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Preeclampsia – Studies on the Placenta and B-type Natriuretic Peptide

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ACTA UNIVERSITATIS UPSALIENSIS UPPSALA 2018

ISSN 1651-6206
ISBN 978-91-513-0411-3
urn:nbn:se:uu:diva-353295

Dissertation presented at Uppsala University to be publicly examined in Audiotorium Minus, Museum Gustavianum, Akademigatan 3, Uppsala, Friday, 5 October 2018 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish. Faculty examiner: PhD Liv Cecilie Vestrheim Thomsen (Department of Clinical Science, University of Bergen, Bergen, Norway).

Abstract

Junus, K. 2018. Preeclampsia – Studies on the Placenta and B-type Natriuretic Peptide. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1487. 68 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0411-3.

Preeclampsia has several pathophysiological pathways, but the placenta has a central role. The pathophysiology appears to differ between the two subtypes – early- and late-onset preeclampsia. In clinically evident preeclampsia, maternal circulatory levels of the cardiac peptide B-type natriuretic peptide (BNP) and its cleavage fragment NT-proBNP are elevated, but whether or not the peptides are involved in the pathophysiology of preeclampsia is unknown. The overall aim of the current work was to expand knowledge of preeclampsia pathophysiology, with a main focus on the relationship between BNP and NT-proBNP, and early- and late-onset preeclampsia.

In Paper I, the placental transcriptional profiles of early- and late-onset preeclampsia were compared by using microarrays and bioinformatics. A total of 196 transcripts were differently regulated in the two groups. Using qRT-PCR, mRNA levels of the two angiogenesis-related transcripts *ACVRL1* and *EGFL7* were confirmed to be lower in early-onset preeclampsia than in both late-onset preeclampsia and early controls.

In Paper II, the circulatory levels of NT-proBNP were higher in both early- and late-onset preeclampsia than in gestational age-matched controls. BNP mRNA and protein were detected by qRT-PCR and immunohistochemistry in placentas from both women with preeclampsia and controls.

In Paper III, circulatory levels of NT-proBNP were measured in the early second-trimester in women who later developed early-onset preeclampsia and in women who continued to have normal pregnancies. No differences were found between the two groups of women.

In Paper IV, the secretion of NT-proBNP, and the mRNA levels of BNP and BNP receptors were investigated in cultured primary trophoblasts. Low levels of NT-proBNP were found in the supernatants of term but not first-trimester trophoblasts. BNP and BNP-receptor mRNA were detected in term trophoblasts.

The results of this work strengthen the concept of the two subtypes of preeclampsia (early- and late-onset) having partly different pathophysiological pathways. The results also indicate that the placenta releases BNP and that BNP may have receptor-mediated effects on the placenta.

Keywords: BNP, B-type natriuretic peptide, early-onset preeclampsia, gene expression, NT-proBNP, placenta, preeclampsia, trophoblasts

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ISSN 1651-6206
ISBN 978-91-513-0411-3
urn:nbn:se:uu:diva-353295 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-353295)

To Irma on her first birthday

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Junus, K., Centlow, M., Wikström, A.K., Larsson, I., Hansson, S.R., Olovsson M. (2012) Gene expression profiling of placentae from women with early- and late-onset pre-eclampsia: down-regulation of the angiogenesis-related genes *ACVRL1* and *EGFL7* in early-onset disease. *Molecular Human Reproduction*, 18(23):146–55

II Junus, K., Wikström, A.K., Larsson, A., Olovsson M. (2014) Placental expression of proBNP/ NT-proBNP and plasma levels of NT-proBNP in early- and late-onset preeclampsia. *American Journal of Hypertension*, 27(9):1225–30

III Junus, K., Wikström, A.K., Larsson, A., Olovsson M. (2017) Early Second-trimester Plasma Levels of NT-proBNP in Women who subsequently develop Early-onset Preeclampsia. *The Journal of Maternal-Fetal and Neonatal Medicine*, 30:2163-5

IV Junus, K., Majali-Martinez, A., Kallak, T.K., Larsson, A., Olovsson M. (2018) Primary Term Trophoblasts Release NT-proBNP. *Manuscript*

Contents

Abbreviations

ACVRL1	Activin A receptor type II-like 1
BNP	B-type natriuretic peptide
CVD	Cardiovascular disease
EGFL7	EGF-like domain, multiple 7
ER	Endoplasmic reticulum
hCG	β-human chorionic gonadotropin
HELLP	Hemolysis, elevated liver enzymes and low platelets
IDO1	Indoleamine 2, 3-dioxygenase 1
ISSHP	the International Society for the Study of Hypertension in Pregnancy
IUGR	Intrauterine growth restriction
MAP	Mean arterial blood pressure
NICE	National Institute for Health and Care Excellence
NPPB	Natriuretic peptide B (gene)
NPR1	Natriuretic peptide receptor A
NPR2	Natriuretic peptide receptor B
NT-proBNP	N-terminal proBNP
PAPP-A	Pregnancy-associated plasma protein-A
PlGF	Placental growth factor
qRT-PCR	Quantitative real-time polymerase chain reaction
ROBO4	Roundabout homolog 4, magic roundabout
sEng	Soluble endoglin
sFlt1	Soluble fms-like tyrosine kinase 1
SFOG	Swedish Society for Obstetrics and Gynecology
SGA	Small for gestational age

Introduction

Every year about 300 000 women die during pregnancy or in childbirth.[1] Hemorrhages, hypertensive disorders, and sepsis are the three major causes of direct maternal deaths.[2] Among the hypertensive disorders of pregnancy, preeclampsia and eclampsia lead to most deaths.[3] The most important actions against maternal death are to increase the availability of health care and to train personnel in rural and low-resource settings.[1] Nevertheless, even in high-resource settings, preeclampsia continuous to be a feared complication of pregnancy, leading to both maternal and neonatal mortality and morbidity.

Preeclampsia

The pregnancy disorder preeclampsia affects 3–5% of pregnant women.[4] It is one of the leading causes of maternal and neonatal mortality and morbidity, especially in low-resource settings.[1-3] Preeclampsia can deteriorate quickly and may affect multiple organ systems. Maternal complications include eclampsia (seizures), HELLP (Hemolysis, Elevated Liver enzymes, and Low Platelets), stroke, lung edema, kidney failure, and liver rupture.[5] Women with previous preeclampsia have a higher risk of cardiovascular disease (CVD) later in life.[6] Fetal complications include intrauterine growth restriction (IUGR), preterm delivery, and stillbirth.[5] Preeclampsia starts to resolve with delivery and the only curative treatment is to induce birth in women with progressing disease, thereby removing the placenta. In spite of the scientific effort to increase knowledge of the syndrome, the pathophysiological mechanisms underlying the complex disorder of preeclampsia are not fully understood.

