}

We found no difference between women with PE and the healthy controls concerning the postpartum changes in VEGF-A.

The baseline characteristics were similar in women with early-onset PE and their controls, as well as in women with late-onset PE and their controls. Our finding that women who later developed early-onset PE had a higher blood pressure in the first trimester compared with women who did not develop PE is in agreement with earlier reports.\textsuperscript{51} All women with early-onset PE were delivered by caesarean section, whereas only 38\% in the early control group had a caesarean delivery. This difference should not have influenced our results, since different modes of delivery seem to have the same or no effect on the sFlt1 concentrations.\textsuperscript{109}

The persistence of an increased plasma concentration of sFlt1 postpartum in women with early-onset but not with late-onset PE may be considered in relation to the finding of a stronger association between early-onset compared with late-onset PE, and CVD later in life.\textsuperscript{58} The reason for this stronger association might be the presence of a richer extra-placental source of sFlt1 in women with early-onset PE that maintains the plasma sFlt1 at a higher concentration, leading to changes in the vascular endothelium that increase the risk of CVD later in life. The main source of sFlt1 during a pregnancy complicated by PE is the hypoxic placenta,\textsuperscript{76} but sFlt1 can also be produced by non-placental cells such as endothelial cells and monocytes.\textsuperscript{53} Some women with a history of PE display increased expression of sFlt1 in peripheral blood mononuclear cells as long as one year postpartum.\textsuperscript{114} This elevated steady state concentration of sFlt1 is probably genetic in origin and not caused by the previous PE. Phenotypic amplification might occur in states of metabolic stress such as pregnancy, predisposing to PE, or with ageing, predisposing to CVD. Our finding is consistent with an increased extra-placental production of sFlt1 in these women, but a limitation with our study is the short observation period postpartum.

**Study V**

The main findings of this study are that early- but not late-onset preeclampsia was associated with placental oxidative stress, a decreased PAI-2 concentration in serum and an increased PAI-1 to PAI-2 ratio.
In this study the degree of oxidative stress was evaluated by measuring the concentrations of the isoprostane 8-iso-PGF$_{2\alpha}$ in the placenta, urine and serum and of vitamins C and E in the serum. The concentrations of isoprostanes in placentas from women with PE have been investigated in two previous studies.$^{155, 162}$ In one of them an increase was found in both the free and total concentrations of isoprostanes in placentas from women with PE compared with placentas from healthy pregnancies.$^{162}$ The other study did not show any increase in the concentrations of free or total placental isoprostanes in PE, but an increased concentration of free isoprostanes in the decidua basalis was reported.$^{155}$ In both studies the mean length of gestation was about 36 weeks in the group of women with PE (women with early- and late-onset PE were not studied separately) and the control groups were all term pregnancies. In another study the production rate of NADPH oxidase-mediated superoxide was compared between early-onset PE and late-onset PE. The authors showed a higher production rate in early-onset PE, indicating that placental oxidative stress is more associated with early-onset than late-onset PE.$^{113}$

We found no difference in the mean concentration of isoprostane 8-iso-PGF$_{2\alpha}$ in urine between women with PE and healthy pregnant women, although there was a tendency toward a higher urine isoprostane concentration in women with PE. There are several previous studies where the investigators likewise were unable to show differences in urine isoprostane concentrations between women with PE and healthy pregnancies.$^{59, 84, 120}$ Our finding of a similar blood concentration of total isoprostane 8-iso-PGF$_{2\alpha}$ in women with PE and healthy pregnancies is also in conformity with many earlier studies.$^{9, 23, 59, 84, 87}$

