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# **Metabolic and Hormonal Factors Related to Hypertensive Pregnancy**

Special Attention to Insulin Resistance, Sympathetic Over-activity,  
and Hyperandrogenism

Anna Tuuri

ACADEMIC DISSERTATION

To be presented, with the permission of the Medicine Faculty of the University of Helsinki, for public examination at Seth Wichmann auditorium, Department of Obstetrics and Gynaecology, Helsinki University Central Hospital  
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# Abstract

Pregnancy-induced hypertension (PIH) with (pre-eclampsia) or without proteinuria has been found to be a state of insulin resistance and sympathetic over-activity. A hyperactive sympathetic nervous system may directly increase the risk of gestational hypertensive disorders, especially in overweight and obese women (Greenwood J 2003, Fischer T 2004, Wang Z 2013) and altered metabolic functions can also result in an increased risk of pre-eclampsia in these women (Catalano P 2010). Angiopoietin-like protein 6 (Angptl6) and adipocyte fatty acid-binding protein 4 (FABP4) seem to be insulin-sensitivity regulators and they could serve as insulin sensitivity biomarkers before and during complicated pregnancy.

## Material and methods

This study consists of five sub-studies with four different study populations.

In the first and second prospective observational studies there were 72 relatively overweight ( $\text{BMI} \geq 24 \text{ kg/m}^2$ ) nulliparous women. In the study we assessed predictive parameters associated with subsequent pre-eclampsia. Insulin sensitivity was assessed via the Matsuda insulin sensitivity index (ISI) and by way of homeostatic model assessment of insulin resistance (HOMA-IR) at 24 weeks of gestation. Maternal serum levels of FABP4, high-sensitivity C-reactive protein (hs-CRP), total testosterone, sex hormone-binding globulin (SHBG) and non-protein bound calculated free testosterone (cfT), and plasma noradrenaline levels were determined at 24 and 32 weeks. In a subgroup of 47 women, serum concentrations of Angptl6 were quantified at 24 and 32 weeks of gestation. Of the study women, 14 subsequently developed non-proteinuric PIH and 12 developed proteinuric PIH (pre-eclampsia), whereas 46 had normotensive pregnancies.

In the third study there were 36 women. Eleven women with pre-eclampsia were studied at 37 gestational weeks and they were compared with 17 normotensive pregnant women studied at 38 gestational weeks and with 8 non-pregnant women. Blood pressure was measured in different body positions and sympathetic activity was assessed by way of noradrenaline and adrenaline levels. Renin activity, natriuretic peptides and endothelin-1 were also assessed.

In the fourth study the subjects were patients diagnosed at 26–32 gestational weeks as having gestational diabetes mellitus (GDM). Diurnal blood glucose profiles were assessed in 178 women with GDM and they were classified as being normotensive ( $n = 102$ ), having chronic hypertension ( $n = 42$ ) (with or without superimposed pre-eclampsia) or PIH ( $n = 34$ ) (with or without proteinuria). Blood glucose profiles were analysed in the groups.

In the fifth study, 18 women with a history of pre-eclamptic first pregnancy and 19 women with prior normotensive first pregnancies were

studied 23–24 years after delivery. Insulin sensitivity was assessed via the Matsuda whole-body ISI and serum concentrations of follicle-stimulating hormone (FSH), SHBG, total testosterone and cT were assessed.

## Results

In the first study systolic blood pressure (SBP) and plasma noradrenaline levels were higher at 32 weeks and FABP4 levels tended to be higher at 24 and 32 weeks in women with subsequent PIH compared with normotensive controls. In logistic regression analysis, baseline FABP4 levels ( $p = 0.04$ ,  $r^2 = 0.060$ ) and SBP after 10 min standing ( $p = 0.02$ ,  $r^2 = 0.085$ ) were associated with the development of PIH.

In the second study, serum Angptl6 levels at 24 weeks, but not at 32 weeks, were significantly higher in women with subsequent PIH compared with normotensive controls (median 252, range 85–536 ng/ml vs. 145, 28–455 ng/ml,  $p = 0.02$ ). Angptl6 levels did not correlate with metabolic factors.

In the third study plasma noradrenaline levels were higher in pre-eclamptic women than in normotensive pregnant women and the difference was most obvious when they were in an upright position.

In the fourth study hypertension complicated 43% of the pregnancies of women with GDM. The glucose profiles were similar in women with normotensive pregnancy, chronic hypertension and PIH, except that in early morning hours (from 04:00 to 08:00 hours) blood glucose concentrations increased in mothers with chronic hypertension, whereas they decreased in the normotensive women.

In the fifth study insulin sensitivity and total and free testosterone were similar in the two groups. However, in women with prior pre-eclampsia and FSH levels below the median, cT levels were higher than in women with prior pre-eclampsia and FSH levels above the median (median 13.4 pmol/l, range 8.0–22.5, vs. 7.1 pmol/l, range 5.1–20.5,  $p = 0.03$ ), while this kind of difference was not seen in women with prior normotensive pregnancies. Of the women with previous pre-eclampsia, 17% (3/18) had metabolic syndrome and 11% (2/18) had polycystic ovary syndrome, vs. 11% (2/19) and 0% of the controls, respectively.

## Conclusions

In this study there was some evidence that pre-eclampsia was associated with sympathetic over-activity and some signs of it could be seen as early as in the third trimester of pregnancy in women with subsequent PIH. However, there was no evidence of increased insulin resistance or hyperandrogenism before PIH or 23–24 years after pre-eclamptic pregnancy. The results suggest that Angptl6 and FABP4, regulators of insulin sensitivity, could be associated with subsequent PIH to some extent, but there was no association between Angptl6 and FABP4 levels and insulin sensitivity markers. The role and significance of these proteins in the pathogenesis of PIH remain

unknown. Our results strengthen the concept of a potential role of angiogenic and angiogenic-like factors in the pathogenesis of pre-eclampsia.

The data support the hypothesis of the presence of sympathetic over-activity in hypertensive pregnancy. It is suggested that Angptl6 and FABP4, regulators of insulin sensitivity, may have a potential role in the development of PIH.

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# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

- I. Tuuri A, Jauhiainen M, Tikkanen M, Kaaja R. Systolic blood pressure and fatty acid-binding protein 4 predict pregnancy-induced hypertension in overweight nulliparous women. Submitted.
- II. Tuuri AL, Jauhiainen MS, Ehnholm CP, Tikkanen MJ, Nicholls MG, Kaaja RJ. Elevated serum angiopoietin-like protein 6 in women with subsequent pregnancy-induced hypertension: a preliminary study. *Hypertens Pregnancy*. 2013;32(3):203-13.
- III. Kaaja R, Leinonen A, Moore P, Yandle T, Frampton C, Nicholls G. Effect of changes in body posture on vasoactive hormones in preeclamptic women. *Journal of Human Hypertension* 2004;18(11): 789-94.
- IV. Leinonen A, Hiilesmaa V, Andersen H, Teramo K, Kaaja R. Diurnal blood glucose profiles in women with gestational diabetes with or without hypertension. *Diabetic Medicine* 2004;21(11): 1181-4.
- V. Tuuri A, Tiitinen A, Hiilesmaa V, Hämäläinen E, Turpeinen U, Tikkanen MJ, Kaaja R. Hormonal and metabolic characteristics of premenopausal women with a history of preeclamptic pregnancy. *Acta Obstet Gynecol Scand* 2010;89(10):1331-7.

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## ABBREVIATIONS

A-FABP	adipocyte fatty acid-binding protein
Ang-1, -2	angiopoietin-1, -2
Angptl	angiopoietin-like protein
Angptl6	angiopoietin-like protein 6
ANOVA	analysis of variance
aP2	adipocyte P2
ARPs	angiopoietin-related proteins
ANS	autonomic nervous system
BMI	body mass index
cfT	calculated free testosterone
CRP	C-reactive protein
CVD	cardiovascular disease
DBP	diastolic blood pressure
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulphate
DM2	diabetes mellitus type 2
ELISA	enzyme-linked immunosorbent assay
FABP4	(adipocyte) fatty acid-binding protein 4
FAI	free androgen index
FFAs	free fatty acids
Flt-1	fms-like tyrosine kinase-1
FSH	follicle-stimulating hormone
GDM	gestational diabetes mellitus
HDL	high-density lipoprotein
HOMA	homeostatic model assessment
HRV	heart rate variability
hs-CRP	high sensitivity C-reactive protein
HUCH	Helsinki University Central Hospital
IDF	the International Diabetes Federation
IL-6	interleukin-6
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LDL	low-density lipoprotein
LPL	lipoprotein lipase
Matsuda ISI	Matsuda whole-body insulin sensitivity index
MSNA	muscle sympathetic nerve activity
OGTT	oral glucose tolerance test
PCOS	polycystic ovary syndrome
PlGF	placental growth factor
PIH	pregnancy-induced hypertension
RAS	renin-angiotensin system
SBP	systolic blood pressure

SD	standard deviation
sFlt-1	soluble fms-like tyrosine kinase-1
SHBG	sex hormone-binding globulin
Tie-1	tyrosine kinase with immunoglobulin and epidermal growth factor-like extracellular domains 1
Tie-2	tyrosine kinase with epidermal growth factor homology
TNF- $\alpha$	tumour necrosis factor alpha
VEGF	vascular endothelial growth factor

## INTRODUCTION

Pregnancy-induced hypertension (PIH) and pre-eclampsia have been found to be characterized by insulin resistance (Seely E 2003, Roberts J 2011), sympathetic over-activity (Schobel H 1996, Greenwood J 2003) and exaggerated systemic inflammation (Wolf M 2001, Seely E 2003), all features of metabolic syndrome (Lambert G 2010). In women, metabolic syndrome is an important risk factor of cardiovascular disease (CVD) (Galassi A 2006). Hypertensive pregnant women are at an increased risk of later CVD and mortality (Bellamy L 2007, Valdiviezo C 2012), regardless of their proteinuric status (Männistö T 2013). In particular, women with both hypertensive disorders of pregnancy and gestational diabetes mellitus (GDM) are at an increased risk of future CVD (Sullivan S 2011).

Pre-pregnancy adiposity is a risk factor of non-proteinuric PIH (Sukalich S 2006) and proteinuric pre-eclampsia (Roberts J 2011, Wang Z 2013). In non-pregnant individuals a key protein linking obesity to various features of metabolic syndrome is adipocyte fatty acid-binding protein 4 (FABP4) (Xu A 2007). This intracytoplasmic lipid chaperone (Furuhashi M 2008) is actively secreted into the blood stream (Xu A 2006) to control liver glucose metabolism (Cao H 2013). Moreover, FABP4 integrates metabolic and inflammatory response systems (Furuhashi M 2011). In pre-eclampsia maternal FABP4 levels are elevated (Fasshauer M 2008, Shangguan X 2009, Scifres C 2012).

Angiopoietin-like protein 6 (Angptl6) is a liver-derived pro-angiogenic protein, which is an insulin-sensitivity regulator independent of its pro-angiogenic effect. This protein tends to prevent the establishment of related metabolic diseases – at least in animal experiments (Oike Y 2005). In human studies, circulating levels of Angptl6 are elevated in insulin-resistant states such as obesity, diabetes (Ebert T 2009) and metabolic syndrome (Namkung J 2011). Like other pro-angiogenic factors (Stepan H 2007), serum Angptl6 might be expected to be reduced in pre-eclampsia, but serum Angptl6 levels have been found to be elevated in pre-eclamptic women (Stepan H 2009). However, in a recent study, maternal Angptl6 levels were elevated during pregnancy, but not more in pre-eclamptic compared with normotensive pregnant women (Xia G 2013).

Overall, data on the role of Angptl6 in pre-eclampsia is scarce and mixed (Stepan H 2009, Xia G 2013) and in non-proteinuric PIH, unknown. The role of FABP4 in the pathogenesis of pre-eclampsia is still incompletely understood (Fasshauer M 2008) and its role in non-proteinuric PIH lacking. It has also remained unclear if increased sympathetic activity precedes pre-eclampsia, whether it represents only a secondary phenomenon (Fischer T 2004) or whether it appears before insulin resistance (Kaaaja R 2006). Metabolic changes at the end of the reproductive years of a woman's life are

less well recognized than in the postmenopausal period (Spencer C 1997), especially in women with a history of pre-eclampsia.

In the first study we aimed to clarify the possible role of FABP4 in pre-eclampsia by assessing maternal FABP4 levels in the second and third trimesters of pregnancy before clinical onset of the condition. We also sought associations between maternal serum FABP4 levels and indices of possible insulin resistance, sympathetic over-activity, inflammation and hyperandrogenism in cases of PIH. In the second study, accordingly, we aimed to clarify if there was an association between maternal serum Angptl6 levels and indices of possible insulin resistance and sympathetic over-activity in cases of PIH. In the first and the second studies we also aimed to assess if maternal serum Angptl6 and FABP4 levels were associated with subsequent development of hypertensive pregnancy.

In the third study we hypothesized that body posture might magnify differences in neurohormonal indices, especially plasma noradrenaline, between pre-eclamptic and normotensive controls. We studied neurohormone indices in connection with different body postures in women with pre-eclampsia, in normotensive pregnant women and in non-pregnant women.

Our fourth study involved testing the hypothesis that diurnal glucose profiles differ in women with GDM and different forms of hypertensive complications. We investigated 24-hour glucose profiles in women with GDM and/or pregnancy-related hypertensive complications.

In the fifth study our hypothesis was that in premenopausal women with a history of pre-eclampsia there are more signs of insulin resistance, metabolic syndrome and polycystic ovary syndrome (PCOS) than in women with a history of normotensive pregnancies. We assessed insulin sensitivity, metabolic syndrome and signs of hyperandrogenism 23–24 years after pre-eclamptic and normotensive pregnancies.

## **Review of the literature**

# 1 HYPERTENSIVE PREGNANCY

## 1.1 DEFINITION AND CLASSIFICATION

Hypertension is a common medical problem encountered during pregnancy, complicating 6–8% of pregnancies (National 2000). Hypertensive disorders during pregnancy are classified into four categories, as recommended by the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy (National 2000), listed in Table 1.

New-onset hypertension, non-proteinuric PIH or proteinuric PIH (pre-eclampsia), develops in up to 5% of previously normotensive women after 20 weeks of gestation (Seely E 2003). In these women, hypertension develops during the second half of pregnancy, usually during the third trimester, and resolves typically within 6 weeks postpartum (Seely E 2003). Pregnancy-induced hypertension is defined as systolic blood pressure (SBP) elevation to  $\geq 140$  mmHg or diastolic BP (DBP) elevation to  $\geq 90$  mmHg on at least two occasions and at least 4–6 h apart in women who were normotensive before 20 weeks of gestation. Proteinuria is defined as the urinary excretion of  $\geq 0.3$  g protein in a 24-hour specimen. This usually correlates with  $\geq 30$  mg protein/dl ( $\geq 1+$  reading on a dipstick) in a random urine determination (National 2000). Non-proteinuric PIH and pre-eclampsia have the same cut-off values for blood pressure, but differ regarding proteinuria.

**Table 1.** *Classification of hypertensive disorders in pregnancy (National 2000).*

	<b>Definition</b>
Proteinuric PIH (pre-eclampsia)	Hypertension developing after 20 weeks of gestation with proteinuria
Non-proteinuric PIH, also termed gestational hypertension or transient hypertension of pregnancy	Hypertension developing after 20 weeks of gestation without proteinuria/other signs of pre-eclampsia
Chronic hypertension	Hypertension before 20 weeks of gestation
Superimposed pre-eclampsia	Pre-eclampsia developing in a women with pre-existing hypertension

PIH; Pregnancy-induced hypertension.

Chronic hypertension is long-term hypertension, antedating pregnancy and persisting afterwards. It is defined as SBP elevated to  $\geq 140$  mmHg systolic or DBP elevated to  $\geq 90$  mmHg. Hypertension that is diagnosed for the first time during pregnancy and that does not resolve postpartum is also classified as chronic hypertension. In superimposed pre-eclampsia, pre-eclampsia develops in a woman with chronic hypertension (National 2000).

The majority of women with pre-existing hypertension do well in pregnancy. However, about 20% of them develop superimposed pre-eclampsia (Seely E 2003). On the other hand, after late first trimester, blood pressure levels may be normal in women with chronic hypertension, since blood pressure usually falls in the late first to early second trimester and rises to pre-pregnancy levels in the third trimester (Seely E 2003).

Women with GDM are at an increased risk of hypertensive disorders during pregnancy (Joffe G 1998, Bryson C 2003, Sullivan S 2011). These women have more chronic hypertension (Suhonen L 1993, Sullivan S 2011) and non-proteinuric PIH (Bryson C 2003) and 10–30% of women with GDM develop proteinuric pre-eclampsia (Yogev Y 2004, Montroro M 2005). The risk of pre-eclampsia among women with GDM increases with the severity of GDM and with pre-pregnancy obesity (Yogev Y 2004). One in five women with GDM developed non-proteinuric or proteinuric PIH in a Finnish study (Suhonen L 1993).

Generally, non-proteinuric PIH is a relatively benign disorder (Seely E 2003). In the absence of severe disease manifestations, discriminating between non-proteinuric PIH and pre-eclampsia may be difficult (Solomon C 2001). Non-proteinuric PIH and proteinuric PIH (pre-eclampsia) are often seen as a continuum of the same disease, since they share many risk factors (Villar 2006) and the pathogenesis of the diseases is similar (Seely E 2003). Although it is unclear whether they are separate conditions (Seely E 2003), proteinuric PIH is associated with endothelial dysfunction (Franceschini N 2005), which is less evident in non-proteinuric PIH (Powers R 2001). On the other hand, sympathetic over-activity has not been found to be greater in pre-eclampsia compared with non-proteinuric PIH (Greenwood J 2003).

Proteinuric PIH is defined as a systemic syndrome, which includes a combination of maternal hypertension and proteinuria. Other systemic manifestations include disseminated intravascular coagulation, haemolysis, elevated liver function test results, and (rarely) seizures (eclampsia) (Turner J 2010). The clinical presentation of pre-eclampsia is highly variable. In serious pre-eclampsia severe hypertension is associated with proteinuria, or significant end-organ disease is associated with even mild hypertension (Turner J 2010). Hypertension is regarded as severe if SBP is sustained at  $\geq 160$  mmHg or DBP is sustained at  $\geq 110$  mmHg, or both (Turner J 2010, Sibai B 2005). The clinical progression of the disease may worsen from mild to severe, or to eclampsia, without warning, or may be severe at the time of diagnosis (Turner J 2010). Instead of the classic “mild” versus “severe” terminology, “early” (< 34 weeks of gestation) versus “late” (symptoms > 34 weeks of gestation) pre-eclampsia terminology is becoming an indicator of disease significance (Tranquilli A 2013).

### 1.1.1 EPIDEMIOLOGY AND CLINICAL RISK FACTORS

Proteinuric pre-eclampsia is probably a multifactorial and heterogeneous disease entity, but the majority of cases occur in previously healthy nulliparous women (Sibai B 2005). The incidence in nulliparous women ranges from 2 to 7% and 75% of them have mild disease (Sibai B 2005). In all pregnancies the incidence ranges up to 5% to 8% (Turner J 2010), although certain subpopulations experience much higher incidence rates (Sibai B 2005). A risk population in Finland showed an 18.3% incidence of pre-eclampsia (Villa P 2013). Table 2 shows maternal and pregnancy characteristics that have been identified as risk factors of pre-eclampsia.