Definition

There is no consensus of opinion on the definition of preeclampsia. Previously, new onset of hypertension and proteinuria after 20 weeks' gestation, was the most common definition of preeclampsia. This lack of consensus is recognized by the International Society for the Study of Hypertension in Pregnancy (ISSHP). Since 2001, ISSHP has published international recommendations for classifying, diagnosing, and managing preeclampsia and other hypertensive disorders of pregnancy.[7-9]

In 2001 ISSHP introduced separate definitions of preeclampsia for clinical and research purposes, where the clinical definition did not require the presence of proteinuria. The research definition included proteinuria as a prerequisite to ensure more specificity.[7] The research definition was virtually identical to the Swedish definition of preeclampsia [10] and is used in the studies included in this thesis.

New onset of hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg) and proteinuria (≥ 300 mg/24 hour or a spot urine protein/creatinine ratio ≥ 30 mg/mmol or at least 1g/L [2+] on a dipstick test), after 20 weeks' gestation.

A single unified definition of preeclampsia was proposed in a revised set of recommendations published in 2014. Acknowledging preeclampsia as a multisystem disorder, proteinuria was now removed from the definition. Instead, hypertension together with signs of other organ dysfunction defines preeclampsia.[8]

New onset of hypertension after 20 weeks' gestation and one or more of the following new onset conditions: proteinuria, renal insufficiency, liver involvement, neurological complications, hematological complications or uteroplacental dysfunction such as IUGR.

Even though this recommendation has been around for a while, there are still differences on how to define preeclampsia. For example, the British National Institute for Health and Care Excellence (NICE) and the Swedish Society for Obstetrics and Gynecology (SFOG) still include proteinuria in the definition of preeclampsia.[10, 11] The Swedish guidelines are under revision and it is likely that proteinuria will be excluded from the criteria in the near future.

Definition of preeclampsia subtypes

Preeclampsia is often described as heterogeneous syndrome which is reflected in the different definitions and various theories on its cause. Therefore, cases of preeclampsia are often classified into subtypes. Previously, the clinical differentiation between mild and severe preeclampsia was recommended. ISSHP defined severe preeclampsia as systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg. Deteriorating clinical conditions were also to be considered, whereas the amount of proteinuria was not considered a reliable marker of severity.[12] Recently, it was stated that classification of preeclampsia as severe or mild is no longer recommended. The background for ISSHP´s new standpoint is that preeclampsia may become a major life-threat to mother and baby at any time, and classification of preeclampsia as mild may therefore be misleading in a clinical setting.[9]

For research purposes, preeclampsia is often classified into early- or late-onset. Early-onset preeclampsia is more often associated with poor placentation, IUGR, and worse long-term maternal cardiovascular outcomes than late-onset preeclampsia.[13] Before 2013, there was no consensus on the definition of early-onset preeclampsia. Definitions ranged from preeclampsia presenting before 28 to 37 gestational weeks. Guidelines from ISSHP now state that early-onset preeclampsia presents before 34 weeks' gestation.[12] Preeclampsia is also often defined as preterm, meaning preeclampsia leading to delivery before 37 weeks' gestation.

Epidemiology and risk factors of preeclampsia

Preeclampsia affects about 3–5% of all pregnancies in Sweden, the US and other Western countries.[4] The incidence varies across the world, and is higher in developing than in developed countries.[14] Being one of the major causes of maternal mortality, it is estimated that 14% of all maternal deaths are due to preeclampsia.[2] Every year, preeclampsia is also responsible for over 500,000 fetal and neonatal deaths.[9] Close monitoring of women with preeclampsia and timing of delivery are crucial in the management of the disorder. Therefore, almost all maternal and neonatal deaths occur in low- or middle-income countries, where the availability of health care is low.[2] Even so, preeclampsia is one of the most common reasons for intensive care during pregnancy in high-income countries.[15] In the Nordic countries the maternal mortality rate is low. In Norway, hypertensive disorders were the most common cause of maternal death between 1996 and 2014,[16] and preeclampsia is the second most common cause of maternal death in the Nordic countries.[17] Delivery resolves preeclampsia, and 8% of preterm births in the US are due to induction of delivery in women with severe preeclampsia.[18] About 10–12% of preeclampsia cases are early-onset.[19, 20]

In addition to the acute consequences of preeclampsia, women with previous preeclampsia have a higher risk of CVD later in life. For all preeclampsia cases, there is a twofold increased risk of post-pregnancy CVD[21], and this association is even stronger in women with early-onset preeclampsia, with an eight- to ninefold increased risk.[22]

Risk factors of preeclampsia include maternal characteristics such as age, ethnicity, and obesity. Underlying diseases including diabetes mellitus, chronic hypertension, renal disease, systemic lupus erythematosus, and antiphospholipid syndrome increase the risk of preeclampsia. Moreover, obstetric factors such as parity, multiple pregnancy, assisted conception, previous preeclampsia, family history of preeclampsia, and a pregnancy interval greater than ten years also increase the risk.[23, 24] Many of the risk factors of preeclampsia are also risk factors of CVD.[25]

Clinical aspects of preeclampsia

Symptoms of preeclampsia vary greatly. Women with preeclampsia are often asymptomatic or have mild symptoms and the disorder is diagnosed during a routine antenatal health visit. However, severe symptoms can also be the first sign of preeclampsia. Symptoms of epigastric pain, nausea, and vomiting can occur. Neurological symptoms include headaches, visual disturbances, eclampsia (seizures), and stroke. The liver can be affected, with increased levels of transaminases, liver dysfunction, or, in rare cases, liver rupture. Kidney failure and lung edema can also be signs of preeclampsia. The coagulation cascade can be affected, with disseminated intravascular coagulation. Placental complications include IUGR and placental abruption. The HELLP syndrome, characterized by hemolysis, elevated levels of transaminases, and thrombocytopenia is associated with preeclampsia, but some HELLP cases have normal blood pressure. HELLP often has an acute onset and can deteriorate rapidly. Apart from IUGR and placental abruption, fetal complications include stillbirth and neonatal death. As the only cure for preeclampsia is delivery of the fetus and the placenta, prematurity-associated complications are also common.[5]

Details in the guidelines for monitoring and managing preeclampsia differs somewhat between countries. However, the main recommendations are basically the same and include clinical assessment, control of blood pressure and proteinuria, blood tests, and fetal assessment.[9-11] Magnesium sulfate is generally recommended for seizure prophylaxis and treatment in severe cases.[26] Hypertension is treated with antihypertensive drugs to decrease the risk of cerebral hemorrhage.[27] When the symptoms are mild, management can often be expectant. Delivery resolves preeclampsia, and is therefore recommended at 37 weeks' gestation if the syndrome is progressing, or before that, if there are either maternal or fetal indications.[9]

Prediction and prevention of preeclampsia

For a long time, there was no established method to predict preeclampsia before clinical signs of the syndrome were evident, nor was it possible to prevent preeclampsia. This is no longer true.