Previous studies have reported reduced concentrations of vitamin C in women with PE,$^{23, 135}$ findings which we not could confirm. In the present study there was a difference in the mean concentrations of vitamin E in early and late controls, with higher concentrations in late compared with early controls. This is consistent with reports from previous longitudinal studies where increasing levels have been found during normal pregnancy.$^{132}$ Earlier studies of vitamin E concentrations in women with PE show divergent results, even after correction of the concentrations for lipoprotein levels. Some, like our current study, show no differences between healthy pregnant women and women with preeclampsia,$^{23, 55}$ but there are also reports of increased concentrations of vitamin E in women with PE.$^{78}$ Differences in study design, population characteristics, overall dietary intake habits, and use of multivitamins are examples of factors likely to have contributed to the variability in results across studies. In our study all pregnant women had participated in the standard antenatal follow-up, as part of which they had received information and recommendations concerning a healthy and appropriate diet.
This study is, to our knowledge, the first study where the PAI-1/PAI-2 ratio has been compared between women with either early- or late-onset PE. We have shown that an increased PAI-1/PAI-2 ratio is associated with early- but not late-onset PE. The reason for the high PAI-1/PAI-2 ratio in women with early-onset PE is a reduced concentration of PAI-2 in women with early-onset PE compared with early controls. This difference in PAI-2 concentration was not seen in women with late-onset PE compared with late controls. PAI-2 is supposed to be almost exclusively produced by trophoblasts\textsuperscript{82} and our PAI-2 results therefore indicate an association between early-, but not late-onset PE and a dysfunctional placenta. The concentrations of PAI-1 did not differ between women with PE and their corresponding control groups. This finding is consistent with some studies,\textsuperscript{93, 133} while other studies report elevated PAI-1 concentrations in women with PE.\textsuperscript{23, 47, 140} Our finding of a higher PAI-1 concentration in early compared with late controls was unexpected. In earlier longitudinal studies an increase in PAI-1 throughout pregnancy has been reported, although slow or non existent in the third trimester.\textsuperscript{23, 133} One reason for the divergent results can be that our measurements were made from serum samples whereas most earlier studies have used plasma samples.\textsuperscript{23, 32, 47, 133, 140} The fact that PAI-1 is involved in various processes in the body and that it can be produced by other cell types than vascular endothelial cells, such as vascular smooth muscle cells, platelets and neutrophils,\textsuperscript{151} makes PAI-1 a rather nonspecific marker of endothelial dysfunction.

The ratio of PAI-1 to PAI-2 is suggested to be a reliable discriminator between women with normal and preeclamptic pregnancies since it reflects both endothelial and placental function, both of which are believed to be altered in PE.\textsuperscript{82, 121} Several reports have shown that the increase of the ratio precede clinical signs of PE with weeks, and the ratio has been suggested to be useful as a possible screening maker for prediction of PE.\textsuperscript{23, 104, 121} In none of the studies early- and late-onset PE were studied separately. Our study adds new information to this matter, and suggests that only early-onset PE can be predicted by using the PAI-1/PAI-2 ratio.

There were some differences between women with PE and their controls regarding their baseline characteristics. Our finding that women who later developed PE had a higher blood pressure in the first trimester compared with women who did not develop PE is in agreement with earlier reports.\textsuperscript{51} Women who later developed early PE had a higher BMI in the first trimester than early controls with term delivery. This was also expected, since obesity is a risk factor for PE.\textsuperscript{35} There are indications that isoprostane concentrations, at least in urine in nonpregnant women, increase with BMI.\textsuperscript{66} This further supports our finding of a non significant difference in urine- and serum isoprostane concentrations between early-onset PE and early controls. The difference should not have influenced our PAI-1/PAI-2 results, since
the PAI-1/PAI-2 ratio is not supposed to be affected by BMI in the second or third trimester\textsuperscript{157}. The length of gestation at delivery was lower in women developing late-onset PE (38 weeks) than in their controls (40 weeks). In normal pregnancy the isoprostane concentration, at least in plasma, seems to be stable through the second half of pregnancy\textsuperscript{23} and the PAI-1/PAI-2 ratio is also stable during the third trimester\textsuperscript{23,133}. Therefore, we do not expect the difference in length of gestation at delivery to have influenced our isoprostane or PAI-1/PAI-2 ratio results.

**General discussion**

As has been pointed out above, pre-eclampsia is clinically a heterogeneous disease and major differences are observed between early- and late-onset manifestations. Our studies III, IV and V were focused on differences between these two variants of PE. For this purpose we separated women with early- and late-onset disease into two distinctly separate groups, with a mean length of gestation at delivery of 29 weeks and 38 weeks respectively. We also collected samples from control groups of pregnant women at corresponding lengths of gestation. In our study groups we were able to confirm differences between early- and late-onset PE regarding several foetal and maternal clinical findings (Tables 6 and 7). Early-, but not late-onset PE was associated with giving birth to an SGA infant. At delivery, women with early-onset disease had a higher systolic blood pressure than women with late-onset disease, even though more women with early-onset PE were being treated with antihypertensive medication. At delivery women with early-onset, but not late-onset PE had elevated plasma concentrations of C-reactive protein and creatinine compared with controls.