**Table 2.** Risk factors of pre-eclampsia (adapted from Sibai B 2005, Turner J 2010)

<p>Maternal obstetric factors:</p> <ul style="list-style-type: none"> <li>• Nulliparity</li> <li>• Extremes of maternal age</li> <li>• Pre-eclampsia in a previous pregnancy</li> <li>• Pregnancies after donor insemination, oocyte donation, embryo donation</li> <li>• Multi-foetal gestation</li> <li>• Molar pregnancy</li> </ul>
<p>Maternal comorbid conditions:</p> <ul style="list-style-type: none"> <li>• Chronic hypertension</li> <li>• Pre-gestational vascular/endothelial/renal disease</li> <li>• Pre-gestational diabetes</li> <li>• Rheumatic disease</li> <li>• Infections</li> </ul>
<p>Maternal genetic factors:</p> <ul style="list-style-type: none"> <li>• Anti-phospholipid antibodies</li> <li>• Factor V Leiden mutation (protein C resistance)</li> <li>• First-degree relative with a pre-eclamptic pregnancy</li> <li>• African-American race</li> <li>• Maternal low birth weight</li> </ul>
<p>Maternal lifestyle factors:</p> <ul style="list-style-type: none"> <li>• Obesity</li> <li>• Smoking (reduced risk)</li> </ul>
<p>Paternal obstetric factors:</p> <ul style="list-style-type: none"> <li>• Limited sperm exposure</li> <li>• Primipaternity</li> <li>• Paternity by male who fathered a previous pre-eclamptic pregnancy in another woman</li> <li>• Paternity by a male born from a pre-eclamptic pregnancy</li> </ul>

Early-onset pre-eclampsia has been more strongly associated with African-American race, chronic hypertension and congenital anomalies

(Lisonkova S 2013), whereas younger maternal age, nulliparity, and diabetes mellitus have been associated with late-onset disease (Lisonkova S 2013).

The role of a high body mass index (BMI) on disease onset has been controversial (Valensise H 2008, Lisonkova S 2013). Obesity seems to increase the risk of all “forms” of pre-eclampsia (Roberts R 2011). Pre-pregnancy adiposity is a strong independent risk factor of both proteinuric and non-proteinuric PIH (Sukalich S 2006, Roberts J 2011). Among obese pregnant women about 10% develop proteinuric PIH (Roberts J 2011). The underlying mechanism connecting obesity and pre-eclampsia is still incompletely understood (Roberts R 2011, Catalano P 2010).

### **1.1.2 PATHOPHYSIOLOGY**

The exact pathophysiology of pre-eclampsia remains undefined and can differ in women with various risk factors (Sibai B 2005). A central mechanism in the pathogenesis of pre-eclampsia is endothelial dysfunction. In pre-eclampsia endothelial dysfunction is involved in placental and systemic circulations (Solomon C 2006), but the target organ is maternal endothelium (Young B 2010). It is thought that endothelial abnormalities impairing renal natriuresis and increasing total peripheral resistance (TPR) cause hypertension (Gilbert J 2008). Other signs of endothelial dysfunction in pre-eclampsia include proteinuria, microangiopathic haemolytic anaemia and organ hypoperfusion (Salomon C 2006). In addition to endothelial dysfunction, perturbation of the renin-aldosterone-angiotensin II axis, excessive oxidative stress, inflammation, immune maladaptation, and genetic susceptibility may all contribute to the pathogenesis of pre-eclampsia (Young B 2010).

The pathophysiology may also differ according to the time of onset of the disorder (Sibai B 2005). Late-onset pre-eclampsia is referred to as maternal pre-eclampsia and early-onset disease is referred to as placental pre-eclampsia. The latter emphasizes the central role of poor placentation in early-onset disease (Redman C 2005).

#### ***Placental pre-eclampsia***

Abnormal placentation is thought to start with early-onset, placental pre-eclampsia (Redman C 2005). The onset of placental pre-eclampsia is typically < 34 weeks of gestation and women have no cardiovascular risk factors (Oudejans C 2007). Placental pre-eclampsia is associated with intrauterine growth retardation and abnormal placental morphology (Oudejans C 2007).

Since hypertension in pre-eclampsia develops during the third trimester and resolves after delivery, the placenta is thought to play a central role in the disease (Redman C 2005, Staff A 2013). A placental or maternal reaction to placentation is the fundamental error in placental pre-eclampsia. A

placental disorder carries a high recurrence risk, and runs in families, with a clear genetic component (Oudejans C 2007).

In normal placentation embryo-derived cytotrophoblasts invade the placental bed, where they come into contact with maternal tissues. The distal ends of the maternal spiral arteries are then transformed into widely dilated, structureless conduits (Redman C 2005) with low resistance.

In pre-eclampsia, in contrast, trophoblast invasion is shallow and spiral arteries are not remodelled appropriately (Kanasaki K 2009). Spiral artery blood flow remains in a state of high resistance and low flow, with resulting hypoperfusion and hypoxaemia of the placenta (Redman C 2005, Roberts J 2005, Kanasaki K 2009). Placental ischaemia is widely regarded as a key factor in pre-eclampsia (Gilbert J 2008). Hypoxia and chronic inflammation induce up-regulation of placental inflammatory cytokines such as tumour necrosis factor-alpha (TNF- $\alpha$ ), and interleukin-6 (IL-6), increase synthesis of anti-angiogenic soluble fms-like tyrosine kinase-1 (sFlt-1) and increase circulating vasoactive factors angiotensin II type 1 receptor autoantibodies (AT1-AA), and thromboxane (TX). Elevations of these soluble factors are thought to result in endothelial dysfunction in maternal vasculature (Gilbert J 2008).

### ***Maternal pre-eclampsia***

In turn, most cases of late-onset pre-eclampsia might rather be considered mainly as maternal disorders (Egbor M 2006, Oudejans C 2007). In this disorder the placenta may lack abnormal placental morphology typical of early onset pre-eclampsia (Egbor M 2006). The clinical disease of maternal pre-eclampsia may arise from interaction between a possibly normal placenta and a predisposed maternal constitution reflecting microvascular disease, as occurs with long-term hypertension, diabetes or other states of metabolic unbalance, or reflecting a maternal genetic predisposition (Oudejans C 2007).

Late-onset disease is associated with more favourable outcomes (Valensise H 2008) and with much milder endothelial dysfunction than in early-onset pre-eclampsia (Gilbert J 2008), supporting the idea that the pathophysiology is different in these two diseases (Valensise H 2008).

### **1.1.3 MATERNAL HAEMODYNAMICS**

During pregnancy profound changes take place in maternal haemodynamics (Table 3). The autonomic nervous system (ANS) plays a central role in adaptation of the cardiovascular system to various haemodynamic needs and is the principal system in short-term cardiovascular control (Ekholm E 1996).

Compared with normal pregnancy, in pre-eclampsia vascular resistance is increased in the foeto-placental circulation, there are constricted vessels

(Kanasaki K 2009), plasma volume is lower, renin expression increased (Shah D 2005) and plasma renin activity lower (Kanasaki K 2009) (Table 3).

**Table 3.** *Maternal haemodynamics in normal and pre-eclamptic pregnancies*

	<b>Normal pregnancy versus non-pregnant</b>	<b>Pre-eclampsia versus normal pregnancy</b>	<b>Reference</b>
BP SBP DBP MAP	Slightly decreased minimal changes evident decrease Decreased	Increased strongly  Increased	Kanasaki K 2009, Rang S 2002
HR	Increased	Decreased	Rang S 2002
CO	Increased	Reduced	Rang S 2002
SVR	Decreased (vascular dilatation)	Increased strongly (vascular contraction)	Rang S 2002, Kanasaki K 2009
Plasma volume	Increased	Decreased	Rang S 2002, Shah D 2005
Uterine blood flow	Increased	Reduced	Thornburg K 2000, Shah D 2005
Renal plasma flow	Increased	Decreased	Thornburg K 2000, Moran P 2003
RAS	Increased	Lower	Shah D 2005

BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate; CO, cardiac output; SVR, systemic vascular resistance; RAS, renin-angiotensin system.

Normal pregnancy is characterized by resistance to the vasoconstrictive effects of angiotensin II. In non-proteinuric and proteinuric PIH, this resistance is blunted, resulting in increased sensitivity to angiotensin II (Kanasaki K 2009, Powe C 2011).

## **1.2 METABOLIC CHARACTERISTICS**

### **1.2.1 INSULIN RESISTANCE**

All pregnancies are characterized by some level of insulin resistance (Seely E 2003). The relative glucose intolerance helps to maintain continuous glucose transfer to the foetus, which requires 80% of its energy as glucose, even if maternal postprandial plasma glucose levels decrease, and during fasting. In early gestation insulin secretion increases, whereas insulin sensitivity is unchanged, decreased or even increased (Barbour L 2007). From mid-pregnancy onwards insulin sensitivity gradually decreases, being lowest in the third trimester. As a result, a 2- to 2.5-fold increase in insulin secretion is necessary to maintain euglycaemia (Barbour L 2007). In late pregnancy the insulin-mediated glucose disposal rate declines and insulin shows reduced ability to suppress lipolysis.

Dietary or endogenous glucose stimulates insulin secretion by the  $\beta$ -cells of the pancreas. Insulin increases glucose uptake by fat and muscle, decreases glucose production by the liver, and inhibits lipolysis. Insulin resistance or decreased insulin sensitivity is characterized by the impaired action of insulin in insulin-sensitive target tissues such as muscle, liver, adipose tissue and endothelium. A compensatory state of insulin resistance is adaptive hyperinsulinaemia, which forestalls the development of hyperglycaemia and subsequent diabetes (Lau D 2005).

Resistance to insulin can be characterized at a pre-receptor level (insulin antibodies), a receptor level (decreased number of receptors on the cell surface), or a post-receptor level (defects in the intracellular insulin signalling pathway) (Catalano P 2010). In pregnancy, decreased insulin sensitivity is characterized as post-receptor defect (Ryan E 1988, Catalano P 2010).

Because insulin resistance and resultant hyperinsulinaemia progress as the placenta develops, insulin resistance is probably mediated by placenta-derived hormones, including human placental lactogen, progesterone, cortisol, estradiol, human placental growth hormone and prolactin (Ryan E 1988, Briana D 2009). Pregnancy-associated insulin resistance has been also related to free fatty acids (FFAs), peroxisome proliferator-activated receptors, leptin, TNF- $\alpha$ , IL-6, adiponectin and resistin (Zavalza-Gómez A 2008). Recently the role of adipokines has been highlighted.

### **1.2.2 PIH AND INSULIN RESISTANCE**

The features of the mother must also be considered in the pathophysiology of pre-eclampsia, since several similar metabolic abnormalities that are thought to predispose women to the development of pre-eclampsia are observed in the non-pregnant state in women who have had pre-eclampsia (Seely E 2003). One of those metabolic factors that may play a role in the

pathogenesis of PIH is insulin resistance (Seely E 2003). Hyperinsulinaemia may directly predispose women to hypertension by increasing renal sodium reabsorption and stimulating the sympathetic nervous system (Seely E 2003). Insulin resistance could contribute to endothelial dysfunction in pre-eclampsia (Seely E 2003, Gilbert J 2008). However, the precise role of insulin resistance in the development of pre-eclampsia and non-proteinuric PIH remains undetermined (Seely E 2003, Gilbert J 2008).

As early as in the first trimester women with subsequent late-onset pre-eclampsia have been found to be more insulin resistant compared with early-onset and normotensive groups (D'Anna R 2006). Maternal mid-trimester insulin resistance has been associated with subsequent pre-eclampsia (Hauth J 2011).

In non-proteinuric (Seely E 2003) and proteinuric PIH, insulin resistance is enhanced (Kaaja R 1999a, Seely E 2003). In mild pre-eclampsia increased insulin levels have been observed independently of a maternal overweight condition (Moran C 2006). In established pre-eclampsia, women have been found to have higher levels of insulin and glucose, increased levels of triglycerides and FFAs and elevated levels of cytokines (e.g. TNF- $\alpha$ ) associated with the insulin resistance (Salomon C 2006).

Pregnant women with both insulin-resistant chronic hypertension and GDM (Buchanan T 1990) may be more insulin-resistant than those with GDM alone (Caruso A 1999). In GDM a central component of the pathophysiology is chronic insulin resistance (Barbour L 2007). However, in one study, women with GDM who developed pre-eclampsia were not more insulin resistant than those who remained non-pre-eclamptic (Montoro M 2005).

Although insulin sensitivity improves postpartum, women have had decreased insulin sensitivity for at least three months after pre-eclamptic pregnancy (Kaaja R 1999a).

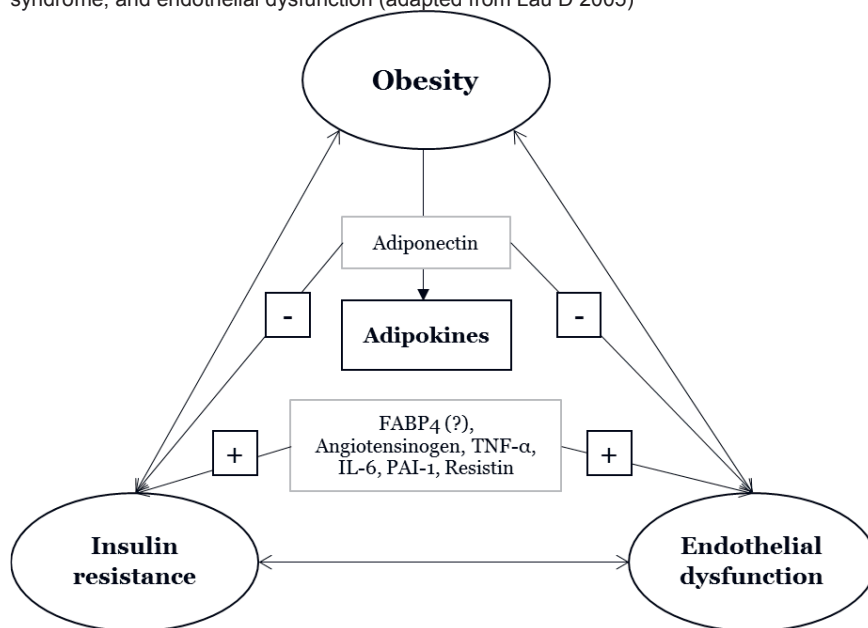
### 1.2.3 FATTY ACID-BINDING PROTEIN 4

#### Adipocytokines

Adipose tissue-derived proinflammatory mediators may directly contribute to insulin resistance, vascular injury and atherogenesis (Lau D 2005). These proinflammatory adipocytokines, or adipokines, include TNF- $\alpha$ , IL-6, leptin, plasminogen activator inhibitor-1 (PAI-1), angiotensinogen, resistin and C-reactive protein (CRP). Adipokines link obesity to insulin resistance in metabolic syndrome and endothelial dysfunction (Figure 1). They may directly influence endothelial function through their proinflammatory properties (Lau D 2005).

Fatty acid-binding protein 4 is a novel adipokine that maintains glucose homeostasis (Furuhashi M 2008). In non-pregnant individuals various features of metabolic syndrome are linked to obesity by FABP4 (Xu A 2007).

**Figure 1** Adipokines are cellular mediators that link obesity to insulin resistance in metabolic syndrome, and endothelial dysfunction (adapted from Lau D 2005)



#### ***Fatty acid-binding proteins (FABPs)***

Lipid-binding proteins facilitate solubility and the translocation of hydrophobic ligands in intra- and extracellular fluids (Zimmerman A2002). Cytoplasmic fatty acid-binding proteins (FABPs) are lipid chaperones that coordinate fatty acid transport and utilization in adipocytes, macrophages, trophoblasts, human placental endothelial capillary cells (Furuhashi M 2008, Scifres C 2011) and microvascular endothelial cells (Elmasri H 2009). At least 9 different FABP isoforms have been identified (Furuhashi M 2011). FABPs derived from metabolically active cells regulate inflammatory and

metabolic responses mainly in adipocytes and macrophages (Furuhashi M 2011). A member of the FABP family (Zimmerman A 2002) expressed in both adipocytes and macrophages is fatty acid-binding protein 4 (Furuhashi M 2008).

### **Fatty acid binding protein 4 (FABP4)**

FABP4, also known as adipocyte fatty acid-binding protein (A-FABP) or adipocyte P2 (aP2), is mainly expressed in adipocytes (Furuhashi M 2008) where it chaperones FFAs in lipolysis (Furuhashi M 2008). Lipolytic signals,  $\beta$ -adrenergic stimuli and signals of fasting regulate synthesis of FABP4 (Cao H 2013).

During fasting a key energy source is serum FFAs (Cao H 2013). From serum, FFAs are taken up in muscle to be oxidized. In muscle, FFAs reduce glucose uptake. In the liver FFAs activate glucose production (Cao H 2013) by increasing gluconeogenesis and lipogenesis. The increase in plasma free fatty acids is especially relevant, as they cause insulin resistance. In muscle, excess fatty acids reduce insulin-mediated glucose utilization and in liver they inhibit insulin-mediated suppression of glucose production (Cao H 2013).

In early gestation free fatty acid concentrations are lower than later in pregnancy. In late pregnancy maternal adipose tissue depots decrease and postprandial FFA levels are increased (Barbour L 2007). In pre-eclampsia triglyceride-rich lipoproteins and circulating FFAs are increased. Levels of FFAs are increased even before the clinical onset of pre-eclamptic symptoms (Kaaja R 2011).

From mature adipocytes FABP4 is actively secreted into the bloodstream (Xu A 2006, Furuhashi M 2008). In FABP4-null mice both basal and hormone-stimulated lipolysis is impaired and they are protected from insulin resistance induced by dietary and genetic obesity, suggesting that this lipid chaperone is also involved in regulating insulin sensitivity (Furuhashi M 2008, Xu A 2012). In primary hepatocytes in vitro and in lean mice in vivo, recombinant FABP4 stimulates glucose production and gluconeogenic activity, providing evidence that FABP4 controls liver glucose metabolism (Cao H 2013).

In mice, FABP4 is also expressed in plaque macrophages (Furuhashi M 2007), where it modulates cholesterol ester accumulation and foam cell formation (Furuhashi M 2011). FABP4 could function as a mediator at the interface of lipid metabolism and inflammatory responses in atherosclerotic disease (Furuhashi M 2011, Pastenkamp G 2012).

### **FABP4, metabolic risk factors and CVD**

In overweight and obese individuals circulating FABP4 levels have been found to be markedly elevated (Xu A 2006) and they correlated strongly with body mass index (Cao H 2013). Elevation of serum FABP4 has also been associated with various obesity-related cardiometabolic risk factors such as

atherogenic dyslipidaemia, insulin resistance (Xu A 2006), hyperglycaemia (Tso A 2007) and hypertension (Ota H 2012), which are key components of metabolic syndrome. High FABP4 concentrations have been reported to be predictive as regards the development of metabolic syndrome, independently of adiposity and insulin resistance (Xu A 2007) and also as regards the development of DM2, again independently of obesity and insulin resistance (Tso A 2007).

In pregnancy-specific glucose disturbance, GDM, maternal FABP4 levels have been found to be increased (Kralisch S 2009). Serum levels of FABP4 have also been associated with atherosclerosis (Peeters W 2011), endothelial dysfunction (Lee M 2011) and macrovascular complications of diabetes (Xu A 2012).

FABP4 possibly potentiates lipid-induced inflammation in obesity-related cardiovascular disease. The inflammatory status of atherosclerotic lesions is a major factor triggering acute cardiovascular events. Plasma FABP4 levels have been reported to be an independent risk factor of vascular inflammation and they correlated positively with those of several proinflammatory markers (high sensitivity CRP (hs-CRP), TNF- $\alpha$  receptors and IL-6) (Xu A 2012). Among subjects who underwent carotid endarterectomy, FABP4 levels were associated with an unstable plaque phenotype and were predictive of the occurrence of an adverse outcome in CVD (Peeters W 2011). In patients with ischaemic stroke FABP4 has served as prognostic indicator of early mortality (Tso A 2011). After adjustment for traditional risk factors, FABP4 levels have been predictive of the development of CVD (Chow W 2013).

### **FABP4 and hypertensive pregnancy**

Maternal circulating FABP4 levels have been reported to be elevated in proteinuric pre-eclampsia (Fasshauer M 2008, Shangguan X 2009), even before the clinical onset of the disease (Scifres C 2012). Maternal serum FABP4 levels have been found to be elevated independently of maternal body mass index (Scifres C 2012).

### **1.3 SYMPATHETIC ACTIVITY**

#### ***Autonomic nervous system (ANS)***

The autonomic nervous system (ANS) mediates neuronal regulation of the internal milieu, primarily the smooth musculature of all organs. The highest integrative organ of the ANS is the hypothalamus. With hypophyseal connections, the hypothalamus also regulates the endocrine glands and coordinates the autonomic nervous and endocrine systems. The peripheral ANS consists of sympathetic, parasympathetic and enteric nervous systems (Schmidt R 1989).