Aspirin lowers the incidence of preeclampsia and the risk of delivering preterm in women at high risk.[28] Many attempts to predict preeclampsia and to identify the women who would benefit from this treatment have been made.[29] A combination of medical history and maternal characteristics in early pregnancy and a false-positive rate set at 11%, have resulted in a prediction rate of 40% for all cases of preeclampsia, 48% for preterm preeclampsia, and 54% for preeclampsia requiring delivery before 34 weeks.[24] Adding measurements of the uterine artery pulsatility index, mean arterial blood pressure (MAP), and

levels of serum pregnancy-associated plasma protein-A (PAPP-A) and placental growth factor (PlGF) at 11 to 13 weeks of gestation to the screening method has resulted in detection of 76% of cases of preterm preeclampsia and 38% of cases of term preeclampsia, at a fixed false-positive rate set at 10%.[30]

This method was used in the multicenter, double-blind, placebo-controlled ASPRE trial to identify women at high risk of developing preterm preeclampsia. The aim was to study if low-dose aspirin from the first-trimester of pregnancy reduces the incidence of preeclampsia. In fact, treatment with low-dose aspirin lowered the incidence of preterm preeclampsia, the figures being 4.3% in the placebo group and 1.6% in the aspirin group.[31] In a secondary subgroup analysis of the data from the ASPRE trial, women with chronic hypertension did not seem to benefit from prophylactic treatment with low-dose aspirin.[32] The number needed for screening in the ASPRE trial was 333. The trial was not designed for studying adverse maternal and neonatal outcomes and it is probable that the number needed for screening to prevent these outcomes is even higher. Before this method can be implemented in a wide setting, careful assessment of cost-benefit needs to be done.

Assay of PlGF alone or together with soluble fms-like tyrosine kinase 1 (sFlt1) is also being evaluated in so-called 'rule in' and 'rule out' tests. Levels of PlGF are decreased and those of sFlt1 are increased in women with preeclampsia. The ratio of sFlt1/PlGF[33] or the level of PlGF alone[34] can be used to rule out preeclampsia requiring delivery within the next 1–2 weeks in women with suspected preeclampsia before 35 weeks' gestation. Since 2016, the 'rule out' test together with standard clinical assessment has been recommended by NICE.[35] In contrast, ISSHP does not recommend the use of these tests until further clinical studies have been done and ISSHP stresses that these tests may mostly be of use in settings where regular antenatal follow up is not possible.[9]

Pathophysiology of preeclampsia

In recent decades scientific efforts have led to an increased understanding of preeclampsia.[36] Nevertheless, the etiology and pathophysiology of the disorder remain unclear. Preeclampsia is therefore often described as a complex and heterogeneous syndrome. Several hypotheses and models have been suggested to explain the cause and the progress of the syndrome.

For a long time, it was generally accepted that preeclampsia was a placental disorder, caused by poor placentation. The placenta but not a fetus, as in a molar pregnancy, is needed to develop preeclampsia, and preeclampsia resolves upon delivery of the placenta.[37] Moreover, preeclampsia is associated with IUGR and small lesions are seen in placentas from pregnancies complicated by preeclampsia. All this evidence was together interpreted as poor placental function leading to preeclampsia.

The role of the placenta in preeclampsia

The female body undergoes major physiological adaptations during pregnancy to nurture the growing child. By producing steroid hormones, peptides, and other factors that are released into the maternal bloodstream, the placenta is of major importance in inducing these maternal adaptations. Other functions of the placenta are the exchange of oxygen and CO_2, and delivery of nutrients and excretion of waste products to and from the growing fetus. It is also an immunological barrier that protects the fetus from being attacked by the maternal immune system.[38]

In normal pregnancy, placental extravillous trophoblasts invade the uterine spiral arteries between gestational weeks 10 and 20. The spiral arteries lose the smooth muscles and become dilated and unresponsive to vasoconstrictors. This results in reduced velocity and pulsatility of the maternal blood flow to the placenta.[39, 40]

The two-stage model of preeclampsia

The two-stage model of preeclampsia,[41] was generally accepted for a long time. The first stage in this model is poor placentation. The trophoblast invasion that normally takes place is impaired and the spiral arteries are not completely remodeled.[37] This results in high-velocity perfusion and intermittent hypoperfusion and reperfusion with consequent oxidative stress and endoplasmic reticulum (ER) stress. These processes damage the placental villous tissue (syncytiotrophoblast stress) and thereby the release of several different trophoblast-derived particles into the maternal circulation.[39] These particles then contribute to an exaggerated maternal inflammatory response and endothelial dysfunction. As a consequence, in stage two, the clinical syndrome of preeclampsia develops.[41-43]

Poor placentation is not only associated with preeclampsia but also with IUGR without coexistent preeclampsia, and preterm birth.[44, 45] IUGR is associated with ER stress, and consequently, syncytiotrophoblasts stress alone cannot be sufficient to cause preeclampsia. To explain this paradox, the two-stage model has been modified, and it is suggested that there is a maternal contribution to preeclampsia.[46] This could be increased sensitivity to the fetal- or trophoblast-derived factors released into the maternal circulation in some women.[47] Factors increasing sensitivity to the trophoblast-derived factors could be genetic, behavioral, and environmental. However, these maternal factors that are by definition risk factors of preeclampsia can sometimes also be risk factors of IUGR and preterm birth.[13, 48] The two-stage model has also been extended to comprise a total of four stages. Before conception, impaired maternal tolerance to paternal antigens (stage 0), and later incomplete tolerance to the fetus (stage 1) may underlie poor placentation (stage 2) and the clinical stage of preeclampsia (stage 3).[49, 50]

The most studied trophoblast-derived factors in preeclampsia that are believed to be part of the link between stages one and two, include the antiangiogenic proteins sFlt1 and soluble endoglin (sEng), and the proangiogenic protein PlGF. In most pregnancies complicated by preeclampsia, maternal levels of both sFLT1 and sEng are increased, whereas those of PlGF are decreased. An imbalance between these anti- and proangiogenic factors is believed to lead to generalized endothelial dysfunction and induction of the maternal signs of preeclampsia.[51] Many other factors have been studied; for example, circulating syncytiotrophoblast-derived extracellular vesicles, [52] and free fetal hemoglobin[53] are increased in preeclampsia, and may lead to amplification of the maternal inflammatory response and oxidative stress.

Placental dysfunction in late pregnancy

Preeclampsia with poor placentation is mostly associated with early-onset and is sometimes referred to as placental preeclampsia. In term preeclampsia, spiral artery remodeling is often normal and in contrast to placental preeclampsia, this type of preeclampsia is sometimes called maternal preeclampsia. It generally presents at or near term, i.e. has a late-onset.[41, 54] In 2015, a hypothesis to explain placental dysfunction in these cases was put forward.[55] Towards the end of pregnancy, and as the placenta grows larger, the intervillous space can be compressed. This may lead to decreased perfusion and oxygen delivery, resulting in syncytiotrophoblast stress, which in turn leads to preeclampsia.[55]

Subtypes of preeclampsia

One reason for the failure to explain preeclampsia is that there may be partly different disease mechanisms leading to a similar clinical presentation with hypertension and organ dysfunction in the second part of pregnancy. The various clinical presentations and outcomes, and the various laboratory findings in preeclampsia support the possibility that there is more than one type of the disorder. It is possible that different subtypes of preeclampsia have been studied together, making it difficult to draw conclusions and to reproduce results.[36]

In 1996 it was first suggested that there may be two separate causes or origins of preeclampsia, a placental and a maternal subtype.[56] In analogy with this, it has also been proposed that early- and late-onset preeclampsia are two different disorders.[48] This dichotomous view may be simplistic, and in most cases there is likely to be a combination of both placental and maternal factors that lead to the different manifestations of preeclampsia.[13, 36, 57] Apart from placental and maternal preeclampsia, there are probably other subtypes that need to be considered, for example, preeclampsia with HELLP, preeclampsia superimposed on chronic disease, and recurrent preeclampsia.