Altogether this thesis supports the theory of the two main pathophysiological pathways to PE as has been suggested by Ness and Roberts\textsuperscript{97}. According to this theory, PE can be mediated either through a “placental pathway” that is associated with enhanced release of some placental factor which stimulates the systemic maternal inflammatory response, or through a “maternal pathway” that is more associated with maternal constitutional factors that exaggerate the response to a normal inflammatory burden, or by a contribution from both pathways.

**The pathophysiology of early-onset pre-eclampsia**

We suggest that early-onset PE is associated with both the placental and maternal pathways, and that the two-stage theory forms a relevant basis for discussing the pathophysiology of the early-onset disease (Fig.9). Our finding of a combination of placental and maternal factors underlying the more severe cases of PE is consistent with the theory of Ness and Roberts\textsuperscript{97}. 59
An association between a dysfunctional/hypoxic placenta (“the placental pathway”) and early-onset PE is supported by our following findings:

- Early-onset PE was associated with giving birth to a child born small for gestational age (studies III-V), i.e. placental dysfunction.
- Women with early-onset PE had a median sFlt1 concentration in plasma that was 43 times higher than that in early controls (study III). Placental hypoxia stimulates production of sFlt1.
- Women with early-onset PE had a median PI GF concentration that was 21 times lower than that in early controls (study III) and in these women the PI GF concentration was so suppressed that we were unable to detect any decrease in PI GF after the delivery of the placenta, which is the major source of PI GF during pregnancy (study IV). Placental hypoxia down-regulates the production of PI GF, but the main cause of the low PI GF concentration is most likely its binding to circulating sFlt1.
- Women with early-onset PE had a mean concentration of 8-iso-PGF2α in the placenta that was higher than that in early controls (study V), indicating an oxidatively stressed placenta in early-onset PE. Placental hypoxia promotes oxidative stress.
- Women with early-onset PE had a lower mean concentration of PAI-2 in the serum compared with early controls, leading to a decreased PAI-1/PAI-2 ratio in early-onset PE (study V). PAI-2 is mainly produced by placental tissue and is considered to be a marker for placental function during pregnancy.

An association between maternal constitutional factors (“the maternal pathway”) and early-onset PE is supported by our following findings:

- women who suffered from gestational hypertensive disease in their first pregnancy were at increased risk of developing ischaemic heart disease later in life (study II). This risk was higher if the hypertensive disease was more severe, usually associated with early-onset PE.

The exact underlying mechanism of the relation between PE and CVD is not known. One main hypothesis is that the maternal endothelial dysfunction is established before pregnancy and thus exists prior to the manifestation of clinical PE, and that the endothelial cells in these women are sensitised and respond to pregnancy in an exaggerated way. With increasing age the same predisposing factors can develop into manifest CVD. One possible common underlying factor could be an altered sFlt1 concentration and our finding of a persistently elevated sFlt1 level one week postpartum in women with early-onset PE is consistent with that theory (study IV). Some of the constitutional factors (e.g. hypertension, diabetes, collagen vascular disease) may
also reduce placental perfusion as a result of an abnormal microvasculature.\textsuperscript{97}

\textbf{Figure 9.} Pathophysiology of early-onset pre-eclampsia. The blue ellipses indicate factors that we found to be associated with early-onset pre-eclampsia.

\textit{The pathophysiology of late-onset pre-eclampsia}

We suggest that late-onset PE is associated with the maternal pathway, but that its association with the placental pathway is weak or non-existent (Fig. 10). The two-stage theory may thus be irrelevant for the pathophysiology of late-onset PE.

A weak or non-existent association between a dysfunctional/hypoxic placenta (“the placental pathway”) and late-onset PE is supported by our following findings:

- Late-onset PE was not associated with giving birth to a child who was small for gestational age (studies III-V).
- Women with late-onset PE had a mild/moderate impairment of the angiogenic balance (studies III & IV). This finding may indicate a weak association with a dysfunctional/hypoxic placenta.
- Women with late-onset PE did not have a higher mean concentration of 8-iso-PGF\textsubscript{2α} in the placenta compared with late controls (study V).
Women with late-onset PE did not have a decreased mean concentration of PAI-2 in the serum or an increased PAI-1 to PAI-2 ratio compared with late controls (study V).