In most situations sympathetic and parasympathetic systems act “synergistically” to regulate organ function and to cope with environmental variations. However, various pathological conditions such as cardiovascular disease are associated with an autonomic imbalance, characterized by a hyperactive sympathetic system and a hypoactive parasympathetic system (Thayer J 2010).

#### ***Sympathetic nervous system***

The sympathetic nervous system is widely recognized as a major regulator of human blood pressure homeostasis and a contributor to the pathogenesis of hypertension. Environmental and internal stresses strongly stimulate adrenomedullary and sympathetic secretion of noradrenaline and adrenaline. The metabolic actions of these catecholamines mainly provide energy for immediate and effective responses in stress or emergency situations. They act as catalysts to mobilize free fatty acids from adipose tissue, and glucose and lactate from glycogen. Furthermore, they inhibit the secretion of insulin. Chiefly adrenaline induces glycogen breakdown in the liver and musculature, and promotes gluconeogenesis in the liver (Schmidt R 1989). Both adrenaline and noradrenaline work as neurotransmitters as well as hormones (Schmidt R 1989). Noradrenaline (in US English norepinephrine) is the most common sympathetic postganglionic neurotransmitter, activating adrenergic receptors on the peripheral target organs or glands (Schmidt R 1989).

#### ***Ghrelin***

Ghrelin is a peptide hormone, which apart from metabolic functions also influences the cardiovascular system. It has been shown to lower peripheral resistance, either directly at the vascular level and/or by centrally modulating sympathetic nervous system activity (Isgaard J 2011). Noradrenaline can stimulate ghrelin secretion, whereas insulin seems to inhibit it (Gagnon J 2012). During pregnancy, decreased, unaltered, or increased plasma levels of ghrelin have been reported (Valsamakis G 2010).

Ghrelin is a leptin inhibitor. Obesity-related hyperleptinaemia might increase central sympathetic nerve activity, which would lead to development

of hypertension, but the evidence is not conclusive (Lambert G 2010, Simonds S 2012, Machleidt F 2013). In animals leptin activates sympathetic nerve activity and in humans, serum leptin concentrations are closely correlated to muscle sympathetic nerve activity (MSNA) (Machleidt F 2013). Acute experimental hyperleptinaemia in humans has an excitatory effect on MSNA without changes in blood pressure, heart rate or plasma catecholamines (Machleidt F 2013). The role of leptin in chronically elevated sympathetic nerve activity, leading to hypertension, has not yet been shown (Simonds S 2012).

### ***Sympathetic activity and insulin resistance***

It is a well-known feature of insulin that it stimulates sympathetic nerve activity, particularly to skeletal muscle tissue (Grassi G 2004). Sympathetic over-activity and insulin resistance are linked in a positive-feedback fashion and two-way reinforcement, but which precedes the other is still unclear (Kaaaja R 2006).

### ***Sympathetic over-activity and normal pregnancy***

In the first trimester of normal pregnancy during orthostatic stress the immediate heart rate increase provoked by standing up is unchanged, but the biphasic heart rate response is reduced, implying a diminished baroreflex-induced slowing of the heart (Ekholm E 1996). In the third trimester the difference between standing and resting heart rate is found to be reduced or unchanged compared with early pregnancy, the post-partum period and the non-pregnant state (Ekholm E 1996).

From the first trimester on, the immediate blood pressure reaction is also changed. On active standing up, systolic blood pressure rises during pregnancy instead of falling and diastolic blood pressure rises more in pregnancy than in the non-pregnant state (Ekholm E 1996). The blood pressure rise after standing up may be explained by increased sympathetic efferent activity to the blood vessels induced by the upright position, or a small decrease in stroke volume (Ekholm E 1996).

Pregnancy does not affect the change in mean arterial pressure caused by standing up (Ekholm E 1996). During orthostatic stress both systolic and diastolic blood pressure change in the same way in pregnant and non-pregnant women (Ekholm E 1996).

In the early weeks of normal pregnancy sympathetic activation in muscle seems to be enhanced compared with the non-pregnant state (Jarvis S 2012). In the 3<sup>rd</sup> trimester there is evidence of moderate sympathetic nervous system over-activity in muscle (MSNA) and in the heart as assessed by heart rate variability (HRV) analysis, but not in overall sympathetic activity as reflected in plasma noradrenaline levels (Kaaaja R 2006). In HRV analysis the vagal component of the ANS seems to dominate in the 1<sup>st</sup> trimester of normal pregnancy and as pregnancy advances there is change towards greater sympathetic and lower vagal modulation (Kuo C 2000).

### **1.3.1 PIH AND SYMPATHETIC OVER-ACTIVITY**

There is evidence that second trimester diastolic, systolic, and/or mean arterial blood pressure can predict non-proteinuric PIH (Ekholm E 1994). Women with later PIH have been seen to lack the physiological drop in blood pressure in mid-pregnancy (Ekholm E 1994).

In orthostatic tests in mid-pregnancy, heart rate and blood pressure reactions have been found not to differ in women with subsequent PIH compared with those remaining healthy (Ekholm E 1994). In women with proteinuric PIH, no differences have been found in heart rate and blood pressure responses compared with healthy pregnant women in the initial phase or in the steady state (Rang S 2002).

In non-proteinuric PIH, direct recordings from peripheral sympathetic nerves have shown an increased sympathetic drive (MSNA) compared with that in normotensive non-pregnant women (Greenwood J 1998, Greenwood J 2001) and women with normal pregnancies (Greenwood J 2001, Greenwood J 2003). After delivery peripheral sympathetic hyperactivity seems to fall to normal in women with normal pregnancy and with non-proteinuric PIH (Greenwood J 2001).

Proteinuric pre-eclampsia has been recognized as a state of sympathetic over-activity - at least at the skeletal muscle level as demonstrated by MSNA (Schobel H 1996, Greenwood J 2003), in heart as demonstrated by HRV (Yang C 2000), and perhaps globally as reflected by increased venous noradrenaline (Manyonda I 1998) and by increased diurnal venous plasma levels of norepinephrine (Kaaja R 1999b). In pregnant women with pre-eclampsia during a preceding pregnancy, pregnancy-induced sympathetic over-activity has been demonstrated by MSNA, which normalized after delivery (Fischer T 2004). However, it is not known whether women have increased sympathetic activity before the onset of pre-eclampsia.

Compared with non-proteinuric PIH, sympathetic over-activity in muscle has not been found to be greater in proteinuric pre-eclampsia (Greenwood J 2003).

## 1.4 ANDROGENS

Throughout normal pregnancy maternal total testosterone levels increase progressively (Bammann B 1980, O'Leary P 1991), whereas concentrations of dehydroepiandrosterone sulphate (DHEAS) decrease (O'Leary P 1991). Serum free testosterone concentrations barely change until the third trimester, when they increase about twofold (Bammann 1980). During pregnancy, circulating sex hormone-binding globulin (SHBG) concentrations are increased as a result of raised oestrogen levels (O'Leary P 1991), which might increase testosterone levels. Increased SHBG levels are expected to reduce, not increase, free testosterone levels.

### ***Androgens in hypertensive pregnancy***

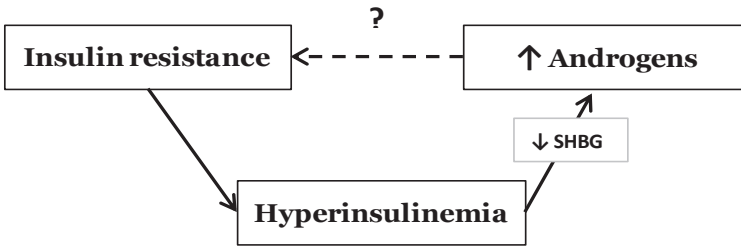
Before clinical pre-eclampsia in the second trimester of pregnancy, maternal total testosterone levels (Carlsen S 2005) and free testosterone indexes (Carlsen S 2005) have been found to be elevated. In the third trimester maternal total testosterone levels (Carlsen S 2005, Salamalekis E 2006), the free testosterone index (Carlsen S 2005) and free testosterone levels (Salamalekis E 2006) were elevated in women with later pre-eclampsia compared with those who remained normotensive. However, first or second trimester concentrations of total testosterone, SHBG and the T/SHBG ratio have not been found to predict development of pre-eclampsia (Tuutti E 2011).

In most studies, during overt pre-eclampsia maternal total testosterone levels have been elevated compared with those in normotensive pregnancies (Acromite M 1999, Serin I 2001, Steier J 2002, Troisi R 2003, Atamer Y 2004). In those studies where free testosterone was also determined, levels were higher in women with pre-eclampsia (Acromite M 1999, Serin I 2001). Maternal estradiol and DHEAS levels have been found to be the same in pre-eclamptic and normotensive women (Acromite M 1999, Serin I 2001, Troisi R 2003, Atamer Y 2004).

### ***Relationship between androgens and insulin resistance***

It is well known that in women hyperinsulinaemia as a consequence of insulin resistance causes hyperandrogenaemia. Hyperinsulinaemia increases ovarian androgen production and decreases hepatic production of SHBG. Decreased SHBG levels lead to increased circulating concentrations of free testosterone. However, the metabolic consequences of androgen excess in women are not so clear, i.e. whether androgens adversely influence insulin action (Carbould A 2008) (Figure 2).

**Figure 2** Hyperandrogenism and insulin resistance (adapted from Carbould A 2008)



In women, androgen administration has been associated with the development of insulin resistance (Golden S 2004). In pre- and perimenopausal women a positive association has been found between the free androgen index (FAI) and insulin resistance (HOMA-IR), fasting insulin and glucose, even after adjustment for BMI (Sutton-Tyrell K 2005). In postmenopausal women the FAI has been associated with hyperinsulinaemia and hyperglycaemia (Golden S 2004) and bioavailable testosterone with insulin resistance (HOMA-IR) (Golden S 2007). In addition to direct effects of androgens on insulin action, androgens potentially influence insulin sensitivity in women indirectly through effects on lipid metabolism and body fat distribution, specifically as regards the development of central obesity (Arner P 2005, Pasquali R 2006, Corbould A 2008).

***Androgens in polycystic ovary syndrome (PCOS)***

Women with polycystic ovary syndrome (PCOS) are 3–4 times more likely to have non-proteinuric PIH and pre-eclampsia during pregnancy. They have been found to be at a higher risk of developing non-proteinuric PIH independently of BMI (Boomsma C 2006). Pregnant women with PCOS have higher androgen levels than their age-matched controls (Sir-Petermann T 2002). In these women higher androgen levels might predispose them to pre-eclampsia.

It is well established that women with PCOS are at an increased risk of developing diabetes mellitus, glucose intolerance, hypertension, hyperlipidaemia (cardiovascular risk factors), metabolic syndrome and cardiovascular disease (Homburg R 2009). In women with PCOS, the prevalence of metabolic syndrome is high across all age groups (Essah P 2006). In affected women, metabolic syndrome is particularly common in those with the highest insulin levels and a high BMI (Ehrmann D 2006).

Hyperandrogenaemia may contribute to the increased risk of CVD in women with PCOS. In adolescent girls with PCOS, hyperandrogenaemia was found to be a significant predictor of metabolic syndrome independently of obesity and insulin resistance (Coviello A 2006). Even if hyperandrogenism partly resolves in women with PCOS before menopause, testosterone levels remain elevated compared with controls and this might increase their risk of

CVD (Winters S 2000, Legro R 2003). Augmented sympathetic nerve activity (MSNA) may contribute to the increased prevalence of CVD in PCOS (Sverrisdottir Y 2008). Serum testosterone concentrations are a strong predictor of sympathetic nerve activity in women with PCOS. The relationship between testosterone and the sympathetic nervous system is unclear (Sverrisdottir Y 2008).

## 1.5 INFLAMMATION

Normal pregnancy provokes an increased maternal systemic inflammatory response, at least in the third trimester (Sibai B 2005). In non-pregnant subjects, a subclinical elevation of high-sensitivity C-reactive protein (hs-CRP) is a marker of systemic low-grade inflammation, and an efficient marker of cardiovascular events (Ridker P 2003).

### ***Inflammation in hypertensive pregnancy***

In pre-eclamptic pregnancy the inflammatory response is more extreme than in normal pregnancy (Sibai B 2005). In overt pre-eclampsia there are elevated levels of CRP (Teran E 2001), hs-CRP (Ertas I 2010), TNF- $\alpha$  and IL-6 (LaMarca B 2007). Cytokines are thought to link placental ischaemia with cardiovascular and renal dysfunction.

In placental, renal, and vascular tissues TNF- $\alpha$  activates the endothelin system, and IL-6 stimulates the renin-angiotensin system (RAS) (LaMarca B 2007). RAS and the sympathetic nervous system interact with proinflammatory cytokines. These cytokines affect angiogenic and endothelium-derived factors regulating endothelial function (LaMarca B 2007). A novel candidate that induces TNF- $\alpha$  is a maternal autoantibody, the angiotensin II type I (AT(1)) receptor agonistic autoantibody (AA) (Irani R 2010).

### ***Metabolic inflammation***

Inflammation is important in the causal pathway through which obesity predisposes women to pre-eclampsia (Wolf M 2001). Abnormal adipokine production and infiltration of inflammatory cells characterize dysfunctional adipose tissue. Visceral fat produces more CRP and inflammatory cytokines than subcutaneous fat, and contributes more to oxidative stress (Roberts J 2011).

A form of metabolically driven, low-grade, chronic inflammation, “metaflammation” is associated with obesity and associated disorders such as insulin resistance and DM2 (Hotamisligil G 2006, Furuhashi M 2011). In the pathogenesis of chronic metabolic diseases such as DM2 and atherosclerosis, lipids and lipid signals are critical in integration of metabolic and inflammatory response systems (Hotamisligil G 2006). In addition to cytokines and lipids (extracellular mediators), intracellular stress

(endoplasmic reticulum stress and excess production of mitochondrial reactive oxygen species) can initiate metaflammatory pathways in metabolically active cells such as adipocytes and macrophages (Furuhashi M 2011). The adipocytokine FABP4 integrates inflammatory and metabolic responses (Furuhashi M 2008).

## **1.6 ANTI-ANGIOGENIC AND PRO-ANGIOGENIC FACTORS**

In pre-eclampsia placental stress stimulates release of several trophoblast-derived factors that contribute to exaggerated maternal inflammatory responses. Inflammation and hypoxia up-regulate angiogenic factors initiating angiogenesis. Angiogenesis is induction and growth of new blood vessels from pre-existing ones. It is a complex, highly regulated system essential for growth of the foetus and placenta, wound healing and tumour development (Charnock-Jones D 2004).

Circulating levels of placenta-derived anti-angiogenic proteins – soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) or soluble fms-like tyrosine kinase-1 (sFlt1) and soluble endoglin (sEng) are increased before the onset of clinical pre-eclampsia (Levine R 2006). Circulating concentrations of pro-angiogenic factors such as free placental growth factor (PlGF) are reduced in pre-eclampsia (Levine R 2004, Staff A 2013). Higher levels of sFlt-1 are proposed to antagonize the vasodilatory effects of PlGF-1 and vascular endothelium-derived growth factor (VEGF), resulting in placental vascular insufficiency and other systemic effects (Solomon C 2006). It is implied that this angiogenic imbalance increases maternal vascular inflammation and generalized endothelial dysfunction (Staff A 2013).

### ***Angiopoietins and Tie receptors***

An important family of vascular endothelium-specific angiogenic growth factors are the angiopoietin (Ang) proteins. Most of them bind to endothelial cell-specific tyrosine kinase (Tie-2) receptors and are related to regulation of angiogenesis. Ang-1 and Ang-2 are produced by the villous trophoblast and are thought to play a substantial role in placental vascular development (Charnock-Jones D 2004, Seval Y 2008). Ang-1 activates and Ang-2 blocks Tie-2 receptors (Seval Y 2008). Circulating concentrations of Ang-1 are increased in (proteinuric) pre-eclampsia and non-proteinuric PIH (Nadar S 2005), but not in the first trimester before later pre-eclampsia (Leinonen E 2010). On the other hand, circulating concentrations of Ang-2 in the second trimester are increased in women with subsequent pre-eclampsia, but the levels seem to decrease from first trimester levels (Leinonen E 2010). During pre-eclampsia and non-proteinuric PIH, circulating concentrations of Ang-2 are decreased (Nadar S 2005). In normal pregnancy at least, maternal Ang-1 and Ang-2 concentrations decrease rapidly after delivery (Keikkala E 2012).

### **Angiopoietin-like proteins**

Angiopoietin-like proteins (Angptls) or “angiopoietin-related proteins” (ARPs) are structurally similar to the angiopoietins: they contain a N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain, and a fibrinogen-like domain, and have angiogenic effects (Oike Y 2004). Angptls do not bind to either the angiopoietin receptor Tie-2 or the related protein Tie-1 (Oike Y 2004) and remain orphan ligands (Hato T 2008). The Angptl family has seven members (Angptl 1–7), which have been found in both humans and mice (Hato T 2008) (Table 4). Angptl1–4 and Angptl6 act on endothelial cells to control angiogenesis. Independently of their angiogenesis-regulating functions, Angptls3, 4 and 6 appear to directly regulate lipid, glucose and energy metabolism (Hato T 2008) (Table 4). Angptl2, 4 and 6 expression is up-regulated by a variety of factors, including hypoxia (Hato T 2008) (Table 4).

**Table 4.** *Biological effects of Angptls on angiogenesis and metabolism (adapted from Hato T 2008)*

	<b>Angiogenesis</b>	<b>Hypoxic induction</b>	<b>Metabolism</b>
<b>Angptl1</b>	Pro/Anti	No	NR
<b>Angptl2</b>	Pro	Yes	NR
<b>Angptl3</b>	Pro	No	Inhibits LPL activity and increases serum TG levels
<b>Angptl4</b>	Pro/Anti	Yes	Inhibits LPL activity and increases serum TG levels
<b>Angptl5</b>	NR	NR	NR
<b>Angptl6</b>	Pro	Yes	Increases energy expenditure
<b>Angptl7</b>	NR	NR	NR

NR no record; Pro, pro-angiogenic; Anti, anti-angiogenic; LPL, lipoprotein lipase; TG, triglyceride.

#### **1.6.1 ANGIOPOIETIN-LIKE PROTEIN 6**

Angiopoietin-like protein 6 (Angptl6) also called angiopoietin-related growth factor (AGF), is a liver-derived protein that independently of its pro-angiogenic effect antagonizes the development of obesity and insulin resistance (insulin-sensitizing effect) and thereby tends to prevent the establishment of related metabolic diseases – at least in animal experiments (Oike Y 2005). Human studies indicate that circulating levels of Angptl6 are elevated in insulin-resistant states such obesity, diabetes (Ebert T 2009) and metabolic syndrome (Namkung J 2011). In animal experiments, Angptl6 improves lipid profiles (Oike Y 2005), but it has no known effect on human lipoprotein metabolism (Miida T 2010).

Like other pro-angiogenic factors including placental growth factor (Stepan H 2007), serum Angptl6 might be expected to be reduced in pre-

eclampsia, but in one study serum Angptl6 levels were elevated in pre-eclamptic women compared with normotensive pregnant women (Stepan H 2009), while in a recent study maternal Angptl levels were elevated in pregnant women compared with non-pregnant women, but there were no differences in pre-eclamptic and normotensive pregnant women (Xia G 2013).

## **1.7 LONG-TERM HEALTH CONCERNS**

### **1.7.1 INSULIN RESISTANCE**

Insulin resistance has been seen during PIH (Kaaja R 1999 a, Seely E 2003) and three months (Kaaja R 1999a) and 18 months after pre-eclamptic pregnancy (Wolf M 2004). However, in one study, five years after pre-eclamptic pregnancy there were no differences in insulin sensitivity in women with previous pre-eclampsia and normotensive control women (Lampinen K 2008). A history of (proteinuric) pre-eclampsia has been associated with mild hyperinsulinemia seventeen years after first pregnancy (Laivuori H 1996), but twenty years after pre-eclampsia a study group of women had only a tendency towards higher fasting insulin levels, but glycosylated haemoglobin levels were significantly higher than in controls (Sattar N 2003). Women with PIH have an increased risk of developing diabetes, even in the absence of GDM (Feig D 2013), but the presence of PIH and GDM raises the risk of diabetes significantly beyond that seen with GDM alone (Feig D 2013).