In summary, there are probably several pathophysiological pathways leading to preeclampsia, indicating that the two-stage model with a single linkage

is too simplistic. The importance of preeclampsia definitions based on a pathophysiological understanding of the different subtypes rather than on phenotypes has been emphasized. For example, trophoblast-derived biomarkers may make it possible to identify placental components of preeclampsia, and PlGF has been proposed as the best one.[57]

The cardiovascular system and preeclampsia

Maternal adaptations to pregnancy occur in the hormonal, immunological, metabolic, neurological, and cardiovascular systems. Cardiovascular adaptations are necessary to enable sufficient uteroplacental circulation and are essential to meet the metabolic demands of the growing fetus.[58] Cardiac output (CO) and plasma volume increase during pregnancy.[59] In contrast, total peripheral resistance (TPR) decreases. These changes are most prominent in the early third trimester, after which CO decreases and TPR increases towards the end of pregnancy. Blood pressure decreases during pregnancy and is lowest in the second trimester.[60]

The maternal heart also undergoes major changes during pregnancy.[61] In a recent large prospective cross-sectional study, the average increase of ventricular mass in healthy women at term pregnancy was 40%, and these changes resolved after pregnancy.[62]

In women with preeclampsia with or without IUGR, and in women with normotensive IUGR pregnancies the physiological cardiovascular changes associated with normal pregnancy are impaired.[63] Pregnancies complicated by preeclampsia are associated with both structural and functional cardiac changes. Preterm preeclampsia is associated with severe left hypertrophy.[64] Both diastolic and systolic functions are impaired in preeclampsia. Global diastolic dysfunction is more often seen in term preeclampsia (40%) than in controls (14%).[65] In preterm preeclampsia, the changes are even more severe than in term preeclampsia.[64] Some of these changes are evident in mid-gestation, before the onset of clinical preeclampsia, and these changes are most severe in women developing preterm preeclampsia.[66]

The cardiovascular changes in preeclampsia were for a long time considered to be an effect secondary to placental dysfunction. Recently, however, it has been suggested that adaptation failure of the cardiovascular system is the primary mechanism leading to placental dysfunction in some cases of preeclampsia.[67, 68]

B-type natriuretic peptide

B-type natriuretic peptide (BNP) was first isolated from porcine brain and was initially named brain natriuretic peptide.[69] Together with atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) it belongs to the family of

natriuretic peptides. The 32-amino-acid peptide hormone BNP is mainly secreted into the circulation from atrial myocytes in the normal heart.[70] In response to ventricular distension or ventricular hypertrophy, however, BNP is predominantly secreted from the cardiac ventricles. It is hypothesized that the production and secretion of cardiac peptides are not only regulated by ventricular stress but also by a complex regulatory system involving both the neurohormonal and inflammatory systems.[71]

The gene natriuretic peptide B (*NPPB*) encodes the precursor proBNP, which comprises 108 amino-acid residues and is cleaved into BNP and an inactive 76 amino-acid N-terminal fragment, NT-proBNP. No functional activity of NT-proBNP has been described but it is useful as a target for measurement in plasma. In recent years it has become clear that there is considerable molecular heterogeneity in NT-proBNP and that there are actually several proBNP-derived fragments and also intact proBNP circulating in plasma, all are referred to as NT-proBNP.[72]

The BNP peptide mainly binds to natriuretic peptide receptor A (NPR1) but to some extent also to natriuretic peptide receptor B (NPR2).[73] These receptors are are guanylyl cyclase-coupled transmembrane receptors and function via the second messenger cyclic guanosine monophosphate (cGMP).[74] The natriuretic receptors are expressed in different tissues including the heart, kidney, testis, and adipose and smooth muscle tissue.[73] Functions of BNP include increased glomerular filtration, and increased diuresis and natriuresis. The BNP peptide also suppresses the renin–angiotensin–aldosterone system, which further enhances natriuresis and leads to a reduction of extracellular volume. Moreover, BNP decreases both systemic and vascular resistance. The BNP peptide also binds to the clearance receptor NPR3, which enables internalization and degradation of BNP. The enzyme neprilysin also inactivates BNP.[75] Circulating levels of BNP are affected by gender, age, hypertension, renal disease, and body weight. Plasma levels of BNP/NT-proBNP are decreased in obesity.[76, 77]

In individuals with congestive heart failure, BNP correlates with echocardiographic findings of ventricular strain, and BNP is a well-established biomarker for diagnosis and prognosis in patients with chronic heart failure.[78]

B-type natriuretic peptide and preeclampsia

During normal pregnancy maternal plasma levels of BNP are higher than in healthy non-pregnant women.[79] There are no differences in BNP [79] or NT-proBNP [80] levels throughout the trimesters in healthy pregnant women. When both pregnancy-induced hypertension and preeclampsia are clinically evident, plasma levels of BNP and NT-proBNP are elevated compared with those in normotensive pregnant women.[81-84] Levels seem to be especially high in women with severe or early-onset disease.[85-87]

As mentioned above, cardiovascular adaptation to pregnancy is impaired in preeclampsia. It has been suggested that increased levels of BNP/NT-proBNP in women with preeclampsia are associated with cardiovascular dysfunction. In fact, elevated levels of BNP in women with preeclampsia are correlated with increased left ventricular mass and relative wall thickness.[81, 87] High NT-proBNP levels in women with preeclampsia have also been associated with strain on the heart caused by high afterload.[84] Furthermore, impaired diastolic left ventricular function in women with early-onset preeclampsia is associated with an increase in plasma levels of NT-proBNP.[85] All these findings indicate that elevated plasma levels of NT-proBNP in pregnancies complicated by preeclampsia may be secondary to an increased cardiac load in these women.