An association between maternal constitutional factors (“the maternal pathway”) and late-onset PE is supported by our following findings:

- Women with gestational hypertensive disease and term delivery (indicating late-onset PE) were at increased risk of developing ischaemic heart disease later in life, although the risk was quite modest compared with that observed after more severe PE (indicating early-onset PE) (study II).

Figure 10. Pathophysiology of late-onset pre-eclampsia. The blue ellipses indicate factors that we found to be associated with late-onset pre-eclampsia.

Altogether our findings point to a heterogeneity and complexity of the pre-eclamptic disease. This is probably the reason why it has been so difficult to find reliable markers for prediction of PE and ways to prevent or treat the disease. In recent years, antioxidant therapy has gained considerable interest as a possible means of preventing PE. In 1999 a small but promising study was reported in which a 21% reduction in the PAI-1/PAI-2 ratio and a reduced risk of developing PE were observed in a high risk population of pregnant women after supplementation with vitamins C and E. Since then two large randomised controlled trials have shown, however, that vitamin C and E supplementation does not seem to prevent PE. This was also true for women with early-onset PE in those trials, which is in disagreement with our hypothesis that oxidative stress might be an important causative factor in the pathogenesis of early-onset PE. The reason why supplementation of vitamins C and E failed could be that dietary supplementation increases the
amount of these antioxidants in the serum, but not in the placental tissue, and therefore does not reduce the placental oxidative stress and the risk of developing PE. Another reason might be that the antioxidant therapy was started too late in these randomized trials. The average lengths of gestation on inclusion were 19 and 17 weeks respectively, which means that placentation was almost already completed when the supplementation began. It might be speculated that the excess antioxidants might prove helpful in the first stage of PE, if administered before conception or early during pregnancy, when the trophoblasts are invading the spiral arteries and the placental characteristics of PE are founded.
Conclusions

- The albumin-creatinine ratio in a random urine sample cannot predict 24-hour urine albumin excretion accurately in women with manifest pre-eclampsia. The albumin-creatinine ratio from a 24-hour collection of urine, on the other hand, is an accurate predictor of the total albumin amount and can be used to minimise errors from incomplete collections. (I)

- Women with pregnancies complicated by gestational hypertensive disease, especially severe and recurrent disease, are at increased risk of developing ischaemic heart disease later in life. Identification of these women provides an opportunity for counseling on lifestyle and risk factor modifications that may lower the risk for future maternal vascular disease. (II)

- There are major clinical and biochemical differences between early- and late-onset pre-eclampsia. (III-V)

- Both early- and late-onset pre-eclampsia are associated with altered angiogenesis, but the association with early-onset pre-eclampsia is much stronger. (III-IV)

- Early- but not late-onset pre-eclampsia showed an association with a dysfunctional placenta (III-V)

- Early- but not late-onset pre-eclampsia is associated with placental oxidative stress. (V)

- Early- but not late-onset pre-eclampsia is associated with an increased PAI-1 to PAI-2 ratio in the serum. (V)
Future plans

The association between pre-eclampsia and the future risk of developing cardiovascular disease is very important. Increased knowledge about this association might help us to identify gender-specific risk factors for cardiovascular disease. Such knowledge might also give these women an opportunity for lifestyle and risk factor modifications to reduce the risk for future maternal vascular disease. Previous studies of this association have been hampered by short follow-up periods, a limited number of evaluated risk factors and the fact that only clinical but not sub-clinical events have been recorded.

We therefore plan to assess the impact of pre-eclampsia on several subclinical and clinical cardiovascular risk factors and events in a cohort of 505 women aged 70-75 randomly selected to participate in the PIVUS study (Prospective Investigation of the Vasculature in Uppsala Seniors). Outcomes in the PIVUS study (e.g. signs of the metabolic risk syndrome, signs of impaired microvascular function, cardiac magnetic resonance imaging (MRI) findings indicating myocardial infarctions, and cerebral MRI findings indicating cerebral infarctions) will be compared between women exposed to gestational hypertensive disease during their first pregnancy and women with a normal first pregnancy.