Even without a history of hypertensive pregnancy, in women at or after menopause insulin resistance increases, and insulin secretion and elimination decrease (Kaaja R 2008 a). Decreased insulin sensitivity in the postmenopausal period has been thought to be due to oestrogen deficiency (Spencer C 1997). In women, advancing age and obesity also decrease insulin sensitivity (Guthrie J 2001, Carr M 2003). It is estimated that middle-aged women gain approximately 0.55 kg/year (Carr M 2003), as a result of increased abdominal obesity (Kaaja R 2008 a). Visceral fat increases with advancing age and after menopause decreased insulin sensitivity becomes significant as women get older (DeNino W 2001). More than menopausal status, an increase in body weight is likely to have a great influence on insulin sensitivity (Guthrie J 2001). Insulin resistance and increased visceral fat accumulation are two of the most important pathophysiological components of metabolic syndrome (Carr M 2003), but it is uncertain whether the starting point is insulin resistance leading to obesity, or vice versa (Kaaja R 2008a).

### **1.7.2 SYMPATHETIC OVER-ACTIVITY**

In women with normal pregnancy and with non-proteinuric PIH, peripheral sympathetic hyperactivity seems to fall to normal after delivery (Greenwood J 2001). However, even in normotensive women with a history of pre-eclampsia there are signs of increased sympathetic activity (Courtat D 2006). Five years after pre-eclampsia women have been found to have higher levels of noradrenaline compared with women with a history of normotensive pregnancy (Lampinen K 2014).

Chronic activation of the sympathetic nervous system has been associated with components of metabolic syndrome such as blood pressure elevation (Esler M 2000), dyslipidaemia (Lambert E 2013), impaired fasting glucose with hyperinsulinaemia (Grassi G 2004) and obesity (Grassi G 1995, Esler M 2001). A hyperactive sympathetic nervous system is clearly associated with metabolic syndrome (Brook R 2000, Grassi G 2005) and it seems that sympathetic hyperactivity is fundamental in the pathophysiology of the condition (Lambert G 2010) and might even precede insulin resistance (Lambert G 2010). Metabolic syndrome is a powerful risk factor of cardiovascular disease (McNeill A 2005) and CVD is also associated with a hyperactive sympathetic system (Thayer J 2010).

### **1.7.3 ANDROGENS**

Even 17 years after pre-eclamptic pregnancy women have been found to have higher serum free testosterone concentrations and FAIs than controls (Laivuori H 1998). In women with pre-eclamptic pregnancy the higher levels of testosterone may contribute to the increased risk of later vascular morbidity. Cardiovascular disease is associated with endothelial dysfunction (Verma S 2002), like pre-eclampsia (Gilbert J 2008). In pregnant rats high androgen levels have been associated with endothelial dysfunction (Chinnathambi V 2013). To the best of my knowledge the literature on androgens in and after non-proteinuric PIH is scarce.

### **1.7.4 METABOLIC SYNDROME AND CARDIOVASCULAR DISEASE**

Pregnancy-induced hypertension and pre-eclampsia in particular, and CVD, share metabolic abnormalities: obesity, hypertension, insulin resistance and dyslipidaemia. Shared risk factors could explain the apparent association between pre-eclampsia and later CVD (Valdiviezo C 2012). These disorders also share common features of pathophysiology: oxidative stress, increased inflammatory activation, enhanced sympathetic activity and endothelial dysfunction.

Five to six years after pre-eclampsia women have been found to have impaired endothelium-independent and endothelium-dependent

vasodilatation (Lampinen K 2006). Endothelial dysfunction is associated with pre-eclampsia (Gilbert J 2008), atherosclerosis (Gimbrone M 1995) and CVD (Verma S 2002). An important risk factor of CVD is metabolic syndrome (Galassi A 2006). The first manifestation of metabolic syndrome may occur in pregnancy, presenting as pre-eclampsia or GDM (Kaaaja R 2005, Kaaaja R 2008b). Women with a history of PIH have an increased prevalence of metabolic syndrome as early as in their mid-30s (Forest J-C 2005). In women, PCOS (Essah P 2006) and visceral obesity increase the risk of metabolic syndrome (Carr M 2003).

Metabolic syndrome is a term describing a cluster of metabolically related cardiovascular risk factors (Alberti K 2006). Insulin resistance, hypertension, dyslipidaemia (increased triglyceride and decreased HDL levels) and obesity characterize the syndrome. Although obesity seems to be strongly related to metabolic syndrome, not all obese subjects develop the condition. The clinical criteria of metabolic syndrome as defined by the International Diabetes Federation (IDF) (Alberti K 2006), the World Health Organisation (World Health Organisation 1999) and the National Cholesterol Education Program Adult Treatment Panel-III (ATP III) (Expert Panel 2001) are given in Table 5. In Study III we used IDF diagnostic criteria (Alberti K 2006) for diagnosis of metabolic syndrome. In IDF criteria abdominal obesity and two additional factors are sufficient for the diagnosis.

The prevalence of metabolic syndrome increases with age, especially after menopause (Kaaaja R 2008a). Menopause itself has many characteristics of metabolic syndrome (Spencer C 1997). Metabolic syndrome could be the possible underlying mechanism common to pre-eclampsia and CVD. Regardless of their proteinuric status (Männistö T 2013), women with hypertensive pregnancies are at increased risk of later cardiovascular disease and mortality (Bellamy L 2007, Valdiviezo C 2012). Women with a history of pre-eclampsia have a 3- to 4-fold increased risk of developing hypertension, a twofold increased risk of ischaemic heart disease and an increased stroke risk compared with women without such a history (Valdiviezo C 2012). Women with prior pre-eclampsia, especially those with early onset disease ( $\leq 37$  weeks of gestation) (Bellamy L 2007) and recurring disease in subsequent pregnancies (Lykke J 2009), carry an enhanced risk of future CVD. The risk of future cardiovascular events increases significantly if pregnancy has been complicated by both hypertensive disorders of pregnancy and GDM (Sullivan S 2011).

**Table 5.** *Clinical criteria defining metabolic syndrome in women*

	<b>IDF</b>	<b>World Health Organisation Definition</b>	<b>ATP III Definition</b>
Reference	(Alberti K 2006)	(WHO 1999)	(Expert Panel 2001)
Needed for diagnosis	Central obesity plus any two of the four additional factors	Fasting hyperinsulinaemia or impaired glucoregulation and > 2 other factors	≥ 3 of 5 risk factors
<b>Central obesity</b>			
Waist circumference	≥ 80 cm for Europid women		> 88 cm for women
WHR		> 0.85 for women and/or	
BMI		BMI > 30 kg/m <sup>2</sup>	
<b>Glucose tolerance</b>			
Fasting plasma glucose	≥ 5.6 mmol/l and/or	≥ 6.1 mmol/l	≥ 6.1 mmol/l
Previously diagnosed type 2 diabetes	Yes		
<b>Blood pressure</b>			
Systolic	≥ 130 mmHg and/or	≥ 140 mmHg and	≥ 130 mmHg and/or
Diastolic	≥ 85 mmHg and/or	> 90 mmHg	≥ 85 mmHg
Treatment of previously diagnosed hypertension	Yes		
<b>Dyslipidaemia</b>			
Triglycerides	≥ 1.7 mmol/l, or specific treatment for this lipid abnormality	≥ 1.7 mmol/l for women	> 1.69 mmol/l
HDL cholesterol	< 1.29 mmol/l in females, or specific treatment for this lipid abnormality	< 1.0 mmol/l for women	< 1.29 mmol/l for women
Microalbuminuria	Not included in criteria	≥ 20 mg/min or albumin/creatinine ≥ 30 mg/g	Not included in criteria

ATP III, Adult Treatment Panel-III; WHR, waist-to-hip ratio; HDL, high-density lipoprotein.

## 2 MEASUREMENT OF INSULIN SENSITIVITY

All the used techniques to estimate insulin sensitivity in vivo during pregnancy have limitations. During pregnancy, there is non-insulin-mediated glucose disposal from the mother to the foetus through placentally facilitated diffusion of glucose. Therefore, all estimates of insulin sensitivity in pregnancy are overestimates of maternal insulin sensitivity, particularly in late gestation (Catalano P 2010).

### ***The hyperinsulinaemic-euglycaemic clamp***

The gold standard method in measuring insulin sensitivity is the hyperinsulinaemic euglycaemic clamp (DeFronzo R 1985, Kaaja R 2006, Catalano P 2010), but it is an invasive method mainly used for research purposes when accuracy and reproducibility of the measures are required (Kaaja R 2006, Catalano P 2010).

In this technique, insulin is infused in order to attain a steady-state insulin concentration. A variable glucose infusion is then used, based on frequent glucose sampling, in order to maintain a constant euglycaemic glucose concentration, i.e. the glucose is clamped at a steady-state level (DeFronzo R 1985, Catalano P 2010).

### ***Minimal model analysis of the intravenous glucose tolerance test (IGTT)***

Like the hyperinsulinaemic-euglycaemic clamp, minimal model analysis of the intravenous glucose tolerance test (IGTT) is an invasive method mainly used for research purposes (Bergman R 1987). Insulin sensitivity is estimated, but it may be less useful in late gestation when insulin sensitivity is decreased, compared with the euglycaemic clamp, because of the increased variability in insulin sensitivity estimation (Catalano P 2010).

### ***Insulin sensitivity indexes (ISIs)***

Fasting glucose and insulin characterize only the insulin sensitivity of the liver (Abdul-Ghani M 2007). Insulin sensitivity indexes are more common clinical methods used to estimate insulin sensitivity. A quantitative insulin sensitivity check index (QUICKI) (Katz A 2000) and a homeostatic assessment model (HOMA) (Matthews D 1985) are insulin sensitivity indexes calculated by using fasting concentrations of plasma glucose and insulin. These indexes have been shown to be good predictors of insulin sensitivity in subjects with normal glucose tolerance and diabetes, but they are inaccurate in patients with impaired glucose tolerance or a new stage of non-diabetic impaired fasting hyperglycaemia (Kaaja R 2006), since at pre-diabetic stages insulin levels may be elevated. Indices involving fasting

concentrations of glucose and insulin primarily reflect hepatic insulin sensitivity, but not insulin action in peripheral tissue (Kaaja R 2006, Abdul-Ghani M 2007).

To evaluate whole-body glucose tolerance in vivo, the most commonly used method is the oral glucose tolerance test (OGTT). The Matsuda and DeFronzo insulin sensitivity index (ISI) is calculated from the 75-g 2-h OGTT (Matsuda M 1999). Matsuda ISIs correlate well ( $r = 0.73$ ,  $p < 0.0001$ ) with ISIs derived from the euglycaemic clamp technique (Matsuda M 1999).

The products of the area under the plasma glucose curve and under the plasma insulin curve have been used as an index of insulin resistance, but the concept has not been validated (Matsuda M 1999).

### 3 MEASUREMENT OF SYMPATHETIC ACTIVITY

#### ***Assessment of the autonomic nervous system***

Autonomic nervous control can be non-invasively studied by analysis of spontaneous heart rate and blood pressure variability from continuous recordings of heart rate and blood pressure, or by analysing blood pressure and heart rate responses to a variety of physiological stresses in cardiovascular reflex tests, such as the Valsalva manoeuvre, the orthostatic stress test, the isometric handgrip test and by measuring heart rate variability (Ekholm E 1996, Rang S 2002). During pregnancy non-invasive methods are especially of interest, since they carry minimal risks to the mother and foetus and can be used repeatedly (Rang S 2002). The limitations of these non-invasive tests are that they do not reveal the level of disturbed autonomic regulation of blood pressure and most non-invasive test methods show large inter-individual variability (Rang S 2002).

#### ***Orthostatic stress test***

In the orthostatic stress test one changes posture rapidly from lying to an upright position, remaining standing still for a desired time (in our study 10 minutes). Blood pressure and heart rate are measured at different time points.

In normal subjects, when posture is changed from supine to upright it causes blood to pool from the thorax into the veins of the lower extremities and trigger reflex changes characterized by sympathetic activation, vagal withdrawal and release of renin and vasopressin (Chen G-Y 1999).

Usually, in non-pregnant persons, moving from a supine to a standing position results in a transient drop in systolic blood pressure ( $\leq 20$  mmHg) and a rise in heart rate ( $\leq 20$  beats/min) (Thulesius O 1976). However, the heart rate response is biphasic and within 30 s after standing up the increased heart rate is followed by a rapid decrease (Ekholm E 1996), stabilized by the end of 3 minutes. An upright position primarily amplifies the sympathetic noradrenergic system of the heart and peripheral blood vessels. The aggravated tachycardia as a reaction to standing up suggests an increased sympathetic outflow to the heart. In the orthostatic test the blood pressure and heart rate differences between supine and upright positions are related to plasma noradrenaline levels (Ewing D 1985).

In normal subjects, compared with the supine position, assuming the right- or left-lateral decubitus position demonstrates higher vagal and lower sympathetic modulation (Kuo C 2000).

### ***Heart rate variability (HRV)***

Heart rate has been proposed as a global index of ANS influence on the heart, and an elevated heart rate may reflect a shift in the autonomic balance toward enhanced sympathetic and suppressed vagal tone. Elevated heart rate has been predictive of cardiovascular disease (Festa A 2000).

### ***Time domain method***

Perhaps the simplest way to measure heart rate variation is a time domain method of assessing heart rate variability (HRV). With these methods either the heart rate at any point in time or the intervals between successive normal complexes are determined (Eur Heart J 1996).

### ***Frequency domain method***

A power spectral analysis of variation in heart rate is one of the frequency domain methods of assessing HRV. It is based on mathematical models to determine the balance between sympathetic and vagal nerve activities in the heart (Kajja R 2006). Power spectral analysis of variation is a dynamic and non-invasive technique, but it seems to have poor correlation with noradrenaline spillover from the heart and nerve firing rates determined by microneurography in situations where stimuli other than arterial and low-pressure baroreceptors (postural stimulation) are engaged (Kajja R 2006).

### ***Measurement of sympathetic activity***

Sympathetic drive in humans can be measured by 1) assay of plasma and urinary catecholamines (noradrenaline and adrenalin), 2) assessment of systemic and regional noradrenaline spillover, and 3) direct sympathetic nerve recordings. However, there is no “gold standard” technique to assess sympathetic activity (Grassi G 1999, Kajja R 2006). Various techniques are complementary.

### ***Muscle sympathetic nerve activity (MSNA)***

So far, in humans the only method for direct recording of regional sympathetic nerve traffic is muscle sympathetic nerve activity (MSNA) measured via microneurography. In MSNA efferent postganglionic muscle sympathetic nerve activity is assessed (from peroneal or brachial nerves) (Grassi G 1999). Direct recordings allow discrimination between the central or peripheral nature of increased plasma noradrenaline levels, and precise estimation of the behaviour of regional sympathetic neural function both under physiological and pathological conditions (Grassi G 1999). The rise in MSNA is a direct measure of central (baroreceptor reflex-controlled) sympathetic vasoconstrictor impulses to the muscle vascular bed. An important limitation in clinical research is that the method is invasive and there is inaccessibility to the sympathetic nerves to internal organs. In this regard an indirect inference of sympathetic activity from regional noradrenaline spillover measurements is more helpful.

### ***Regional and systemic noradrenaline spillover***

A release of sympathetic neurotransmitter to plasma from an individual organ is regional spillover, which is studied using the principle of isotope dilution, with intravenous infusion of tritiated noradrenaline and sampling from venous drainage of the organ in question (mainly heart and kidney). The whole body noradrenaline spillover rate gives a measure of overall sympathetic nervous activity (Grassi G 1999). Spillover methods with radioactive agents are contraindicated during pregnancy.

### ***Plasma noradrenaline***

Measurement of plasma noradrenaline concentration in venous blood is the most commonly employed index of sympathetic activity in man, but it is relatively insensitive (Grassi G 1999). Most of the noradrenaline secreted from the sympathetic fibres is destroyed or taken up again and only a minute fraction escapes from the neuroeffector junctions (Grassi G 1995). The sympathetic nervous system typically shows regional differentiation, so that sympathetic outflow to some organs may be activated while that to other regions may be unchanged or inhibited (Grassi G 1999). The plasma noradrenaline concentration gives no indication of the source of the hormone and does not discriminate between central (increased secretion) and peripheral (decreased clearance) mechanisms of augmentation (Grassi G 1999, Kaaja R 2006). Furthermore, venous noradrenaline assays have shown limited reproducibility, and sensitivity in detecting increased sympathetic nerve activity is not optimal (Grassi G 1999, Kaaja R 2006). Plasma noradrenaline has a diurnal rhythm and high-performance liquid chromatography (HPLC) is used to measure diurnal plasma noradrenaline levels (Kaaja R 1999b).

### ***Plasma adrenaline***

The adrenal medulla acts as a modified sympathetic ganglion and adrenal neurons directly release catecholamines (noradrenaline and adrenaline) into the blood stream, mainly adrenaline. Therefore, plasma adrenaline levels mainly reflect adrenal medulla production of the hormone (Schmidt R 1989) and they are generally taken as a valid index of adrenal medullar production of the hormone.

## 4 AIMS OF THE STUDY

To seek associations between maternal serum FABP4 levels in the second and third trimesters of pregnancy and indices of possible insulin resistance, sympathetic over-activity, inflammation and hyperandrogenism in cases of PIH (I).

To assess if maternal serum levels of Angptl6 and FABP4 in the second trimester of pregnancy are associated with subsequent development of hypertensive pregnancy and indices of possible insulin resistance, sympathetic over-activity, inflammation and hyperandrogenism in cases of PIH (II).

To evaluate sympathetic activity by way of neurohormonal indices in pre-eclamptic pregnancy in connection with different body postures (III).

To assess diurnal glucose profiles in cases of GDM with and without hypertensive complications (IV).

To assess if there are signs of insulin resistance and hyperandrogenism in the premenopausal period in women with a history of pre-eclampsia (V).

## 5 SUBJECTS AND METHODS

All five studies were approved by the Ethics Committee of the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital (HUCH). The approval number for Study III is 296/E8/2000 and that for Studies IV and V, 045/97. All women gave informed consent except in Study I, where a standard clinical procedure was followed and therefore no patient consent was needed.

### 5.1 WOMEN WITH SUBSEQUENT PIH (I, II)

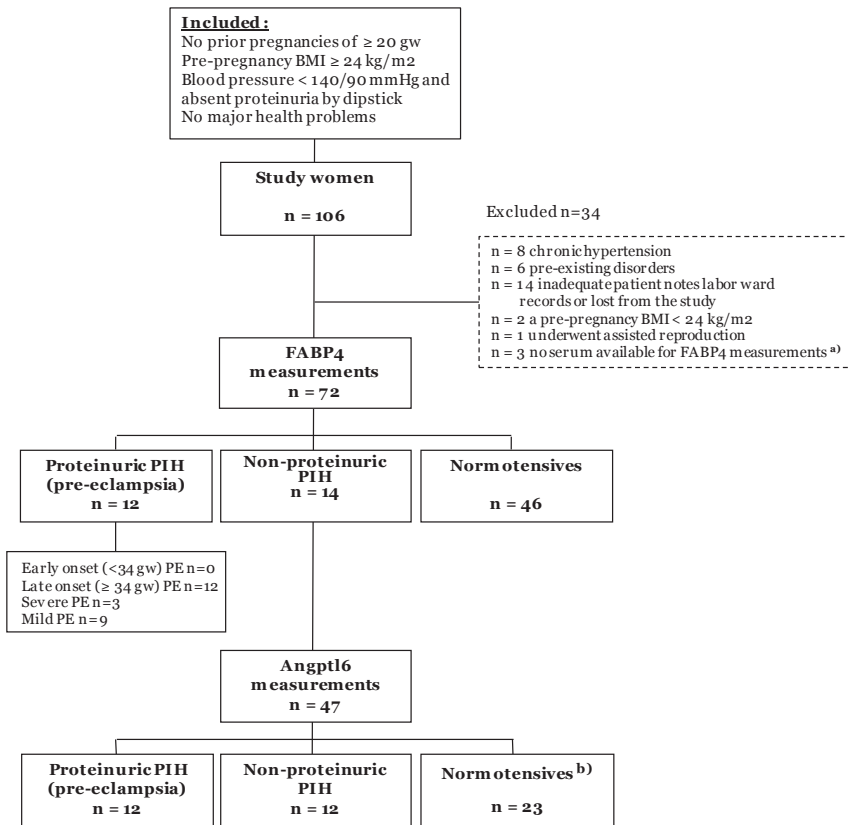
**Studies I and II** were prospective observational studies. In Study I 72 women and in Study II a subset of 47 women were investigated. They were at a relatively high risk of developing pre-eclampsia, because of relative overweight (BMI  $\geq 24$  kg/m<sup>2</sup>) and nulliparity (Figure 3). All women had a singleton pregnancy. Women were recruited from consecutive referrals for a routine first-trimester ultrasonographic scan at Helsinki University Central Hospital maternal polyclinic.