Recently a study was carried out to test whether or not circulating NT-proBNP levels together with the sFlt□1/PlGF□ratio would be a better short□term predictor of delivery in women with signs of preeclampsia than the sFlt□1/PlGF□ratio alone. Indeed, this combined model yielded a higher AUC (0.845 [95% CI, 0.787–0.896]), than that for the sFlt□1/PlGF□ratio alone (AUC 0.786 [95% CI, 0.722–0.844]). In the same study, median levels of NT-proBNP differed in pregnancies complicated by isolated IUGR and pregnancies complicated by preeclampsia. This was not the case as regards the sFlt□1/PlGF□ratio.[88]

It is not known how the high levels of BNP in preeclampsia affect the cause of the disorder. Nor is it known what effect BNP has in the reproductive system. Amnion cells produce BNP and high levels of BNP are present in amniotic fluid in the first and second trimesters of pregnancy.[89] Biologically active NPR1 and NPR2 are present in the decidua vera, chorion laeve, myometrium, and placenta, indicating direct effects of natriuretic peptides on pregnancy.[90]

Levels of NT-proBNP in amniotic fluid correlate with those in the fetal circulation, but neither correlated with the NT-proBNP levels in the circulation of healthy pregnant women.[91] Nor does cord blood NT-proBNP levels correlate with those in the maternal circulation at term or near-term delivery.[92] Fetal sex does not influence the levels of cord blood NT-proBNP.[93, 94] Whether there is a correlation between fetal and maternal circulating levels of NT-proBNP, and if fetal sex affects circulatory levels of NT-proBNP, in women with preeclampsia are not elucidated.

Rationale

At the time this project was initiated, the importance of studying subtypes of preeclampsia was beginning to emerge. It was also recently discovered that circulating levels of BNP and NT-proBNP were elevated in preeclampsia at the time of diagnosis. Whether or not BNP was part of a causal pathway or a consequence of organ involvement was not known. At the time, the focus of

the cardiac association with preeclampsia was mostly on the long-term risk of CVD. Considering the endocrine functions of the placenta, and the various trophoblast-derived factors elevated in preeclampsia, we hypothesized that the placenta releases BNP/NT-proBNP and is partly responsible for the high levels of these factors present in the circulation of women with preeclampsia.

Aims

The overall aim of this work was to expand knowledge of preeclampsia pathophysiology by studying the two subtypes – early- and late-onset preeclampsia, and the peptides BNP and NT-proBNP. Specific aims were:

I To compare placental transcription profiles in women with early- and late-onset preeclampsia.

II To compare plasma levels of NT-proBNP in women with early- and late-onset preeclampsia and gestational-age-matched controls and to evaluate the possibility of BNP/NT-proBNP being a partly placenta-derived peptide by investigating BNP mRNA and protein in the placenta.

III To compare early second-trimester plasma levels of NT-proBNP in women developing early-onset preeclampsia with women with uncomplicated pregnancy outcomes.

IV To see if NT-proBNP is released from primary first-trimester and term trophoblasts.

Materials and Methods

The studies were approved by the Regional Ethics Review Boards in Uppsala and/or Lund, Sweden (Reference numbers: 01-254, 2010/129 and 2016/138). Informed consent was obtained from all women before inclusion in the studies. Preeclampsia was defined as new onset of hypertension (> 140/90 mmHg) observed on at least two separate occasions six hours or more apart, combined with proteinuria (≥ 2 on a dipstick, or a 24-hour urine sample measuring ≥ 300 mg).

Study design and study populations

Table 1. *Overview of the studies.*

Paper	Study design	Subjects	Methods	Outcomes
I	Cross-sectional	8 women with early- and 7 with late-onset preeclampsia. 4 early and 6 late controls.	Microarray and qRT-PCR	Placental whole genome mRNA expression in early- and late-onset preeclampsia
II	Cross-sectional	18 women with early- and 20 with late-onset preeclampsia. 22 early and 14 late controls.	Measurement of plasma NT-proBNP, qRT-PCR, immunohistochemistry and placental explants.	Plasma levels of NT-proBNP, placental *NPPB* mRNA and BNP protein expression.
III	Case-control	16 women with preeclampsia developing and 43 with uncomplicated pregnancy outcomes	Measurement of plasma NT-proBNP	Early second-trimester plasma levels of NT-proBNP
IV	Case-control	7 women with elective abortions and 10 women with elective C-sections.	Isolation, purification and culture of primary trophoblasts, qRT-PCR	Levels of NT-proBNP in trophoblast supernatants, *NPPB*, *NPR1*, and *NPR2* mRNA levels in primary term trophoblasts

Paper I

This cross-sectional study involved four groups of women. For the microarray experiment, eight women with early-onset (presenting at < 32 gestational weeks) and seven women with late-onset (presenting at ≥ 35 gestational weeks) preeclampsia were included. For verification with quantitative real-time (qRT) polymerase chain reactions (PCRs) two additional groups of women were included: four early controls, without signs of infection, that were admitted because of imminence of premature delivery, and six late controls who were recruited among healthy pregnant women delivering at term. Women with early-onset preeclampsia and the early control group were recruited from the University Hospital in Uppsala, Sweden. Women with late-onset preeclampsia and the late control group were recruited from the University Hospital in Lund, Sweden. Women with multiple pregnancies, ongoing upper urinary tract infection, chronic hypertension, diabetes mellitus, or renal disease were not included in any of the study groups.

Paper II

Four groups of women were included in this cross-sectional study. Data from the same cohort have been published before.[95-98] All women were recruited from the University Hospital in Uppsala, Sweden. For the plasma analysis, 18 women with early-onset preeclampsia (presenting at < 32 gestational weeks) and 20 women with late-onset preeclampsia (presenting at ≥ 35 gestational weeks) were included. The early control group consisted of 22 women who were recruited during a routine visit to an antenatal clinic at 25–31 completed gestational weeks. All these women had uncomplicated pregnancy outcomes. The late control group consisted of 14 women with uncomplicated pregnancy outcomes who delivered at term. Women with multiple pregnancies, ongoing upper urinary tract infection, chronic hypertension, diabetes mellitus, or renal disease were not included in any of the study groups. For the gene expression analysis, the study groups in Paper I were used.

Paper III

This was a case-control study with 16 women who later developed early-onset (≤ 34 gestational weeks) preeclampsia and 43 women with uncomplicated pregnancy outcomes. All women were selected from the population-based Uppsala University Hospital Biobank of Pregnant Women. Pregnant women above 18 years of age, attending their second-trimester routine ultrasonographic examination were approached for inclusion in the biobank. A venous blood sample was collected from the participating women, and brief maternal demographic data was collected. Cases and controls were matched for

parity and date of inclusion in the biobank. Women who had chronic hypertension, preterm birth, or delivered small for gestational age (SGA) infants were not included as controls. SGA was defined as having a birth-weight at, or more than, two standard deviation scores below the mean, based on the intrauterine growth curves published by Marsal and colleagues.[99] Women with multiple pregnancies, diabetes mellitus, or renal disease were not included in either group.

Paper IV

The study was designed as a case-control study, where primary trophoblasts were cultured in 5% or 20% oxygen.

Primary first-trimester trophoblasts were isolated from seven healthy women undergoing elective vaginal termination of pregnancy (legal abortion) in Graz, Austria. The study was approved by the institutional review board and ethics committee of the Medical University of Graz. All women gave written informed consent for collection and investigational use of tissues. Data from the same experiments have been published before.[100, 101]

Primary term trophoblasts were isolated from ten women who delivered by cesarean section at the University Hospital in Uppsala, Sweden due to breech presentation, more than two previous cesarean sections, or maternal request due to fear of giving birth. Women who had chronic diseases, gestational diabetes, or a BMI of > 30 kg/m^2 were not included. Neither were women with multiple pregnancies, preterm birth, or SGA infants.