The role of the placenta in the pathophysiology of early- and late-onset PE is also of great interest. Increased knowledge about the placental function and role in connection with different pregnancy complications is probably of great importance for the development of more appropriate ways to predict, prevent and manage pre-eclampsia.

With a combination of MRI and magnetic resonance spectroscopy (MRS), a method is now available for the first time by which direct information about placental perfusion and placental metabolism can be obtained in vivo. Placental perfusion, ATP-concentrations and pH-values will be estimated in clinical cases of early-onset and late-onset PE and also in cases of intrauterine growth restriction (without maternal signs of PE). The cases will be compared with each other and with control groups at corresponding lengths of gestation.

En allmänt accepterad orsaksmodell till preeklampsi är en s.k. 2-stegsmodell. Det första steget startar mycket tidigt i graviditeten och består av att blodförsörjningen till och utvecklingen av placenta (placentationen) är störd. Som en konsekvens av detta frigörs en eller flera faktorer från placenta till moderns cirkulation och dessa anses starta preeklampsins andra steg med kliniska symptom. Dessa faktorer är inte kända men flera av dem är relaterade till angiogenes (kärlnybildning) och oxidativ stress (en obalans mellan reaktiva syreföreningar och antioxidanter).

Man har nyligen visat att det sannolikt finns en starkare koppling mellan störd placentation och preeklampsi som debuterar tidigt under graviditet jämfört med preeklampsi som debuterar sent. När man undersöker faktorer som kan vara kopplade till olika orsaksmekanismer som kan tänkas utlösa preeklampsi så anser vi att kvinnor med tidigt respektive sent debuterande preeklampsi bör studeras som två separata grupper.

Studie I
Sambandet mellan albumin och kreatinin-kvoten (ACR) i ett enstaka urinprov och den totala albuminmängden i en 24-timmars urinsamling undersöcktes hos kvinnor med preeklampsii. Detta för att hitta en metod som skulle innebära en förenkling av diagnostiken vid preeklampsii. Vi fann dock att korrelationen var rätt dålig och vi anser inte att ACR i ett enstaka urinprov kan ersätta 24-timmars urinsamling hos kvinnor med preeklampsii.

Studie II
Kvinnor som fött sitt första barn i Sverige 1973-82 undersöcktes beträffande risken att insjukna eller dö i ischemisk hjärt sjukdom (t.ex. hjärtinfarkt) under perioden 1987-2001. Vi fann att risken att utveckla ischemisk hjärt sjukdom var 1,6-2,8 gånger större om kvinnan utvecklat förhöjt blod tryck under sin graviditet jämfört med de kvinnor som hade normalt blod tryck under sin graviditet. Den högre siffran gäller för kvinnor som hade en svårare form av förhöjt blod tryck i sin första graviditet samt kvinnor som utvecklat förhöjt blod tryck under både sin första och andra graviditet.

Studie III-V
Huvudsyftet med studie III-V var att undersöka olika faktorer kopplade till angiogenes och oxidativ stress och deras samband med tidigt debuterande (debut under graviditetsvecka 24-32) och sent debuterande (debut under graviditetsvecka 35-42) preeklampsii. Vi fann att kvinnor med tidigt debuterande preeklampsii hade kraftigt förändrade nivåer av tillväxtfaktorn placental growth factor och dess receptor sFlt1, medan de med sent debuterande preeklampsii endast hade lätt till måttligt förändrade nivåer. Vi fann också ett samband mellan tidigt debuterande preeklampsii och en förhöjd oxidativ stress i placenta samt en förhöjd kvot mellan plasminogen activator inhibitor typ 1 och 2 i serum (en markör för störd placentation och preeklampsii). Dessa samband fanns inte hos kvinnor med sent debuterande preeklampsii.

Sammanfattningsvis visar studie III-V många skillnader mellan tidigt och sent debuterande preeklampsii med en starkare koppling mellan tidigt debuterande preeklampsii och störd placentation. Vi anser att våra fynd är viktiga inför fortsatta studier syftande till att hitta effektiva metoder för att förutsäga, förebygga samt behandla preeklampsii.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)