All women underwent the same study protocol at a single site (Table 6), and also underwent routine maternity ward follow-up. The first examination was at 24 weeks of gestation and the study included 106 women. Table 6 shows detailed information of the orthostatic test, a standard 2-hour 75-g OGTT, and laboratory analysis at 24 and 32 weeks of gestation. At baseline, a venous sample was drawn from an antecubital vein after at least 12 hours fasting. Mean arterial pressure (MAP) was calculated as  $([2 \times \text{DBP}] + \text{SBP})/3$ . Delta systolic BP ( $\Delta\text{SBP}$ ) was calculated as SBP after standing minus SBP after rest. Insulin resistance was estimated by using the whole-body insulin sensitivity index (ISI) (Matsuda M 1999) and the homeostatic model assessment – insulin resistance (HOMA-IR) (Matthews D 1985) at 24 weeks of gestation. The Matsuda whole-body ISI was calculated as follows:  $10\,000 / \sqrt{([\text{fasting plasma glucose} \times \text{fasting serum insulin}] \times [\text{mean OGTT glucose concentration} \times \text{mean OGTT insulin concentration}])}$  (Matsuda M 1999). HOMA-IR was calculated as follows:  $(G_o \times I_o)/22.5$ , where  $G_o$  is fasting glucose (mmol/l) and  $I_o$  is fasting insulin ( $\mu\text{U/ml}$ ) (Matthews D 1985). The first trimester of pregnancy was defined as  $\leq 13$  w + 2 d of gestation and the second trimester as  $\leq 26$  w + 4 d of gestation.

Diagnoses of non-proteinuric PIH and proteinuric PIH (pre-eclampsia) were based on criteria of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy (National 2000) (Table 1). PIH was defined as systolic BP (SBP) elevation to  $\geq 140$  mmHg or diastolic BP (DBP) elevation to  $\geq 90$  mmHg after 20 weeks of gestation with (pre-eclampsia) or without proteinuria. Proteinuria was

defined as  $\geq 0.3$  g protein/24 hours or  $\geq 1+$  in dipstick testing. Pre-eclampsia was regarded as serious if severe hypertension was associated with proteinuria or if hypertension was associated with severe proteinuria ( $\geq 5$  g per day). Hypertension was regarded as severe if SBP was sustained at  $\geq 160$  mmHg or DBP sustained at  $\geq 110$  mmHg, or both (Sibai B 2005). Chronic hypertension was diagnosed as SBP elevation to  $\geq 140$  mmHg or DBP elevation to  $\geq 90$  mmHg, or both, repeatedly before 20 weeks of gestation. Normotensive women had normal BP ( $< 140/90$  mmHg) and no proteinuria during pregnancy.

**Figure 3** Flow chart concerning research subjects in Studies I and II



a) For 1 woman with proteinuric PIH and 2 normotensive women.

b) Angpt16 measurements were made only in a subset of normotensives and 1 outlier was excluded. After Angpt16 measurements patient notes were supplemented for one woman with non-proteinuric PIH and 4 normotensives and diagnoses were supplemented for one woman with proteinuric PIH and 2 women with non-proteinuric PIH (1 woman with subsequent non-proteinuric PIH was among those whose patient notes were supplemented).

**Table 6.** Analyses and gestational weeks at time of analysis in Studies I and II.

<b>At 24 weeks of gestation</b>		<b>75-g 2-h OGTT<sup>*)</sup></b>				<b>At 32 weeks</b>	
<b>Orthostatic test</b>		<b>After 10 min of standing</b>		<b>0.5-h</b>		<b>Fasting</b>	
<b>After 30 min resting in supine position</b>		<b>After 10 min of standing</b>		<b>0-h</b>	<b>1-h</b>	<b>2-h</b>	
Fasting							
S-FABP4	BP	BP		P-Glucose	P-Glucose	P-Glucose	S-FABP4
S-total testosterone	HR	HR		S-Insulin	S-Insulin	S-Insulin	S-Angptf6
S-SHBG	P-Noradrenaline	P-Noradrenaline		S-Angptf6 <sup>**)</sup>	S-Angptf6 <sup>**)</sup>	S-Angptf6 <sup>**)</sup>	S-hs-CRP
S-hs-CRP	P-Adrenaline	P-Adrenaline					S-total testosterone
S-total cholesterol	P-Ghrelin	P-Ghrelin					S-SHBG
S-HDL cholesterol							P-Glucose
S-triglycerides							P-Noradrenaline
S-LDL cholesterol							P-Adrenaline
P-Glucose							P-Ghrelin
S-Insulin							

<sup>\*)</sup> in accordance with American Diabetes Association criteria (The Expert Committee 2003)

<sup>\*\*)</sup> Angptf6 analyses were done in a subset of 47 women.

## **5.2 WOMEN DURING PRE-ECLAMPSIA (III)**

Study III was a case-control study of 36 women with: pre-eclampsia (n=11) studied at 37 gestational weeks, normotensive pregnancy (n=17) studied at 38 gestational weeks, and non-pregnant controls (n=8). Patients with signs of foetal distress, severe pre-eclampsia (BP  $\geq$  170/110 mmHg, proteinuria  $\geq$  5 g/24 h), severe intrauterine growth retardation, diabetes, secondary hypertension or medication were excluded. Of the 36 study women, 6 pre-eclamptic and 9 normotensive pregnant women were studied at Helsinki University Hospital, Finland, while the others were studied at Christchurch Hospital, New Zealand.

Measurements of arterial pressure, heart rate and neurohormones were carried out with subjects in the left-lateral position, then supine, left-lateral, in an upright posture and the left-lateral position again. The measured neurohormones were: noradrenaline, adrenaline, renin activity, natriuretic peptides and endothelin-1. Blood pressure status was classified as recommended by the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy report in 1990 (National 1990).

## **5.3 WOMEN WITH GDM (IV)**

Study IV was a prospective follow-up study of 178 women with gestational diabetes, who delivered at the Department of Obstetrics and Gynaecology, HUCH, over 1 year (January 1, 1995 to December 31, 1995). In the same time period the total number of deliveries at HUCH was 5343.

The diagnosis of gestational diabetes was based on 75-g 2-h OGTTs, carried out at 28–32 weeks of gestation among pregnant women with one or more of the following risk factors of GDM: glucosuria, obesity (BMI  $\geq$  25 kg/m<sup>2</sup>), previous delivery of a macrosomic infant ( $\geq$  4500 g), previous GDM, maternal age  $\geq$  40, estimated foetal weight  $\geq$  2 SD units in current pregnancy. At that time normal upper limits for capillary blood or venous plasma glucose values were: fasting 4.8 mmol/l, 1-h 10.0 mmol/l, and 2-h 8.7 mmol/l (Hyvönen K 1991).

Glucose intolerance suggested by OGTT results was confirmed usually within 2 weeks with a blood glucose profile, measuring blood glucose every 4 h over 24 h. Five measurements were fasting/pre-prandial (08.00, 16.00, 00.00, 04.00, 08.00 h) and two were post-prandial (1 h after meal) at 12.00 and 20.00 h. After 24-h glucose profiles, of the 178 women with GDM, 55 were started on insulin treatment (White A/B) and 123 women had only dietetic treatment (White A).

Women were classified as being normotensive (n = 102), having chronic hypertension (n = 42) (with or without superimposed pre-eclampsia on chronic hypertension) or PIH (n = 34) (with or without proteinuria) (National 1990).

Diurnal blood glucose profiles were compared in these groups. Changes in blood glucose levels were also calculated in obese (BMI  $\geq$  28 kg/m<sup>2</sup>) and non-obese women (BMI < 28 kg/m<sup>2</sup>), regardless of hypertension.

## 5.4 WOMEN IN THE PERIMENOPAUSAL PERIOD (V)

Study V was a case-control study of 18 women with a history of pre-eclamptic first pregnancy and 19 women with prior normotensive first pregnancy studied 23–24 years after delivery (Table 7). Both the study women and the controls had delivered at the Department of Obstetrics and Gynaecology, Helsinki University Hospital, Finland, from the 16<sup>th</sup> of March 1975 to the 4<sup>th</sup> of January 1980, and were all Caucasian.

**Table 7.** Subjects in Study V

Study group	No of subjects	Inclusion criteria	Exclusion criteria
A history of pre-eclamptic pregnancy	18	Pre-eclampsia during index pregnancy	<ul style="list-style-type: none"> <li>• Pre-existing medical disorders before index pregnancy</li> <li>• Assisted reproductive techniques</li> <li>• Multiple pregnancies</li> <li>• Hormone replacement therapy <sup>a)</sup></li> </ul>
Prior normotensive pregnancy	19	Normal blood pressure and no proteinuria during index pregnancy	

GDM; gestational diabetes mellitus, PIH; pregnancy-induced hypertension, OGTT; oral glucose tolerance test

<sup>a)</sup> Oestrogen substitution and contraceptive pills increase SHBG levels and inhibit testosterone production through LH suppression.

The diagnosis of proteinuric PIH (pre-eclampsia) was based on criteria of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy (National 2000) (Table 1). 75-g 2-h OGTTs were carried out and Matsuda's whole-body insulin sensitivity index (Matsuda ISI) was calculated. Based on a 75-g 2-h OGTT, glucose tolerance was categorized to normal, diabetes, and abnormal glucose tolerance according to American Diabetes Association criteria (The Expert Committee 2003) (Table 8). Abnormal glucose tolerance included both impaired fasting glucose and impaired glucose tolerance. In 2-hour OGTTs, plasma glucose was assessed at 0, 1 and 2 hours after a 75-g oral glucose load.

**Table 8.** Glucose tolerance evaluated via 2-hour 75-g OGTTs according to American Diabetes Association criteria (Expert committee 2003)

	Normal	Abnormal glucose tolerance	Diabetes
fP-gluc	< 6.1 mmol/l	6.1 – 6.9 mmol/l	≥ 7.0 mmol/l
	and	and/or	and/or
2-hour P-gluc	< 7.8 mmol/l	7.8 – 11.0 mmol/l	≥ 11.1 mmol/l

Areas under the curve (AUCs) were calculated for glucose and insulin. Glucose AUCs (mmol/l \* minutes) at 120 min were evaluated according to the formula  $15 * (G_0 + 2 * G_{30} + 3 * G_{60} + 2 * G_{120})$ , where G is a glucose value at each time point, and insulin AUCs ( $\mu\text{U}/\text{ml} * \text{minutes}$ ) at 120 min were evaluated according to the formula  $30 * (I_0 + 2 * I_{60} + I_{120})$ , where I is a insulin value at each time point (Matthews J 1990).

Serum concentrations of follicle-stimulating hormone (FSH), sex hormone-binding globulin (SHBG), total and calculated free testosterone, dehydroepiandrosterone sulphate (DHEAS), luteinizing hormone (LH), total cholesterol, high-density lipoprotein (HDL) cholesterol, hormone-sensitive high-density lipoprotein (HDL2) cholesterol, HDL3 cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol and plasma insulin were assessed. Diagnoses of metabolic syndrome were based on IDF criteria (Alberti K 2006) (Table 5). A PCOS phenotype was defined using the Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group (2004) criteria as the presence of two or more of the following features: (1) history of oligomenorrhoea/amenorrhoea, (2) clinical and/or biochemical signs of hyperandrogenism (hirsutism, acne and/or S-testosterone > 2.7 nmol/l) and (3) polycystic ovaries detected in ultrasonography (Rotterdam 2004).

## 5.5 BLOOD SAMPLE ANALYSES

Venous blood samples were drawn into heparinized (serum) tubes and EDTA (plasma) tubes on ice, centrifuged for 10 min and the samples stored at -20 or -80 °C until analysis.

### ***Adrenaline, noradrenaline and ghrelin***

For measurements of adrenaline, noradrenaline (Eisenhofer G 1986) and ghrelin (Davis M 2004) plasma was transported on ice to Endolab, Christchurch Hospital in New Zealand. All individual samples were analysed in a single assay. The intra-assay coefficients of variation for these assays varied between 2.4% (for noradrenaline) and 8.3% (for ghrelin) (I, II). The intra-assay coefficient of variation for noradrenaline in Study II was 6%.

### ***Angiotensin-like protein 6 (Angptl6)***

Serum Angptl6 concentrations were analysed using a commercial ELISA method according to the protocol described in the package insert (Adipogen ANGPT6 Human ELISA Kit, TwinPlex, Cat. no. AG-45A-0016TP-Kio1). Validation of the Angptl-6 ELISA assay was based in part on the use of quality control samples included in each ELISA kit. The expected range  $\pm$  SD for Angptl6 in the quality control sample was  $20 \pm 5$  ng/ml. The mean value of this sample in our assay was 22.9 ng/ml with intra- and inter-assay precision values (coefficients of variation) of 1.2% and 2.4%, respectively. The standard curves for the ELISA assays, i.e. Angptl6 concentrations plotted against absorbance values showed good stability and reproducibility ( $r = 0.983 \pm 0.01$  (SD)).

### ***Fatty acid binding protein 4 (FABP4)***

Serum FABP4 concentrations were analysed by using a non-competitive ELISA (Biovendor RD 191036200R). Intra- and inter-assay variations were about 2.5% and 5.5%, respectively. The manufacturer reports a normal range of  $19.6 \pm 8.1$  ng/ml (mean  $\pm$  1 SD) for 35- to 52-year-old women.

### ***Follicle-stimulating hormone (FSH) and luteinizing hormone (LH)***

Serum concentrations of FSH and LH were determined by time-resolved immunofluorometric assays from Perkin-Elmer, Wallace Ltd. (AutoDelfia, Turku, Finland).

### ***High sensitivity CRP (hs-CRP)***

A high-sensitivity CRP (hs-CRP) assay measures low plasma levels of CRP. Hs-CRP was measured by particle-enhanced immunoturbidimetric assay (CRP (Latex) HS, Tina-quant C-reactive protein (latex) high sensitivity assay, Roche Diagnostics) on a Modular automatic analyzer (Hitachi Ltd., Tokyo, Japan). The intra-assay coefficients of variation (CVs) were 1.1% at 0.70 mg/l and 0.7% at 5.9 mg/l and the inter-assay CVs were 8.2% at 0.44 mg/l ( $n=81$ ) and 3.3% at 2.94 mg/l ( $n= 81$ ). The method is accredited by FINAS Accreditation (SFS-EN ISO/IEC 17025:2005 and SFS-EN ISO 15189:2007).

### ***Lipids***

Total cholesterol and total HDL cholesterol concentrations were measured by using commercial kits (Boehringer, Mannheim, Germany). Triglyceride concentrations were analysed in a Technicon autoanalyzer (AutoAnalyzer II; Technicon Instruments, Tarrytown, NY). For hormone-sensitive high-density lipoprotein cholesterol (HDL2 cholesterol) and HDL3 cholesterol analysis, HDL cholesterol was separated from serum by precipitation of the other lipoproteins with heparin-manganese chloride (Gidez L 1982). The supernatant was further fractioned into HDL2 and HDL3 cholesterol by

precipitation with 0.11% dextran sulphate. Concentrations of LDL cholesterol were calculated by using the Friedewald equation (Friedewald W 1972).

### **Total serum testosterone**

In Study I serum testosterone concentrations were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Before analysis, 30 µl of 0.2 µM deuterated testosterone as internal standard (IS) (D3-testosterone, Sigma) in 50% (vol./vol.) methanol was added to 250 µl of serum before extraction with 4 ml of diethyl ether. After mixing for 3 min and centrifugation the upper layer was collected and evaporated to dryness under nitrogen. The residue was dissolved in 250 µl of 50% methanol. Calibrators containing 0.2–100 nM testosterone (Fluka) were prepared in 50% methanol. Twenty-five µl of sample extracts and calibrators were analysed on an LC-MS/MS system equipped with an API 2000 triple quadrupole mass spectrometer (PE Sciex, Foster City, CA). Peripherals included an Agilent series 1200 high-performance liquid chromatography system with a binary pump (Waldbronn, Germany). Separation was performed on a SunFire C18 column (2.1 × 50 mm; Waters, Milford, MA). The mobile phase was a linear gradient consisting of methanol and 130 µM ammonium acetate in water, at a flow rate of 250 µl/min. The gradient was: 0 min, 50% methanol; 2 min 95% methanol; 3 min 95% methanol; and 3.5–10 min 50% methanol. The column was directly connected to the electrospray ionization probe. Testosterone was detected as protonated ions in the positive mode with the following transitions: m/z 289 to m/z 97 & 109 and IS, m/z 292 to m/z 97. Data were acquired and processed via Analyst Software (Ver 1.4; Sciex). Mass calibration and resolution adjustments (at 0.7 atomic mass units at full width and half height) on both the resolving quadrupoles were optimised using a polypropylene glycol solution with an infusion pump.

In Study V, for measurements of total testosterone we used a specific liquid chromatography-tandem mass spectrometric method (LC-MS/MS) with an API 2000 triple quadrupole mass spectrometer (PE Sciex, Foster City, CA, USA) and deuterated testosterone as an internal standard (Turpeinen U 2008).

### **Calculated androgen indexes (I, V)**

Free, non-protein bound, calculated free testosterone (cfT) (I, V) was calculated by using an equation published by Anderson et al.:  $100 * S\text{-Testo} * (\text{testo-V}\%) = 10 * S\text{-Testo} * (2.28 - 1.38\chi * \log[S\text{-SHBG}/10])$  (Andersson D 1975). In Study III we used the following formula to calculate the free androgen index (FAI):  $100 * S\text{-Testo (ng/dl)}/28.84 * S\text{-SHBG (nmol/l)}$  (Sutton-Tyrell K 2005).

**Table 9.** Characteristics of the assays used in the studies, and reference ranges.

Analyte	Source of reagents	Principle of assay	Coefficient of variation		Study
			Intra-assay	Inter-assay	
S-Adrenaline		Liquid chromatography			I, II, III
S-Angptf6	Adipogen ANGPLT6 Human ELISA Kit, TwinPlex, Cat. no. AG-45A-0016TP-Kio1	ELISA	1.2%	2.4%	I, II
S-Cholesterol	Boehringer, Mannheim, Germany	Enzymatic method			I, II, V
S-DHEAS	Siemens Medical, LA, California, USA	Chemiluminescent enzyme immunoassays			V
S-FABP4	Biovendor RD 191036200R	ELISA	2.5%	5.5%	I
S-FSH	Perkin-Elmer, Wallac Ltd. (AutoDelfia, Turku, Finland)	Immunofluorometry			V
P-Ghrelin		RIA	8.3%		I, II
P-Glucose	Glucoguant, Roche Diagnostics	Hexokinase method			I, II, IV, V
S-HDL	Boehringer, Mannheim, Germany	Enzymatic method			I, II, V
P-Insulin		Immunofluorometry			I, II, V
S-LH		Immunofluorometry			V
P-Noradrenaline	Perkin-Elmer, Wallac Ltd. (AutoDelfia, Turku, Finland)	Liquid chromatography	2.4% (IV, V) 6% (II)		I, II, III
S-hs-CRP	CRP (Latex) HS, Tina-quant C-reactive protein (latex) high sensitivity assay, Roche Diagnostics	Immunturbidimetric	0.7–1.1%	3.3–8.2%	I
S-SHBG	Perkin-Elmer, Wallac Ltd. (AutoDelfia, Turku, Finland)	Immunofluorometry			I, V
P-Testosterone	PE Sciox, Foster City, CA, USA	LC-MS/MS			I, V
S-Triglycerides	AutoAnalyzer II; Technicon Instruments, Tarrytown, NY	Enzymatic method			I, II, V

LC-MS/MS, liquid chromatography-tandem mass spectrometric method

## 5.6 STATISTICAL METHODS

Statistical analyses were performed by using an NCSS 2000 statistical package (NCSS, Inc., Kaysville, UT, USA), except in Study III. Table 10 shows the statistical analyses used in the studies.