Methods

RNA extraction

In Papers I and II, placental total RNA was extracted and washed using TRIZOL® and E.Z.N.A. total RNA Kits or RNeasy Mini Kits (early control group). In Paper IV, total RNA from primary term trophoblasts was extracted using RNeasy Mini Kits. RNA concentrations were measured with a NanoDrop instrument and RNA integrity was assessed by electrophoresis, using an Agilent 2100 Bioanalyzer.

Microarrays

Microarray chips were printed with a whole genome oligo set, containing approximately 27,000 genes at the Swegene DNA Microarray Resource Center, Lund University, Sweden. Before hybridization, complementary DNA (cDNA) probes were prepared and labeled with the fluorescent dyes Cy3 and

Cy5. Cy3-labeled cDNA from each experimental sample was pooled with reference Cy5-labeled cDNA and hybridized onto a microarray chip. A microarray scanner was used to read the fluorescence intensities of the hybridized arrays at 635 (Cy5) and 532 (Cy3) nm. Spot intensities were visualized with GenePix Pro 4.1 software and spots affected by veils, grains of dust or other contaminants were removed from further analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

In Paper I, four genes were selected for verification of the microarray results. The selection was based on possible association with preeclampsia, and was carried out after a literature search concerning the significantly altered gene transcripts. One gene associated with placental development was also selected. The genes were: Activin A receptor type II-like 1 (*ACVRL1*), EGF-like-domain, multiple 7 (*EGFL7*), Roundabout homolog 4, magic roundabout (*ROBO4*), and Indoleamine 2, 3-dioxygenase 1 (*IDO1*). In Paper II, mRNA levels of *NPPB* was measured.

In Papers I and II, relative mRNA levels were measured using the SYBR Green method. Melt curve analysis was carried out after each experiment to ensure specific amplification. TATA box-binding protein (*TBP*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), and succinate dehydrogenase complex, subunit A (*SDHA*) were included as endogenous controls and have been reported as being stable in preeclamptic placenta.[102] In Paper IV, mRNA levels of *NPPB*, *NPR1*, and *NPR2* were measured using the TaqMan method. *SDHA* was used as an endogenous control.[102]

Ct extractions and efficiency calculations for all the PCRs were done with LinRegPCR software. Mean normalized expression (MNE) in Papers I and II, and normalized gene expression (2-DeltaCt) in Paper IV were then calculated.

Immunohistochemistry

A total of eight villous placental samples were randomly selected from the women included in the plasma analysis in Paper II: three from women with early-onset preeclampsia, three from women with late-onset preeclampsia, and one each from the women in the early and late control groups. Human heart tissue was used as a positive control. Placental tissue samples were collected from different cotyledons in the central part of each placenta immediately after delivery. The samples were embedded in paraffin and stored. Before incubation the sections were deparaffinized, rehydrated, washed, and incubated in citrate buffer. Nonspecific binding was blocked and the sections were incubated overnight at 4 °C with mouse monoclonal anti-proBNP. The sections were then incubated with biotinylated anti-mouse IgG for one hour at room temperature. Thereafter streptavidin-conjugated horseradish peroxidase

complex was added. The sections were then rinsed, exposed to chromogen and counterstained with Mayer´s hematoxylin. Using light microscopy, the brown reaction product was observed. Negative control was obtained by omitting the primary antibody.

Placental explants

In Paper II, placental villous tissue from two women with uncomplicated pregnancies was collected from the placental mid-layer at five different but central locations within each placenta. The samples were thoroughly rinsed in sterile phosphate-buffered saline (PBS) and cut into pieces of approximately one millimeter in diameter, and thereafter cultured for 24 hours at 37 °C in culture medium. The medium was then collected, filtered, and centrifuged. The supernatant was collected and stored at -20 °C until analysis.

Primary trophoblast cultures

In Paper IV, primary trophoblasts from first-trimester and term pregnancy were studied. First-trimester trophoblast isolation was carried out using trypsin, dispase and DNase digestion. Cells were then purified on a density gradient. Non-trophoblastic cells were depleted by magnetic immunopurification with the anti-leukocyte marker CD45 and the anti-fibroblast marker CD90. As a quality control, functional activity was assessed by β-human chorionic gonadotropin (hCG) secretion and purity by immunohistochemical staining for cytokeratin 7 and human leukocyte antigen (HLA)-G. Trophoblasts were cultured at 37 °C with 5% CO_2 and 5, 12, or 20% O_2 for 48 or 72 h. Supernatants were then frozen at -20 °C until further analysis.

For term trophoblast isolation, approximately 50 g of placental tissue was digested in a solution containing HBSS, HEPES, Trypsin and DNase I. Cells were then fractionated on the basis of density using a Percoll® gradient. The fraction containing trophoblasts was then washed and cells were counted and stored in liquid nitrogen. Non-trophoblastic cells were then depleted with magnetic beads conjugated to mouse anti-human HLA. The trophoblast containing supernatant was centrifuged and the cell pellet dissolved in DMEM cell-culture medium supplemented with penicillin/streptomycin and fetal calf serum. Cell viability was tested by measuring hCG secretion. Only cells from purifications that had increased hCG production at 96 h were used in further analysis.

For experiments, cells were pooled from two purifications and then plated in duplicates or triplicates at a density of 3×10^5 cells/cm^2. Cultures were maintained at 37 °C in humidified air with 5% CO_2 and either 5% or 20% O_2 for 48 h. Supernatants were collected at 48 h and immediately frozen at -20°C until further analysis. Plates with no cells, containing only DMEM++ from the

same batch used in the experiments, were kept in the incubators for 48 h and were used as negative controls.

NT-proBNP analysis

Levels of NT-proBNP were measured at the Department of Clinical Chemistry, Uppsala University Hospital, Uppsala, Sweden with the accredited method in use at the time for the analysis.

In Papers II and III, venous blood samples were collected in sterile EDTA tubes. The samples were immediately put into a refrigerator and were centrifuged for 10 minutes at 1500 × *g* within four hours of collection. The plasma was then stored at -70 °C until analyzed. In Paper II, levels of NT-proBNP in plasma, and supernatants from the explant experiments, were measured with a Modular E170 analyzer. In Paper III, plasma levels of NT-proBNP were measured with a Cobas E601 instrument. In Paper IV, NT-proBNP levels in the supernatants from the first-trimester cell experiments were measured on a Cobas E601 instrument and the supernatants from the term experiments were measured using the Architec 8200 system.