**Table 10.** Statistical analyses used in the studies.

<b>Analysis</b>	<b>Study</b>
Descriptive statistics	I–V
Power calculations	V
Analysis of distribution	
• Visual observation	III, IV, V
• Shapiro–Wilk’s <i>W</i> -test	I, II
<b>Comparison of data between groups</b>	
• Chi-squared test	IV
• Fisher’s exact probability test	I, II, V
Non-parametric tests	
• Mann–Whitney <i>U</i> -test	I, II, V
• Kruskal–Wallis one-way ANOVA with multiple comparison <i>Z</i> -value test	I, II, IV
• Repeated measures ANOVA	III, IV
• Analysis of variance (ANOVA)	III
Correlation between different parameters	
• Pearson’s correlation	I, II
• Logistic regression	I, II
• Multiple regression	I, II, IV

In Study V, power calculation showed that 18 cases and 19 controls would be needed for a power of 80% at a *p* level of 0.05 to detect a 5-unit difference in mean Matsuda whole-body insulin sensitivity index, with the null hypothesis that both group means are equal to the mean of controls (= 9.5) (Table 10).

Normality of distribution among continuous variables was analysed either by visual observation (III, IV, V) or Shapiro–Wilk’s *W*-test (I, II) (Table 10). For continuous variables, the non-parametric Mann–Whitney *U*-test and Kruskal–Wallis one-way ANOVA with multiple comparison *Z*-value tests were used to compare groups (I, II, IV). For categorical data, the chi-squared test (IV) and Fisher’s Exact test (I, II, V) were used to compare groups.

Correlation between FABP4 levels (I), Angptl6 levels (II) and clinical parameters were performed by using Pearson’s correlation method. Logistic regression analysis was performed to explore the variables associated with indices of pre-eclampsia, using PIH as the dependent variable. Multiple regression analysis was performed in Study I using change of blood glucose (from 04.00 to 08.00 h) as the dependent variable and chronic hypertension

and obesity ( $\text{BMI} \geq 28 \text{ kg/m}^2$ ) as independent variables. In Studies I and II multiple regression analysis was performed by using PIH as the dependent variable.

Probability values of  $< 0.05$  were considered statistically significant in all analyses.

## 6 RESULTS

Detailed results are given in the original publications and therefore only the main results are presented here.

### 6.1 CHARACTERISTICS OF THE STUDY WOMEN

#### ***Women with subsequent PIH (I, II)***

There were no significant differences in clinical characteristics of the study groups (Table 11). Detailed data is in original papers summarized according to pregnancy outcome.

All women were normotensive in the first trimester, but women who developed PIH had significantly higher SBPs and DBPs from the first to the third trimesters of pregnancy compared with women who stayed normotensive (data not shown).

**Table 11.** *Characteristics of the study women according to pregnancy outcome in Studies I and II.*

	PIH	Normotensive	p
<b>Study I</b>			
n	26	46	
Age at 24 weeks of gestation (years)	30.9 ± 6.3	31.1 ± 4.6	0.8
Pre-pregnancy BMI (kg/m <sup>2</sup> )	28.6 (24.7–36.5)	27.1 (24.2–36.3)	0.2
BMI at time of delivery (kg/m <sup>2</sup> )	33.9 ± 3.4	33.2 ± 3.6	0.3
Weight gain during pregnancy (kg)	12.9 ± 8.2	14.4 ± 6.9	0.5

Values are shown as mean ± SD, median (range).

PIH, pregnancy-induced hypertension; BMI, body mass index.

All 12 women with proteinuric PIH had late-onset pre-eclampsia (gestational weeks ≥ 34, data not shown). Of the three women with severe pre-eclampsia, all fulfilled the criteria for severe hypertension. Three women with pre-eclampsia and one woman with non-proteinuric PIH received antihypertensive medication (data not shown). Weeks of gestation at delivery and birth weight did not significantly differ between the groups (data not shown).

#### ***Women during pre-eclampsia (III)***

The women in the three groups were comparable in clinical characteristics (Table 12). The two pregnant groups were comparable as regards weeks of gestation at time of testing and delivery (data not shown).

**Table 12.** Characteristics of the study groups in Study II

	Pregnant			p <sup>a)</sup>
	Pre-eclamptic	Normotensive	Non-pregnant	
n	11	17	8	
Age (years)	26.7 ± 3.3	28.5 ± 4.2	24.1 ± 4.8	NS
BMI (kg/m <sup>2</sup> )	23.3 ± 4.2	26.3 ± 3.6	24.5 ± 4.8	NS
Gestational weeks at testing	36.8 ± 0.9	37.5 ± 0.9		NS

Values are shown as mean ± SD.

BMI, body mass index.

<sup>a)</sup> Analysis of variance (ANOVA)

### **Women with GDM (IV)**

In 1995 GDM was diagnosed in 178 women, with eight twin pregnancies (4% of all the mothers). Hypertension complicated 43% of the cases. Hypertensive women were comparable with normotensive women in regard to maternal age and parity (Table 13). Mothers with chronic hypertension with or without superimposed pre-eclampsia were heavier (mean BMI ± SD, 31.6 ± 7.4 kg/m<sup>2</sup>) than their normotensive controls (25.7 ± 6.0 kg/m<sup>2</sup>, p < 0.0001) (Table 13). Seventeen women received anti-hypertensive medication during their pregnancies. There were no differences in maternal use of anti-hypertensive therapy between hypertension groups. None of the women received corticosteroid treatment prior to the blood glucose measurements.

**Table 13.** Characteristics of the study groups in Study IV

	Normotensive	Proteinuric and non-proteinuric PIH	CHT and superimposed PE	p
n (%)	102 (57.3%)	34 (19.1%)	42 (23.6%)	
Age (years)	32.7 ± 5.3	33.8 ± 6.0	32.8 ± 5.3	NS
BMI (kg/m <sup>2</sup> )	25.7 ± 6.0	28.9 ± 7.0	31.6 ± 7.4	< 0.0001 <sup>a)</sup>
Nulliparity n (%)	24 (23.5%)	13 (38.2%)	14 (33.3%)	NS

Values are shown as mean ± SD. PIH, pregnancy-induced hypertension; CHT, chronic hypertension; PE, pre-eclampsia; BMI, body mass index. <sup>a)</sup> Kruskal–Wallis one-way ANOVA with multiple comparison Z-value test in mothers with chronic hypertension with or without superimposed PE compared with normotensive mothers

**Women in the perimenopausal period (V)**

The baseline characteristics of the study groups were comparable (Table 14). Five of the women had had early-onset pre-eclampsia (weeks of gestation < 34) and 13 had had late-onset pre-eclampsia (weeks of gestation  $\geq$  34). Sixteen women had severe pre-eclampsia. All women with early-onset pre-eclampsia had a severe disease.

**Table 14.** Characteristics of the study groups in Study V

	Pre-eclampsia	Controls	p <sup>a)</sup>
n	18	19	
Age at the time of the study	48.9 (44–54)	49.3 (42–55)	NS
Age at the time of index pregnancy	24.2 (20–31)	25.8 (17–31)	NS
Years since delivery	23.4 (21–27)	23.7 (23–26)	NS
BMI at the time of the study (kg/m <sup>2</sup> )	26.4 $\pm$ 4.5 (21–39)	25.1 $\pm$ 5.2 (18–39)	NS

Values are shown as median (range) or mean  $\pm$  SD (range).

BMI, body mass index.

<sup>a)</sup> Mann–Whitney *U*-test.

## 6.2 INSULIN RESISTANCE

**Women with subsequent PIH (I, II)**

Whole-body ISI and HOMA-IR at 24 weeks and fasting, 1-hour and 2-hour insulin levels were the same in the two groups, irrespective of subsequent hypertension (data not shown). At 24 weeks the whole-body ISI did not correlate with markers of sympathetic over-activity; noradrenaline at supine resting, noradrenaline after 10 min standing, SBP after 10 min standing or with  $\Delta$ SBP (data not shown). At 24 weeks of gestation no correlation was observed between insulin sensitivity biomarkers FABP4 or Anptl6 and whole-body ISI.

At 32 weeks of gestation fasting blood glucose levels were the same in the two groups, irrespective of subsequent hypertension (data not shown).

**Women with GDM (IV)**

Areas under the glucose curve (glucose AUCs) in the hypertensive groups were equal to those in their normotensive controls (Table 15). Diurnal blood glucose levels at all time points (from 08.00 to 08.00 h) were similar in hypertensive groups and in normotensive controls (data not shown). In women with chronic hypertension we observed a significant rise in blood glucose values in the early morning hours (from 04.00 to 08.00 h), whereas they decreased in the normotensive women (Table 15).

**Table 15.** Blood glucose values in women with GDM with and without hypertension in Study IV

	Normotensive	Proteinuric and non-proteinuric PIH	CHT and superimposed PE	p
Blood glucose at 08.00 (mmol/l)	4.7 (4.3_5.2)	4.7 (4.4–5.5)	4.9 (4.6–5.3)	0.08
Blood glucose at 04.00 (mmol/l)	4.7 (4.3–5.1)	4.6 (4.2–5.0)	4.7 (4.3–5.0)	0.6
Blood glucose at 08.00 (mmol/l)	4.5 (4.3–4.9)	4.5 (4.2–4.9)	4.8 (4.6–5.1)	0.051
Area under glucose curve (mmolh/l)	103 (95–109)	101 (97–112)	100 ( 96–108)	0.9
Average change (from 04.00 to 08.00 h) (mmol/l)	-0.1 (-0.3 to 0.2)	-0.1 (-0.2 to 0.4)	0.2 (-0.1 to 0.4)	0.03 <sup>a)</sup>

Values are shown as median (interquartile range (IQR)).

PIH, pregnancy-induced hypertension; PE, pre-eclampsia; CHT, chronic hypertension.

<sup>a)</sup> Kruskal–Wallis one-way ANOVA with multiple comparison Z-value test in mothers with chronic hypertension with and without superimposed PE compared with normotensive mothers.

In the obese women (BMI  $\geq$  28 kg/m<sup>2</sup>, n = 109) the early morning blood glucose rise was higher [median, IQR, 0.1 (-0.2 to 0.5) mmol/l] compared with the non-obese women (BMI < 28 kg/m<sup>2</sup>, n = 69), [0 (-0.3 to 0.2) mmol/l, p = 0.01]. In regression analysis, both BMI and chronic hypertension were significantly associated with a blood glucose rise from 04.00 to 08.00 h. However, when both factors were included in the multiple regression model, neither showed a significant independent effect.

### ***Women in the perimenopausal period (V)***

Insulin sensitivity (Matsuda whole-body ISI, glucose AUC and insulin AUC) was similar in women with a history of pre-eclamptic pregnancy and those with a history of normotensive pregnancy. Insulin AUC was higher in obese (BMI  $\geq$  30 kg/m<sup>2</sup>) and overweight (BMI 25–30 kg/m<sup>2</sup>) women with prior pre-eclampsia than in normal weight (BMI < 25 kg/m<sup>2</sup>) women with prior pre-eclampsia (p = 0.008). Among the controls such a difference was not seen (data not shown). Only in women with a history of pre-eclamptic pregnancy did waist circumference correlated negatively with insulin sensitivity (p = 0.03).

### **Metabolic syndrome**

The waist-hip ratio, blood pressure, heart rate and lipid and lipoprotein levels in the groups were comparable (data not shown). In the pre-eclampsia group, previously diagnosed hypertension was more common than in controls (44 % [8/18] versus 5% [1/19],  $p = 0.008$ ).

At the time of the study there was no difference in metabolic syndrome between the groups (Table 16). Of women with previous pre-eclampsia, 17% (3/18) had metabolic syndrome versus 11% (2/19) of the controls (Table 16). Of the study women, 22% (4/18) were obese (BMI  $\geq 30$  kg/m<sup>2</sup>) versus 16% (3/19) of the controls. All women with metabolic syndrome were obese or at least overweight.

**Table 16.** *Metabolic syndrome among premenopausal women with a pre-eclamptic history. Diagnostic criteria were based on the New International Diabetes Federation definition (IDF) (Alberti K 2006).*

	<b>Pre-eclampsia</b>	<b>Controls</b>	<b>p<sup>a)</sup></b>
n	18	19	
Waist circumference $\geq 80$ cm	8/18 (44%)	9/19 (47%)	NS
Triglycerides $\geq 1.7$ mmol/l	1/18 (6%)	2/19 (11%)	NS
HDL cholesterol $< 1.29$ mmol/l	3/18 (17%)	2/19 (11%)	NS
Systolic BP $\geq 130$ mmHg	11/18 (61%)	6/19 (32%)	NS
Diastolic BP $\geq 85$ mmHg	7/18 (39%)	6/19 (32%)	NS
Medical treatment of previously diagnosed hypertension	4/18 (22%)	1/19 (5%)	NS
FPG $\geq 5.6$ mmol/l	1/18 (6%)	1/19 (5%)	NS
Previously diagnosed DM2	1/18 (6%)	0/19	NS
Metabolic syndrome	3/18 (17%)	2/19 (11%)	NS

Values are shown as n (%). HDL, high-density lipoprotein; BP, blood pressure; FPG, fasting plasma glucose; DM2, diabetes mellitus type 2.

<sup>a)</sup> Fisher's exact test.

## **6.3 FATTY ACID-BINDING PROTEIN 4**

In Study I, at 24 weeks and at 32 weeks mean fasting FABP4 levels tended to be higher in women who subsequently developed PIH than those in the normotensive group (Table 17). Since maternal FABP4 concentrations were not normally distributed, Table 17 also displays median fasting FABP4 levels at 24 weeks and at 32 weeks of gestation without a trend of significantly increased FABP4 levels in women with subsequent PIH. In the subgroup of proteinuric PIH (pre-eclampsia), FABP4 levels at 24 weeks and at 32 weeks were the same as in normotensive women (data not shown).

**Table 17.** Serum fatty acid-binding protein4 (FABP4) levels at 24 and at 32 weeks of gestation

	PIH	Normotensives	p <sup>a)</sup>
n	26	46	
Fasting FABP4 at 24 wk (ng/ml)	29.4 ± 19.2 20.1 (13.1–72.9)	21.7 ± 9.2 19.2 (10.5–49.7)	0.2
Fasting FABP4 at 32 wk (ng/ml)	45.1 ± 46.5 25.9 (12.4–213.7)	26.4 ± 10.9 23.0 (13.2–59.5)	0.4
FABP4 difference from 24 wk to 32 wk (ng/ml)	13.7 ± 34.8 1.4 (-21.1–144.1)	4.6 ± 11.2 3.7 (-27.0–29.0)	0.8

Values are shown as mean ± SD and median (range).

PIH, pregnancy induced hypertension; FABP4, adipocyte fatty acid-binding protein 4.

a) The Mann–Whitney *U*-test was used to compare PIH cases and normotensive controls.

### ***Univariate correlations***

Fasting levels of FABP4 at 24 weeks correlated inversely and significantly with weight gain during pregnancy ( $r = -0.49$ ,  $p = 0.02$ ), SBP after 10 min standing ( $-0.46$ ,  $0.03$ ) and  $\Delta$ SBP ( $-0.56$ ,  $0.006$ ) in women with PIH. In women who remained normotensive, fasting levels of FABP4 at 24 weeks correlated positively with pre-pregnancy BMI ( $r = 0.42$ ,  $p = 0.004$ ), DBP in the 1st trimester ( $0.39$ ,  $0.007$ ) and hs-CRP levels ( $0.30$ ,  $0.04$ ).

### ***Logistic and multiple regression analyses***

In logistic regression we examined clinical factors (FABP4 at 24 and 32 weeks of gestation, FABP4 difference, hs-CRP at 24 and 32 weeks of gestation, hs-CRP difference, testosterone at 24 and 32 weeks of gestation, age at 24 weeks of gestation, pre-pregnancy BMI, weight gain during pregnancy, fP-glucose and ISI at 24 weeks of gestation, SBP after 10 min standing,  $\Delta$ SBP), and pre-eclampsia and PIH were used as the dependent variables. In this analysis, apart from SBP after 10 min standing ( $p = 0.015$ ,  $r^2 = 0.085$ ), baseline FABP4 ( $p = 0.04$ ,  $r^2 = 0.060$ ) was the only variable associated with the development of PIH. In multiple regression analysis, both SBP after 10 min standing ( $p = 0.002$ ,  $r^2 = 0.22$ ) and FABP4 at 24 weeks of gestation ( $p = 0.003$ ,  $r^2 = 0.22$ ) correlated positively with the development of PIH.

## 6.4 SYMPATHETIC OVER-ACTIVITY

### ***Women with subsequent PIH (I, II)***

In Study I, plasma noradrenaline levels after supine resting at 24 weeks were the same in the study groups (Table 18), but after 10 min standing plasma noradrenaline levels tended to be higher as early as at 24 weeks of gestation in women with subsequent PIH (Table 18, A.T. *et al.* unpublished data). At 32 weeks, women with subsequent hypertension had higher noradrenaline levels than normotensive women and the noradrenaline difference from 24 weeks to 32 weeks was significantly higher, accordingly (Table 18).

Plasma adrenaline levels differed neither at 24 weeks nor at 32 weeks of gestation between the groups (Table 18, A.T. *et al.* unpublished data). In Study II, in the subgroup of Angptl6 measurements at 32 weeks of gestation, fasting plasma adrenaline levels were significantly higher in women with non-proteinuric PIH (median 105, range 57–222 pmol/l,  $p = 0.02$ ) compared with normotensive controls (median 73, range 25–136) (data not shown).

Plasma ghrelin levels were the same in the study groups at 24 and 32 weeks of gestation. Even in the modified orthostatic test the upright position did not affect ghrelin levels (Table 18, A.T. *et al.* unpublished data).

At 32 weeks, fasting levels of FABP4 did not correlate with serum noradrenaline levels (data not shown).

**Table 18.** Serum noradrenaline, adrenaline and ghrelin levels at 24 and at 32 weeks of gestation

	PIH	Normotensive	P <sup>a)</sup>
n	26	46	
Noradrenaline (pmol/l) at 24 weeks of gestation after supine resting	1340 (554–2300)	1190 (355–3790)	0.1
Noradrenaline (pmol/l) at 24 weeks of gestation after 10 min standing	2905 (1330–6470)	2450 (979–4420)	0.07
Noradrenaline difference from supine to upright position (pmol/l)	1485 (361–4680)	1220 (214–2899)	0.2
Noradrenaline (pmol/l) at 32 weeks of gestation	1985 (1070–3680)	1540 (712–3770)	0.04
Noradrenaline difference from 24 to 32 weeks of gestation (pmol/l)	748.7 ± 523.3 600 (100–1890)	453.6 ± 801.2 380 (-1230–2320)	0.05
Adrenaline (pmol/l) at 24 weeks of gestation after supine resting	33 (14–147)	47 (19–120)	0.2
Adrenaline (pmol/l) at 24 weeks of gestation after 10 min standing	49 (19–243)	54 (17–130)	0.9
Adrenaline difference from supine to upright position (pmol/l)	10 (-18–156)	10 (-38–47)	0.8
Adrenaline (pmol/l) at 32 weeks of gestation	90 (36–222)	80 (25–166)	0.9
Adrenaline difference from 24 to 32 weeks of gestation (pmol/l)	46 (-34–168)	41 (-89–100)	0.5
Ghrelin (pmol/l) at 24 weeks of gestation after supine resting	127 (29–655)	122 (26–597)	0.4
Ghrelin (pmol/l) at 24 weeks of gestation after 10 min standing	149 (77–1610)	135 (59–642)	0.8
Ghrelin difference from supine to upright position (pmol/l)	17 (-520–966)	12 (-65–510)	0.8
Ghrelin (pmol/l) at 32 weeks of gestation	132 (39–1160)	107 (57–316)	0.2

Values are shown as mean ± SD and median (range). PIH, pregnancy-induced hypertension.

<sup>a)</sup> The Mann–Whitney *U*-test was used, comparing PIH vs. normotensive controls.

In Study I, in the orthostatic test SBP and DBP in supine resting and after 10 minutes standing were significantly higher in women who developed PIH (Table 19, A.T. *et al.* unpublished data). Mean arterial pressures (MAPs) in supine resting and after 10 minutes standing were significantly higher in women who developed PIH (Table 19, A.T. *et al.* unpublished data). Heart

rate in supine resting, after 10 minutes standing and heart rate difference did not differ between women with subsequent hypertension compared with normotensive women (A.T. *et al.* unpublished data).

At 24 weeks of gestation, in the modified orthostatic test, plasma noradrenaline levels correlated with systolic blood pressure after 10 min standing and with mean arterial pressure after 10 min standing in all patients, but only with systolic blood pressure after 10 min standing in women with subsequent proteinuric PIH (A.T. *et al.* unpublished data).

**Table 19.** *The orthostatic test at 24 weeks*

	<b>PIH</b>	<b>Normotensive</b>	<b>p<sup>a)</sup></b>
n	26	46	
SBP supine resting (mmHg)	116 (88–150)	99 (80–122)	< 0.001
DBP supine resting (mmHg)	69 ± 9	62 ± 11	0.007
SBP after 10 min standing (mmHg)	117 ± 12	108 ± 13	0.009
DBP after 10 min standing (mmHg)	79 ± 9	72 ± 8	0.005
MAP supine resting (mmHg)	83 ± 10	75 ± 10	0.002
MAP after 10 min standing (mmHg)	91 ± 8	84 ± 9	0.004

Values are shown as means ± SD or median (range).

PIH, pregnancy-induced hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure.

MAP =  $([2 \times \text{DBP}] + \text{SBP})/3$ .

<sup>a)</sup> The Mann–Whitney *U*-test was used to compare PIH cases with normotensive controls.

In the subgroup of Angptl6 measurement, the difference in supine resting DBP between women who developed PIH and women who remained normotensive did not reach statistical significance (II).

### **Women during pre-eclampsia (III)**

At baseline, after 30 min in the left-lateral position noradrenaline levels were lowest in normotensive pregnant women (Table 20). At all time points, normotensive pregnant women had significantly lower noradrenaline levels than non-pregnant women ( $p < 0.05$ ) (Table 20). In pre-eclamptic women noradrenaline levels were higher than in normotensive pregnant women at all time points ( $p < 0.01$ ) (Table 20).

The change from left-lateral to upright position and from upright back to left-lateral position resulted in greater changes in noradrenaline levels in pre-eclamptic women than in normotensive pregnant women (Table 20).

**Table 20.** Catecholamine levels in Study III

	Pregnant			p	Change in body position
	Pre-eclamptic	Normotensive	Non-pregnant		
n	11	17	8		
Baseline LL noradrenaline (pmol/l)	1267 ± 429	702 ± 365	1247 ± 554	<0.01 <sup>a)</sup> <0.05 <sup>b)</sup>	NS
Supine noradrenaline (pmol/l)	1321 ± 498	850 ± 570	1430 ± 437	<0.01 <sup>a)</sup> <0.05 <sup>b)</sup>	NS
LL noradrenaline (pmol/l)	1175 ± 416	698 ± 483	1255 ± 245	<0.01 <sup>a)</sup> <0.05 <sup>b)</sup>	NS
Upright noradrenaline (pmol/l)	2564 ± 1162	1566 ± 901	2634 ± 889	<0.01 <sup>a)</sup> <0.05 <sup>b)</sup>	<0.05 <sup>d)</sup>
LL noradrenaline (pmol/l)	1206 ± 468	718 ± 425	1269 ± 345	<0.01 <sup>a)</sup> <0.05 <sup>b)</sup>	<0.05 <sup>d)</sup>
Baseline LL adrenaline (pmol/l)	131 ± 62	54 ± 37	57 ± 23	<0.05 <sup>c)</sup>	NS
Supine adrenaline (pmol/l)	135 ± 74	62 ± 43	48 ± 30	NS	NS
LL adrenaline (pmol/l)	114 ± 87	138 ± 267	65 ± 49	NS	NS
Upright adrenaline (pmol/l)	237 ± 206	125 ± 83	225 ± 380	NS	NS
LL adrenaline (pmol/l)	112 ± 79	71 ± 56	63 ± 22	NS	NS

Values are shown as mean ± SD. LL, left-lateral position.

<sup>a)</sup> Fisher's least significance difference test comparing pre-eclamptic with normotensive pregnant women

<sup>b)</sup> Fisher's least significance difference test comparing normotensive pregnant women with non-pregnant women.

<sup>c)</sup> Fisher's least significance difference test comparing pre-eclamptic with normotensive groups

<sup>d)</sup> ANOVA for repeated measures comparing pre-eclamptic with normotensive pregnant women. First a change from left-lateral to an upright position and then from upright back to a left-lateral position.

At baseline, after 30 min in the left-lateral position adrenaline levels were higher in pre-eclamptic patients than in the other two groups (Table 20).

Changes in adrenaline levels with body position were not different between the groups.

Plasma renin activity was higher in pre-eclamptic than in normotensive pregnant women at all time points ( $p < 0.05$ ) (Table 21). Pre-eclamptic women had higher levels of plasma renin activity than non-pregnant women ( $p < 0.001$ ) and changes in plasma renin activity were greater in pre-eclamptic than in normotensive pregnant women moving from the left-lateral to an upright position ( $p < 0.05$ ) (Table 21).

**Table 21.** Plasma renin activity observed in Study III

	Pregnant			p	Change in body position
	Pre-eclamptic	Normotensive	Non-pregnant		
n	11	17	8		
Baseline LL plasma renin activity (nmol/l/h)	2.6 ± 1.3	1.8 ± 0.7	0.9 ± 0.2	<0.05 <sup>a)</sup> <0.001 <sup>b)</sup> <0.05 <sup>c)</sup>	NS
Supine plasma renin activity (nmol/l/h)	2.7 ± 1.4	2.0 ± 0.9	0.9 ± 0.3	<0.05 <sup>a)</sup> <0.001 <sup>b)</sup> <0.05 <sup>c)</sup>	NS
LL plasma renin activity (nmol/l/h)	2.9 ± 1.3	1.9 ± 0.9	0.9 ± 0.3	<0.05 <sup>a)</sup> <0.001 <sup>b)</sup> <0.05 <sup>c)</sup>	NS
Upright plasma renin activity (nmol/l/h)	3.9 ± 2.2	2.3 ± 0.8	1.1 ± 0.3	<0.05 <sup>a)</sup> <0.001 <sup>b)</sup> <0.05 <sup>c)</sup>	<0.05 <sup>d)</sup>
LL plasma renin activity (nmol/l/h)	3.3 ± 1.6	1.9 ± 0.8	1.0 ± 0.3	<0.05 <sup>a)</sup> <0.001 <sup>b)</sup> <0.05 <sup>c)</sup>	NS

Values are shown as mean ± SD.

LL, left-lateral position.

<sup>a)</sup> Fisher's least significance difference test comparing pre-eclamptic with normotensive pregnant women

<sup>b)</sup> Fisher's least significance difference test comparing pre-eclamptic with non-pregnant women

<sup>c)</sup> Fisher's least significance difference test comparing normotensive pregnant women with non-pregnant women.

<sup>d)</sup> ANOVA for repeated measures comparing pre-eclamptic with normotensive pregnant women. Change from left-lateral to upright position.

Plasma natriuretic peptide and endothelin-1 levels were similar in pre-eclamptic and normotensive pregnant women and changes with posture were not different between the three groups (data not shown).

A change from a left-lateral position to a supine position decreased SBP in pre-eclamptic women and increased it in the normotensive groups (data not shown). The difference between pre-eclamptic and normotensive pregnant women was statistically significant ( $p < 0.001$ ). When the women returned to a left-lateral position an opposite change appeared in SBP; in pre-eclamptic women SBP increased and it decreased in the normotensive groups, the difference between the two pregnant groups being significant ( $p < 0.01$ ; data not shown).

## 6.5 ANDROGENS

### ***Women with subsequent PIH (I, II)***

In Study I maternal serum testosterone, SHBG and calculated free testosterone (cfT) levels at 24 and at 32 weeks of gestation were the same regardless of hypertension during pregnancy (Table 22).

**Table 22.** Serum testosterone levels at 24 and at 32 weeks of gestation

	<b>PIH</b>	<b>Normotensive</b>	<b>p<sup>a)</sup></b>
n	26	46	
S-Testosterone (nmol/l) at 24 wk	2.6 (1.2 – 5.9)	3.0 (1.1 – 19.6)	0.4
S-Testosterone (nmol/l) at 32 wk	2.7 (1.6 – 6.8)	2.9 (1.0 – 16.8)	0.8
cfT (pmol/l) at 24 wk	1.4 (0.7 – 3.3)	1.6 (0.6 – 10.8)	0.4
cfT (pmol/l) at 32 wk	1.5 (0.9 – 3.8)	1.6 (0.6 – 9.2)	0.8
S-SHBG (nmol/l) at 24 wk	370 (240 – 660)	411 (230 – 630)	0.3
S-SHBG (nmol/l) at 32 wk	432 (320 – 720)	486 (334 – 890)	0.3

Values are shown as means  $\pm$  SD and median (range).

PIH, pregnancy-induced hypertension; cfT, calculated free testosterone; SHBG, sex hormone-binding globulin.

<sup>a)</sup> The Mann–Whitney *U*-test was used in comparisons.

### ***Univariate correlation***

We found no significant correlation between maternal testosterone at 24 or at 32 weeks versus pre-pregnancy BMI, BMI at time of delivery, weight gain during pregnancy, fasting blood glucose and whole-body ISI at 24 weeks in women who developed PIH or in those who remained normotensive (data not shown). At 24 and at 32 weeks of gestation there was no correlation between maternal FABP4 levels and testosterone levels (data not shown).

### ***Women in the perimenopausal period (V)***

The free androgen index (FAI) and total and calculated free testosterone levels were similar in the two groups (Table 23). Of the women with previous pre-eclampsia, 11% (2/18) had PCOS, versus none of the controls.

Only 11% (2/18) of the women with prior pre-eclampsia and 35% (6/17) of the control women had some climacteric symptoms. To evaluate menopausal transition we calculated median FSH levels in the patient and control groups. The women were then categorized as being above or below the median FSH level in each group. In women with prior pre-eclampsia and FSH below the median, calculated free testosterone levels were higher than in women with prior pre-eclampsia and FSH above the median (median 13.4 pmol/l, range 8.0–22.5 vs. 7.1 pmol/l, 5.1–20.5,  $p = 0.03$ ). In women with prior normotensive pregnancies and FSH levels below the median and FSH above the median calculated free testosterone levels were the same (data not shown). In women with prior pre-eclampsia and normotensive pregnancies and FSH below medians calculated free testosterone and total testosterone levels were similar (data not shown).

**Table 23.** Androgenic characteristics and PCOS in premenopausal women with a pre-eclamptic history

	<b>Pre-eclampsia</b>	<b>Controls</b>	<b>p<sup>a)</sup></b>
n	18	17 <sup>c)</sup>	NS
S-Testosterone (nmol/l)	0.8 (0.4 – 2.3)	0.7 (0.3 – 1.7)	NS
cfT (pmol/l)	9.8 (5.1 – 22)	8.4 (3.5 – 21)	NS
S-SHBG (nmol/l)	50.5 (26 – 125)	61.0 (13 – 174)	NS
DHEAS (µmol/l)	2.6 (0.9 – 6.5)	2.5 (0.7 – 4.7)	NS
FAI	1.7 (0.6 – 4.6)	1.3 (0.4 – 3.0)	NS
FSH (IU/l)	5.9 (2.9 – 93)	6.0 (2.1 – 63)	NS
FSH <10 IU/l, n (%)	14/18 (78%)	11/17 (65%)	NS <sup>b)</sup>
S-Testosterone (nmol/l) if FSH <10 IU/l	0.8 (0.4 – 2.3)	0.8 (0.3 – 1.7)	NS
S-Testosterone (nmol/l) if FSH >10 IU/l	0.6 (0.4 – 1.2)	0.6 (0.4 – 0.9)	NS
cfT (pmol/l) if FSH <10 IU/l	10.5 (6.6 – 22)	9.0 (3.5 – 21)	NS
cfT (pmol/l) if FSH >10 IU/l	6.1 (5.1 – 20)	6.2 (4.8 – 11.4)	NS
PCOS, n (%)	2/18 (11%)	0/17	NS <sup>b)</sup>

Values are shown as median (range).

cfT, calculated free testosterone; SHBG, sex hormone-binding globulin; DHEAS, dehydroepiandrosterone sulphate; FAI, free androgen index; FSH, follicle-stimulating hormone.

<sup>a)</sup> The Mann–Whitney *U*-test and <sup>b)</sup> Fisher’s exact test.

<sup>c)</sup> Two women were excluded from hormone analyses because one used estradiol valerate-cyproterone acetate tablets and one used cyclic norethisterone.

## 6.6 INFLAMMATION

### *Women with subsequent PIH (I, II)*

In Study I, in women at 24 and 32 weeks of gestation there were no differences in hs-CRP levels between the study groups (women with subsequent PIH vs. normotensive controls) (Table 24).

**Table 24.** Serum high-sensitivity C-reactive protein (hs-CRP) levels at 24 and at 32 weeks of gestation

	<b>PIH</b>	<b>Normotensive</b>	<b>p<sup>b)</sup></b>
n	26	46	
hs-CRP at 24 wk (mg/l) <sup>a)</sup>	4.8 (0.7–9.8)	3.9 (0.7–9.8)	0.2
hs-CRP at 32 wk (mg/l) <sup>a)</sup>	4.8 ± 2.4	3.6 ± 2.2	0.06

Values are shown as means ± SD and median (range).

PIH, pregnancy-induced hypertension; hs-CRP, high-sensitivity C-reactive protein.

<sup>a)</sup> From hs-CRP calculations we excluded women with hs-CRP levels of > 10 mg/l, because of the risk of acute infection (at 24 weeks of gestation five women with PIH and eight normotensive women and at 32 weeks of gestation four with PIH and seven normotensive women).

<sup>b)</sup> The Mann–Whitney *U*-test was used in comparisons.

### *Univariate correlations*

There were no correlations between maternal FABP4 levels and hs-CRP levels at 24 weeks or at 32 weeks. Serum hs-CRP levels at 24 weeks, but not at 32 weeks, correlated positively with pre-pregnancy BMI ( $r = 0.39$ ,  $p = 0.05$ ), but inversely with weight gain during pregnancy ( $r = -0.44$ ,  $p = 0.03$ ) in women with subsequent PIH. On the other hand, in women with normotensive pregnancies, at 32 weeks (but not at 24 weeks) serum hs-CRP levels correlated positively with pre-pregnancy BMI ( $r = 0.35$ ,  $p = 0.04$ ) and BMI at time of delivery ( $r = 0.41$ ,  $p = 0.01$ ).

## 6.7 ANGIOPOIETIN-LIKE PROTEIN 6

In Study II, at 24 weeks fasting Angptl6 levels were significantly higher in women who subsequently developed PIH with or without proteinuria compared with normotensive controls (Table 25, Figure 4). At 32 weeks fasting Angptl6 levels tended to be higher in women who developed PIH than in those in the normotensive group, but the difference was not statistically significant (Table 25, Figure 4). The change in fasting Angptl6 levels between 24 and 32 weeks of gestation tended to be greater in women who developed PIH than in the normotensive group, but the difference was not statistically significant (Table 25).

**Table 25.** Serum angiotensin-like protein 6 (Angptl6) levels at 24 and at 32 weeks of gestation

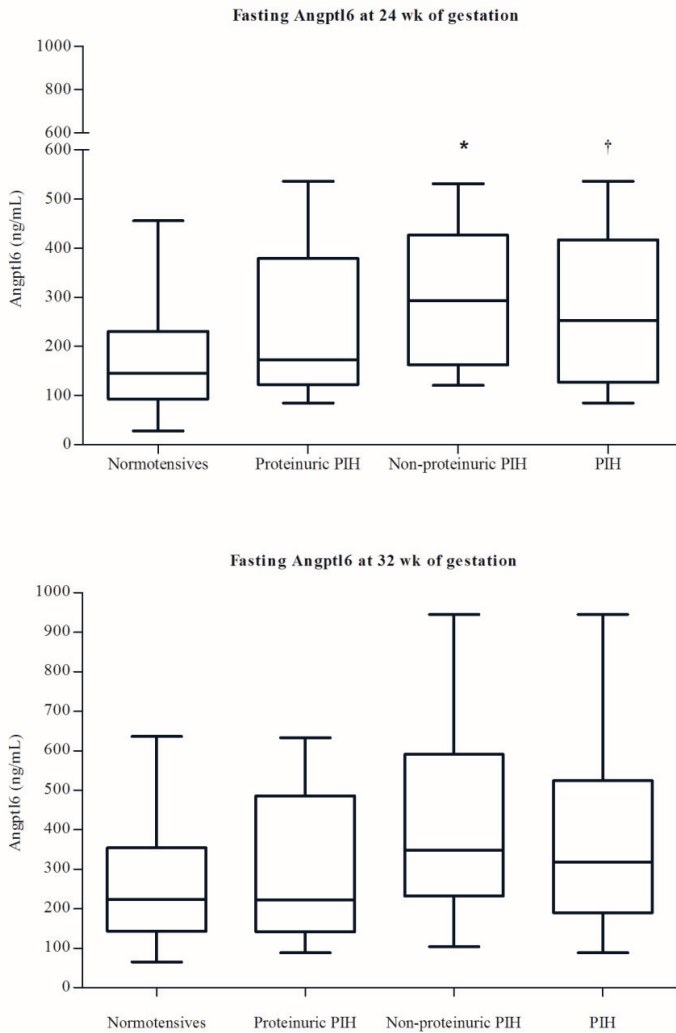
	PIH	Normotensive	p-value <sup>a)</sup>
n	24	23	
Fasting Angptl6 at 24 wk (ng/ml)	252 (85 – 536)	145 (28 – 455)	0.02
2-h Angptl6 at 24 wk (ng/ml)	232 (28 – 816)	221 (44 – 549)	NS
Fasting Angptl6 at 32 wk (ng/ml)	318 (89 – 945)	224 (65 – 636)	NS
Angptl6 difference from 24 to 32 wk of gestation (ng/ml)	108 (-159 to 574)	39 (-224 to 497)	NS

Values are shown as median (range).

PIH, pregnancy-induced hypertension; Angptl6, angiotensin-like protein 6.

<sup>a)</sup> The Mann–Whitney *U*-test was used in comparisons.

In the subgroup of subsequent non-proteinuric PIH, at 24 weeks of gestation fasting Angptl6 levels were significantly higher than in the normotensive controls (median 293 ng/ml, range 121–531, vs. 145 ng/ml, range 28–455,  $p = 0.03$ ), but a similar difference in the subgroup of subsequent proteinuric PIH (173 ng/ml, range 85–536) did not reach statistical significance (Figure 4). At 32 weeks of gestation the differences in Angptl6 levels in the subgroups of non-proteinuric PIH (median 348 ng/ml, range 104–945) and proteinuric PIH (222 ng/ml, 89–633) compared with normotensive controls (224 ng/ml, 65–636) did not reach statistical significance (Figure 4).



**Figure 4** Box plots of serum Angptl6 levels at 24 weeks of gestation and at 32 weeks in women with non-proteinuric PIH, proteinuric PIH (pre-eclampsia), all cases of PIH and in normotensive women. Medians, interquartile ranges and ranges are displayed. \*  $p=0.03$  for women with non-proteinuric PIH compared with normotensive controls, †  $p=0.02$  for women with PIH compared with normotensive controls.

We calculated a receiver operating characteristic (ROC) curve for PIH and proteinuric PIH (pre-eclampsia) to see if assay of Angptl6 could be used as a diagnostic test. It turned out that Angptl6 measurements failed to predict future PIH/pre-eclampsia. For PIH the area under the curve was 0.35 and for pre-eclampsia, 0.46.