Statistics and bioinformatics

Background characteristics, and levels of mRNA and NT-proBNP

Statistical analyses were carried out using an SPSS for Windows software package. Significance tests were two-tailed, and p-values < 0.05 were considered statistically significant. Categorical background variables were compared using Pearson's Chi-Square test or Fisher's exact test. In Papers I and II, continuous variables were compared in the four study groups. Means were compared by analysis of variance (ANOVA) followed by Tukey's test for multiple pair-wise comparisons. Medians were compared by using the Kruskal-Wallis test followed by the (post hoc) Mann–Whitney *U*-test for independent samples. Bonferroni correction was applied to the *post hoc* test to evaluate differences in mRNA and NT-proBNP levels between individual groups. In Paper III, the Student's t-test (means) or the Mann–Whitney *U*-test for independent samples (medians) was used to compare continuous variables in cases and controls. No comparisons between NT-proBNP or mRNA levels between the two study groups were carried out in Paper IV because of the small sample size.

Microarray statistics

The data generated from the microarray experiment was uploaded to the Bio Array Software Environment (BASE), for statistical analysis. Spots with intensities below 100 optical density units in both of the two channels were excluded. Individual spot intensities were also required to be twice as high as the background to be included. Data were normalized for labeling and reference bias by using lowess normalization and global median centering.[103, 104] Fluorescence ratios were calculated as Cy3/Cy5 and log2-transformed. Placental gene transcription in early- *vs.* late-onset preeclampsia was compared by significance analysis of microarrays, with the false discovery rate set to 5% and the *q*-value set to < 0.05. Only spots that were reported in all 15 arrays were included in the group comparison analysis. Primary microarray data were submitted to the Gene Expression Omnibus, accession number GSE22526.

Bioinformatic analysis

To extract biological meaning from the list of up- and down-regulated genes in Paper I, the Database for Annotation, Visualization and Integrated Discovery (DAVID) was used. Significant changes were uploaded to the database and analyzed with the whole human genome as background. Genes were classified (gene ontology) on the basis of biological process, molecular function and cellular component. Pathways in which significantly changed gene expressions were involved were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG). Annotations for protein domains were extracted from InterPro. A *p*-value of ≤ 0.05 and a fold change (FC) of ≥ 2 were used as cut-offs.

Summary of results

Paper I

The transcriptional profiles of placental tissue in women with early- and late-onset preeclampsia were studied. For the verification experiments, two control groups were also included. Background characteristics are presented in Table 2. A total of 196 genes were transcriptionally altered between the groups. Of these genes, 45 had a fold change of ≥ 2 (Table 3).

Several biological processes were affected, among them *angiogenesis* and *oxygen transport* (Table 4). Pathway analysis indicated clustering of altered genes in the *Alzheimer's disease pathway*.

Out of the differently transcribed genes three angiogenesis-related genes and one gene associated with placental development were selected for verification of the microarray results with qRT-PCR. The microarray data was confirmed in the qRT-PCR experiments, and mRNA levels of *ACVRL1* (p = 0.003), *EGFL7* (p = 0.037), *ROBO4* (p = 0.015) and *IDO1* (p = 0.011), were found to be down-regulated in early- compared with late-onset preeclampsia (Figure 1).

During normal placental development mRNA levels vary.[105] When comparing samples from early- and late-onset preeclampsia the findings may therefore be due to the difference in gestational age between the two groups. To investigate if the discovered alterations in transcription levels were related to gestational age, two control groups were included in the qRT-PCR analysis. The mRNA levels of *ACVRL1* (p = 0.011) and *EGFL7* (p = 0.007) were lower in early-onset preeclampsia than in early controls, while *ROBO4* and *IDO1* showed similar levels in these two study groups. There was no difference in mRNA levels between late-onset preeclampsia and late controls (Figure 1).

Table 2. Background characteristics of the women in Paper I.

	Early PE (*n* = 8)	**Late PE (*n* = 7)**	**Early control (*n* = 4)**	**Late control (*n* = 6)**
Maternal age, years	31.5 (29-39)	30 (22-45)	34 (30-39)	24 (21-30)
Nullipara	6 (75%)	6 (86%)	3 (75%)	5 (83%)
Systolic BP, mmHg	150 (140-186)	160 (140-168)	123 (102-126)[a]	120 (100-140)[c]
Diastolic BP, mmHg	99.5 (81-111)	105 (95-110)	79 (68-84)[b]	70 (60-80)[c]
Hypertensive treatment	7 (87.5%)	0 (0%)[a]	0 (0%)[a]	0 (0%)
GA at delivery, days	205 (172-214)	279 (259-285)[a]	170.5 (160-182)[b]	281.5 (267-298)
Caesarean section	8 (100%)	1 (16%)[b]	0 (0%)[b]	2 (33%)
Infant birth weight, g	990 (428-1320)	3290 (2695-3760)[a]	649.5 (490-963)	3612 (3300-4000)
Placental weight, g	237.5 (82-325)	650 (500-850)[a]	235 (130-280)	605 (450-800)

Data is presented as median (range) or *n* (%). PE, preeclampsia; BP, blood pressure; GA, gestational age. The following comparisons were made: early PE with late PE; early PE with early control; late PE with late control.

[a]Significantly different from early PE $p < 0.01$

[b]Significantly different from early PE $p < 0.05$

[c]Significantly different from late PE $p < 0.01$

Table 3. Genes with altered transcription in the microarray analysis (q-value < 5%, log2-fold change ≥ 2). Genes up-regulated or down-regulated in early- compared with late-onset PE are listed, by increasing q-value.

Gene Symbol	**GenBank Accession**	**log2-Fold Change**	***q*-value (%)**
Up-regulated in early PE			
TCL6	NM_020553	2.00	0.00
C15orf29	NM_024713	2.60	0.00
RAB11FIP5	NM_015470	2.56	0.00
N/A	AF090099	2.27	0.00
LIFR	NM_002310	3.46	0.00
CREBZF	NM_001039618	2.09	0.00

DEPDC1B	NM_018369	2.83	0.00
DAB2	NM_001343	3.19	0.00
N/A	AL365412	2.21	0.00
HOPX	NM_139212	4.29	0.00
MAP3K9	NM_033141	2.33	1.67
CYP19A1	NM_000103	3.31	1.65
EXPH5	NM_015065	3.18	1.71
PSPC1	NR_003272	2.23	2.10
DAB2	NM_001343	2.19	2.10
CCDC21	NM_022778	2.04	2.47
TREML2	NM_024807	3.11	2.89
N/A	AL121777	3.78	3.84
HES2	NM_019089	2.07	3.86
N/A	AK023465	3.44	4.10
TRIM25	NM_005082	2.09	4.70
CGA	NM_000735	2.28	4.83
Down-regulated in early PE			
KRT3	NM_057088	-2.27	0.00
FADS3	NM_021727	-2.08	0.00
ACTA2	NM_001613	-2.78	0.00
IDO1	NM_002165	-2.17	0.00
CORO6	NM_032854	-2.56	0.00
APOLD1	NM_030817	-4.17	0.00
TMEM175	NM_032326	-2.22	0.00
HBB	NM_000518	-7.69	0.00
KLF2	NM_016270	-2.78	0.00
AFF3	NM_002285	-2.33	0.00
SDPR	NM_004657	-2.38	1.53
EGFL7	NM_201446	-2.04	1.93
TMEM100	NM_001099640	-2.78	1.93
CLEC3B	NM_003278	-2.04	1.93
ACVRL1	NM_000020	-2.04	1.93
C4orf31	NM_024574	-2.04	1.93
DDIT4	NM_019058	-2.56	1.93
RCAN1	NM_004414	-2.08	1.93
IDO1	NM_002164	-2.27	2.20
SNCA	NM_000345	-2.38	3.79
N/A	AK024295	-2.13	4.70
MAGEA11	NM_005366	-2.08	4.87
HBA2	NM_000517	-3.70	4.87

Table 4. Gene ontological analysis of the genes in Table 3 with the whole human genome as background. Gene ontology groups with $p < 0.05$ and fold enrichment ≥ 2 are listed by increasing *p*-value.