### ***Univariate correlations***

In all women, fasting levels of Angptl6 at 24 weeks did not correlate with pre-pregnancy BMI or BMI at the time of delivery. In women with PIH the correlation between fasting levels of Angptl6 at 24 weeks and pre-pregnancy BMI was inverse and significant ( $r = -0.48$ ,  $p = 0.02$ ), but there was no correlation with BMI at the time of delivery. Weight gain during pregnancy (all women) did not correlate with fasting Angptl6 levels at 24 weeks of gestation, but in women with subsequent PIH the correlation was positive and significant ( $r = 0.45$ ,  $p = 0.04$ ). At 32 weeks, Angptl6 and weight gain during pregnancy still correlated positively in women with PIH ( $r = 0.52$ ,  $p = 0.03$ ), but not in all women together.

At 24 weeks of gestation serum fasting Angptl6 levels correlated with diastolic blood pressure after 10 min standing and with mean arterial pressure after 10 min standing (modified orthostatic test), but serum fasting Angptl6 levels did not correlate with subsequent blood pressure parameters.

### ***Logistic and multiple regression analyses***

Using logistic regression analysis we examined the association with clinical factors, with pre-eclampsia and PIH being used as the dependent variables. In this analysis PIH was associated with SBP after 10 min standing ( $p = 0.01$ ,  $r^2 = 0.13$ ) and with fasting Angptl6 levels at 24 weeks of gestation ( $p = 0.02$ ,  $r^2 = 0.11$ ). In multiple regression analysis only SBP after 10 min standing correlated positively ( $p = 0.02$ ,  $r^2 = 0.21$ ) with the development of PIH.

## 7 DISCUSSION

### 7.1 INSULIN RESISTANCE

#### ***Before pregnancy-induced hypertension***

Our study showed no signs of increased insulin resistance at 24 weeks of gestation in nulliparous women who were subsequently hypertensive compared with women who remained normotensive. This is in contrast to previous studies where women with subsequent pre-eclampsia were already more insulin resistant in the first trimester (D'Anna R 2006) and in mid-trimester (Hauth J 2011). The lack of expected insulin resistance in our study could be explained by different timing of hypertension and differences in ethnicity (Hauth J 2011), parity (D'Anna R 2006, Hauth J 2011) and age. One of the previous studies was a case-control study with older and multiparous women (D'Anna R 2006). The other consisted of a secondary analysis of a randomized trial after antioxidant treatment and with younger women than in our study (Hauth J 2011). In our study women had late-onset hypertension without increased insulin resistance, at least in the second trimester of pregnancy.

#### ***During pregnancy-induced hypertension***

In our fourth study women with GDM showed a greater rise in blood glucose in the early morning hours among those with chronic hypertension, whereas blood glucose levels decreased in the normotensive women. This is in agreement with the results of an earlier study where it was suggested that pregnant women with both chronic hypertension and GDM may be more insulin-resistant than those with GDM alone (Caruso A 1999). The early morning glucose rise in women with chronic hypertension may have resulted from the dawn phenomenon seen in subjects with type 2 diabetes (Porcellati F 2013). In type 2 diabetes fasting hyperglycaemia has been attributed to a transient increase in hepatic glucose production at dawn in the absence of compensatory insulin secretion (Porcellati F 2013). The role of the dawn phenomenon in pregnant women with glucose intolerance is still unknown (Mandujano A 2013).

We did not study insulin sensitivity during overt pre-eclampsia, but the insulin resistance seen during normal pregnancy (Barbour L 2007) has been found to be further enhanced in cases of non-proteinuric (Seely E 2003) and proteinuric PIH (pre-eclampsia) (Kaaja R 1999a, Seely E 2003). However, women with GDM who later develop pre-eclampsia have not been reported to be more insulin resistant than those who remain non-pre-eclamptic (Montoro M 2005).

It seems that increased insulin resistance is a relatively late phenomenon in GDM women who develop hypertensive pregnancy. Apart from the dawn

phenomenon, the early morning rise in women with chronic hypertension may have resulted from greater insulin resistance and sympathetic overactivity in these women. Hypertensive pregnant women have been found to have higher noradrenaline levels in the early morning hours (Kaaja R 1999b), being a sign of increased sympathetic activity. We must be cautious in our interpretations, as we measured neither insulin sensitivity nor sympathetic activity (MSNA or plasma noradrenaline) in our study.

### ***After pregnancy-induced hypertension***

In our fifth study, 23–24 years after pre-eclamptic pregnancy, there were no signs of increased insulin resistance in the premenopausal period, in contrast to earlier assumptions.

Although insulin sensitivity improves postpartum, insulin resistance has been seen three months (Kaaja R 1999a) and 18 months after pre-eclamptic pregnancy (Wolf M 2004), but it seems to taper off later on, especially in normal-weight women. In one study, five years after pre-eclamptic pregnancy there was no difference in insulin sensitivity between normal-weight women with previous pre-eclampsia and normotensive control women (Lampinen K 2008). In another study a history of (proteinuric) pre-eclampsia has been associated with mild hyperinsulinemia seventeen years after first pregnancy (Laivuori H 1996), but twenty years after pre-eclampsia women had only a tendency toward higher fasting insulin levels, although glycosylated haemoglobin levels were significantly higher than in controls (Sattar N 2003). In our study insulin resistance did not differ 23–24 years after pre-eclamptic vs. normotensive pregnancy. Our result could be explained by the fact that in our study women were mostly non-obese. In an earlier study, five years after pregnancy, abdominal circumference and insulin sensitivity correlated only in women with a history of pre-eclampsia (Lampinen K 2008). This was also observed in our study. Although waist circumference was still within normal limits in both groups, and similar, it correlated with insulin sensitivity only in women with a history of pre-eclamptic pregnancy. Thus, this group of women is likely to be prone to insulin resistance and metabolic syndrome if they gain weight, especially in abdominal region.

Regardless of their proteinuric status (Männistö T 2013), hypertensive pregnant women are at an increased risk of later cardiovascular disease (Bellamy L 2007, Valdiviezo C 2012). An important risk factor of CVD is metabolic syndrome (Galassi A 2006). However, in our study of mostly non-obese women with a history of pre-eclamptic pregnancy, metabolic syndrome was not more common in the premenopausal period than in women with a history of normotensive pregnancy.

It might be that after pre-eclamptic pregnancy only some women have persistent insulin resistance and other risk factors common to pre-eclampsia and CVD. Women with a history of recurrent PIH have been found to be at a higher risk of developing modifiable CVD risk factors such as elevated BMI,

elevated blood pressure, an unfavourable lipid profile and diabetes mellitus (Lykke J 2009, Magnussen E 2009). This risk is also increased in those with early onset pre-eclampsia (Bellamy L 2007).

Metabolic risk factors are not likely to entirely explain the elevated risk of later CVD in women with a history of PIH. Five years after pre-eclamptic pregnancy, women have been found to have altered vasorelaxation (Lampinen K 2008) and increased levels of plasma noradrenaline (Lampinen K 2013). In our study insulin resistance was not increased, at least in the premenopausal period, as a sign of increased risk of later CVD in women with prior pre-eclampsia.

## 7.2 FATTY ACID-BINDING PROTEIN 4

Our first study showed that in relatively overweight pregnant women, maternal serum FABP4 concentrations measured at 24 weeks of gestation were associated with the subsequent development of PIH. However, neither at 24 weeks nor at 32 weeks of gestation did serum FABP4 concentrations significantly differ in women who subsequently developed PIH compared with those who remained normotensive.

In contrast to the results of an earlier study where maternal FABP4 levels were higher even before the clinical onset of pre-eclampsia (Scifres C 2012), in our study maternal FABP4 levels were similar in the proteinuric subgroup. We did not study FABP4 levels during overt pre-eclampsia.

To the best of our knowledge, this is the first time that maternal FABP4 levels have been studied in women without proteinuria and before the onset of hypertensive pregnancy. Our result strengthens the idea of FABP4 as a modulator in metabolic alterations and PIH. However, our study revealed no signs of increased insulin resistance at 24 weeks of gestation in women with subsequent PIH.

During pregnancy maternal insulin sensitivity is reduced (Seely E 2003) and lipolysis in adipose tissue increased (Diderholm B 2005). Increased maternal FABP4 levels have been found to be associated with insulin-resistant proteinuric PIH (pre-eclampsia) (Fasshauer M 2008, Shangguan X 2009, Scifres C 2012) and FABP4 could be a marker of increased lipolysis and inflammation. During pregnancy FABP4 could link various metabolic factors to obesity, as in non-pregnant individuals (Xu A 2006, Xu A 2007). Circulating FABP4 levels are higher in overweight/obese than in lean individuals (Xu A 2006). In our study pre-pregnancy BMI correlated positively with serum FABP4 levels. In PIH, FABP4, pre-pregnancy adiposity, and inflammation are most probably metabolically linked together, but the specific role of FABP4 in obesity and PIH is still unclear, and it possibly has a mechanism of its own.

The small number of women and lack of normal-weight controls limit the statistical significance of our results and ability to establish diagnostic

thresholds for maternal serum Angptl6 and FABP4 levels and subsequent development of PIH. Including patients with different types of pregnancy-induced hypertension (pre-eclampsia and non-proteinuric PIH) in a single group could lead to bias. In our study both proteinuric and non-proteinuric PIH were late-onset, mostly mild diseases without demonstrable foetal involvement and the women delivered at term, so women with and without proteinuria were analysed as one group, since the pathogenesis is similar (Solomon C 2001, Seely E 2003). In late-onset disease endothelial dysfunction is much milder than in early-onset pre-eclampsia (< 34 gestational weeks) (Gilbert J 2008) and the pathophysiology is different in these two conditions (Valensise H 2008).

### **7.3 SYMPATHETIC OVER-ACTIVITY**

Our first study gave support to the idea that sympathetic activation may precede hypertensive pregnancy. It showed that plasma noradrenaline levels were higher at 32 weeks of gestation in women with subsequent PIH, but at 24 weeks of gestation only a tendency toward higher levels of noradrenaline was seen after stimulation of the sympathetic nervous system by 10 min standing. An upright position at 24 weeks of gestation did not exaggerate the difference in noradrenaline levels either. At 24 weeks of gestation SBP after 10 min standing was associated with the development of PIH. These changes are likely to be associated with increased sympathetic activity in PIH.

In our study, at 24 weeks of gestation plasma noradrenaline levels correlated with systolic blood pressure after 10 min standing (modified orthostatic test), but only in women with subsequent proteinuric PIH. At 24 weeks of gestation we observed no association between factors related to sympathetic activity (such as supine resting noradrenaline levels, noradrenaline levels after 10 min standing, SBP after 10 min standing and  $\Delta$ SBP) and whole-body ISI. As mentioned earlier, at 24 weeks of gestation whole-body ISI was not different in women with subsequent PIH and those who remained normotensive. Thus, sympathetic over-activity seems to precede the possible insulin resistance in hypertensive pregnancy, but most certainly further larger prospective studies are needed, with more accurate and sensitive methods of assessing insulin sensitivity (hyperinsulinaemic-euglycaemic clamp and IGTTs) and sympathetic activity (MSNA). However, the use of such invasive methods during pregnancy is limited.

Our third study showed that women with overt pre-eclampsia had higher plasma noradrenaline levels compared with those in normotensive pregnant women and the difference was even more obvious when the women were in the upright position. Our result is in accordance with those in earlier studies (Schobel H 1996, Manyonda I 1998, Kaaja R 1999b) and it strengthened the role of sympathetic over-activity in pre-eclampsia.

In overweight and obese pregnant women, a hyperactive sympathetic nervous system may directly increase the risk of gestational hypertensive disorders (Greenwood J 2003, Fischer T 2004, Wang Z 2013). In obesity hyperleptinaemia could be related to raised sympathetic activity (Lambert G 2010, Simonds S 2012, Machleidt F 2013), which could predispose women to pregnancy-related hypertension. In our study, at 24 and 32 weeks of gestation levels of the leptin inhibitor ghrelin were the same in women with subsequent hypertension and in those with normotensive pregnancy and an upright position did not affect ghrelin levels.

Earlier studies have shown that during proteinuric PIH (pre-eclampsia) there are increased levels of venous noradrenaline (Manyonda I 1998) and increased diurnal venous plasma levels of noradrenaline (Kaaja R 1999b), reflecting some evidence of global sympathetic over-activity in this condition. However, a plasma noradrenaline measurement has limited sensitivity as an index of sympathetic activity (Grassi G 1999), as it shows only a momentary reflection of neurotransmitter levels and is subject to changes in noradrenaline clearance, and localisation of sympathetic over-activity is not revealed (Grassi G 1995).

Sympathetic over-activity seems to play an important role in the pathophysiology of hypertensive pregnancy, as it precedes and is seen to prevail after hypertensive pregnancy (Lampinen K 2013), but further studies are required.

## 7.4 ANDROGENS

In our first study maternal serum testosterone levels were the same in the second and the third trimester of pregnancy in women with subsequent PIH and in normotensive women. These results are in agreement with those of a previous study on pre-eclamptic women (Tuutti E 2011). Data on androgens in non-proteinuric PIH is scarce. We did not study maternal testosterone levels during pre-eclamptic pregnancy. In most studies during overt pre-eclampsia maternal testosterone levels have been elevated compared with those in normotensive pregnant women (Acromite M 1999, Serin I 2001, Atamer Y 2004).

In our study, 23–24 years after pre-eclamptic pregnancy, premenopausal women did not show signs of increased androgen levels compared with women with a history of normotensive pregnancy. Thus, the increased risk of CVD in women with a history of hypertensive pregnancy, at least in pre-eclamptic women, seems not to be related to hyperandrogenism. In our study SHBG levels were not decreased, and calculated free testosterone levels and the free androgen index were still within normal limits. Women with a history of pre-eclamptic pregnancy had higher calculated free testosterone levels if FSH was at a premenopausal level, but in women with a history of normotensive first pregnancy the same phenomenon was not seen. Our study

did not confirm the hypertestosteronism which has been reported 17 years after pre-eclamptic pregnancy (Laivuori H 1998).

In healthy women androgen levels start to decrease during the reproductive years before menopause (Davis S 2002). There is an age-related reduction in both ovarian and adrenal androgen production. During menopausal transition circulating SHBG levels fall (Burger H 2000) and as a result free androgen levels rise, but total testosterone levels do not change significantly (Burger H 2002). With age, testosterone levels fall gradually (Burger H 2002). In our study in women with FSH levels above 10 IU/l, androgen levels were the same in women with a history of pre-eclampsia and in women with a history of normotensive pregnancy.

One reason why elevated serum testosterone levels were not observed in the perimenopausal period might be our method of testosterone measurement. The present mass spectrometric method (LC-MS/MS method) of testosterone measurement (Turpeinen U 2008) is more precise for low testosterone levels (below 6 nmol/l) than the immunological method used before (Laivuori H 1998). The problems of direct immunoassays for serum testosterone without extraction and pre-purification steps have been documented (Rosner W 2007). Using a GC-MS reference method for testosterone it has been shown that all commercially available immunoassays give unreliable results for low testosterone levels in women (Taieb J 2003), probably as a result of several interfering factors, such as SHBG and cross-reactivity among antisera for testosterone. This has been confirmed by comparison of immunoassays with LC-MS/MS methods (Wang C 2004). The lack of sensitivity and specificity, together with the bias associated with direct testosterone immunoassays makes them all unsuitable for accurate analysis of testosterone in women.

Another reason for our result might be that our women with previous pre-eclampsia tended more often to have PCOS than the controls, but the number of patients was low. It is known that before menopause hyperandrogenism partly resolves in women with PCOS (Winters S 2000, Legro R 2003). This could explain why hyperandrogenism in general was not seen in the present study.

## **7.5 INFLAMMATION**

In our fifth study, women with subsequent PIH and those with normotensive pregnancies had similar hs-CRP levels at 24 and at 32 weeks of gestation.

Even normal pregnancy provokes an increased maternal systemic inflammatory response, at least in the third trimester, and in pre-eclamptic pregnancy the inflammatory response is more extreme (Sibai B 2005). In previous studies on overt pre-eclampsia, levels of CRP (Teran E 2001), hs-CRP (Ertas I 2010), TNF- $\alpha$  and IL-6 have been reported to be elevated (LaMarca B 2007). In our study, women with subsequent PIH were not

heavier than their controls and they had no signs of insulin resistance or higher levels of FABP4, which might explain our result.

We found no correlation between maternal FABP4 levels and hs-CRP levels at 24 or 32 weeks in women with subsequent hypertension or in controls. In a previous study on non-pregnant women, plasma FABP4 levels correlated positively with those of several proinflammatory markers (hs-CRP, TNF- $\alpha$  receptors and IL-6) (Xu A 2012). At the same time as FABP4 is secreted from adipocytes, inflammation might increase in adipose tissue. In our study FABP4 levels were similar between groups, as with the inflammation marker hs-CRP.

Pre-pregnancy adiposity is a strong independent risk factor of PIH (Sukalich S 2006, Roberts J 2011) and inflammation is important in the causal pathway through which obesity predisposes pregnant women to pre-eclampsia (Wolf M 2001). FABP4 has been shown to be critical for regulating both metabolic and inflammatory responses (Furuhashi M 2011), but our results did not confirm that during pregnancy.

## 7.6 ANGIOPOIETIN-LIKE PROTEIN 6

In our second study it was shown for the first time that maternal Angptl6 levels were already significantly elevated at 24 weeks of gestation, before the onset of PIH. These levels increased in all groups over the next 8 weeks, but by 32 weeks differences between groups were no longer statistically significant. At 24 weeks fasting Angptl6 levels were especially higher in the non-proteinuric subgroup, compared with normotensive controls.

Previous studies have shown that circulating Angptl6 levels are elevated in pre-eclampsia compared with those in normotensive pregnant women (Stepan H 2009), but in a recent study there were no differences in pre-eclamptic and normotensive pregnant women (Xia G 2013). However, maternal Angptl6 levels have been found to be elevated in pregnant women compared with non-pregnant women (Xia G 2013).

The explanation for elevated serum Angptl6 levels in pre-eclampsia and the elevation preceding onset of PIH is not known. Even though there were no signs of increased insulin resistance at 24 weeks of gestation in our study, increased Angptl6 values in PIH and pre-eclampsia might be a response to insulin resistance, perhaps tending to inhibit its further progression to overt diabetes mellitus. Secondly, elevated Angptl6 levels before onset of pre-eclampsia might reflect early signs of placental hypoxia and of endothelial and angiogenic dysfunction (Hato T 2008). A third possibility could be that both metabolic alterations and placental hypoxia are interconnected and responsible for the rise in serum Angptl6 levels.

Future studies with larger cohorts are needed to clarify the predictive role of Angptl6 in PIH. However, even with limited power, our study results strengthen the potential role of angiogenic and angiogenic-like factors in the

pathogenesis of pre-eclampsia, but they showed no association between levels of the insulin sensitivity marker Angptl6 and insulin sensitivity during pregnancy.

## **8 CONCLUSIONS**

The following conclusions can be drawn from the five studies:

Relatively overweight women who subsequently developed PIH had no evidence of insulin resistance, hyperandrogenism or inflammation at 24 or 32 weeks of gestation compared with those who remained normotensive (I).

In pre-eclampsia plasma noradrenaline levels were elevated (III). The rise in noradrenaline levels could already be seen at 32 weeks of gestation in women with subsequent PIH, at least in relatively overweight women (I).

At 24 weeks of gestation, serum FABP4 concentrations and SBP after 10 minutes' standing in an orthostatic test were associated with the subsequent development of PIH (I).

Serum Angptl6 levels in the second trimester of pregnancy were higher in women who subsequently developed PIH (II).

In diurnal glucose profiles there was a greater increase in blood glucose from 4 am to 8 am in women with GDM and chronic hypertension compared with women who remained normotensive (IV).

At climacteric age, women with prior pre-eclampsia did not present with an increased incidence of metabolic syndrome, altered insulin resistance or hyperandrogenism (V).

The present work supports the presence of sympathetic over-activity in hypertensive pregnancy. It is suggested that two regulators of insulin sensitivity, Angptl6 and FABP4, may have potential roles in the development of PIH.

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## ORIGINAL PUBLICATIONS