GO Term	**GO ID**	***p*-value**	**Fold enrichment**
Biological process			
regulation of locomotion	GO:0040012	0.0064	24.0
locomotion	GO:0040011	0.0066	23.6
developmental process	GO:0032502	0.0088	2.17
multicellular organismal development	GO:0007275	0.0090	2.51
angiogenesis	GO:0001525	0.022	12.39
oxygen transport	GO:0015671	0.029	65.64
blood vessel morphogenesis	GO:0048514	0.030	10.68
blood circulation	GO:0008015	0.030	10.61
circulatory system process	GO:0003013	0.030	10.61
gas transport	GO:0015669	0.032	59.08
negative regulation of cellular process	GO:0048523	0.033	3.13
anatomical structure formation	GO:0048646	0.034	9.90
negative regulation of cell migration	GO:0030336	0.035	53.71
blood vessel development	GO:0001568	0.037	9.43
vasculature development	GO:0001944	0.038	9.28
negative regulation of biological process	GO:0048519	0.039	3.00
negative regulation of cell motility	GO:0051271	0.042	45.44
negative regulation of locomotion	GO:0040013	0.043	43.76
Molecular function			
tetrapyrrole binding	GO:0046906	0.00201	15.29
heme binding	GO:0020037	0.00201	15.29
oxygen binding	GO:0019825	0.00222	41.39
iron ion binding	GO:0005506	0.01732	7.00
oxygen transporter activity	GO:0005344	0.03199	59.54
transcription regulator activity	GO:0030528	0.04214	2.58
Cellular component			
hemoglobin complex	GO:0005833	0.02894	66.07

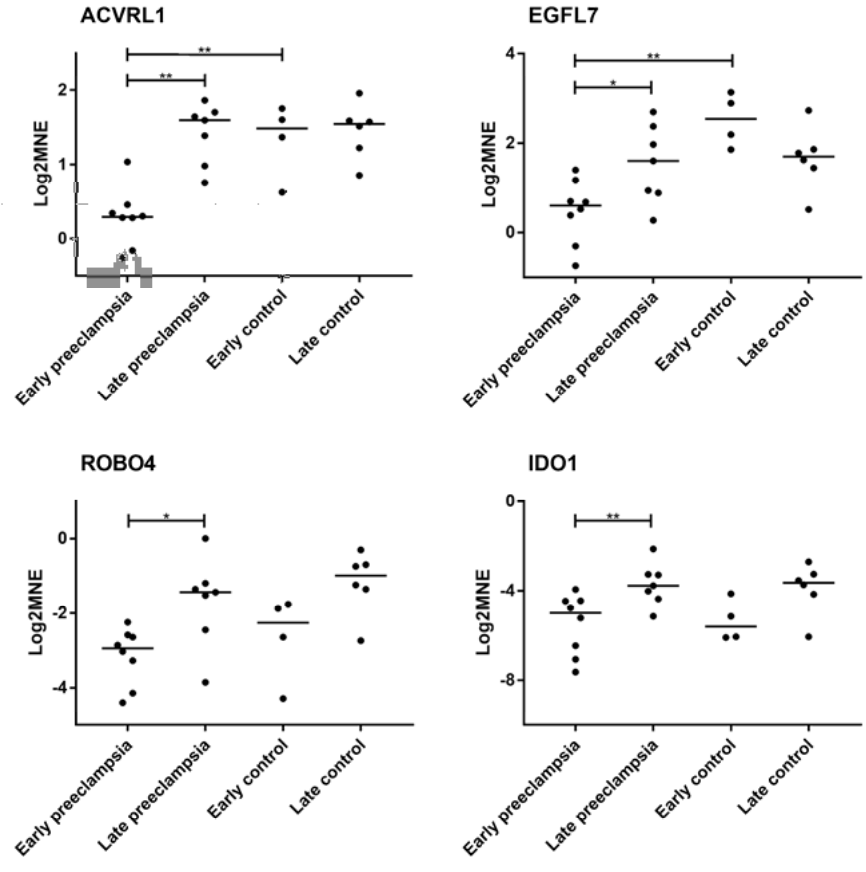

Figure 1. Mean normalized expression (MNE) of placental mRNA. Medians are marked with horizontal lines. * $p < 0.05$, ** $p < 0.01$.

Paper II

Maternal plasma levels of NT-proBNP were measured in women with early- and late-onset preeclampsia, and in two gestational-age-matched control groups. Background characteristics of the included women are presented in Table 5.

Table 5. Background characteristics of the women in the NT-proBNP analysis in Paper II.

	Early PE (*n* = 18)	**Late PE (*n* = 20)**	**Early control (*n* = 22)**	**Late control (*n* = 14)**
Maternal age, years	31 ± 4	30 ± 6	31 ± 4	33 ± 6
Nullipara	12 (67 %)	11 (55%)	9 (41%)	4 (29%)
BMI at first visit, kg/m^2	27 ± 5	27 ± 6	23 ± 3[b]	25 ± 3
Systolic BP (mmHg)	164 ± 17	148 ± 14[b]	124 ± 16[a]	124 ± 12[c]
Diastolic BP (mmHg)	99 ± 6	99 ± 7	80 ± 11[a]	79 ± 12[c]
At sampling				
Gestational age, days	198 ± 24	265 ± 11[a]	197 ± 13	277 ± 11
Hypertensive treatment	15 (83%)	10 (50%)[b]	0 (0%)[a]	0 (0%)[d]
GFR, ml/min/1.73m^2	57 ± 12	45 ± 13[b]	81 ± 10[a]	63 ± 12[c]

Data is presented as mean ± SD or *n* (%). PE, preeclampsia; BMI, body mass index; BP, blood pressure; GFR, glomerular filtration rate. The following comparisons were made: early PE with late PE; early PE with early control; late PE with late control.

[a]Significantly different from early PE $p < 0.001$

[b]Significantly different from early PE $p < 0.05$

[c]Significantly different from late PE $p < 0.001$

[d]Significantly different from late PE $p < 0.01$

Women with both early- and late-onset preeclampsia had higher median levels of NT-proBNP than their gestational-age-matched controls (365 [14–9815] *vs.* 48 [10–170] and 176 [33–2547] *vs.* 41 [14–172] ng/l; both $p < 0.001$; Figure 2). A tendency towards higher levels of NT-proBNP in women with early- *vs.* late-onset preeclampsia was observed ($p = 0.057$; Figure 2).

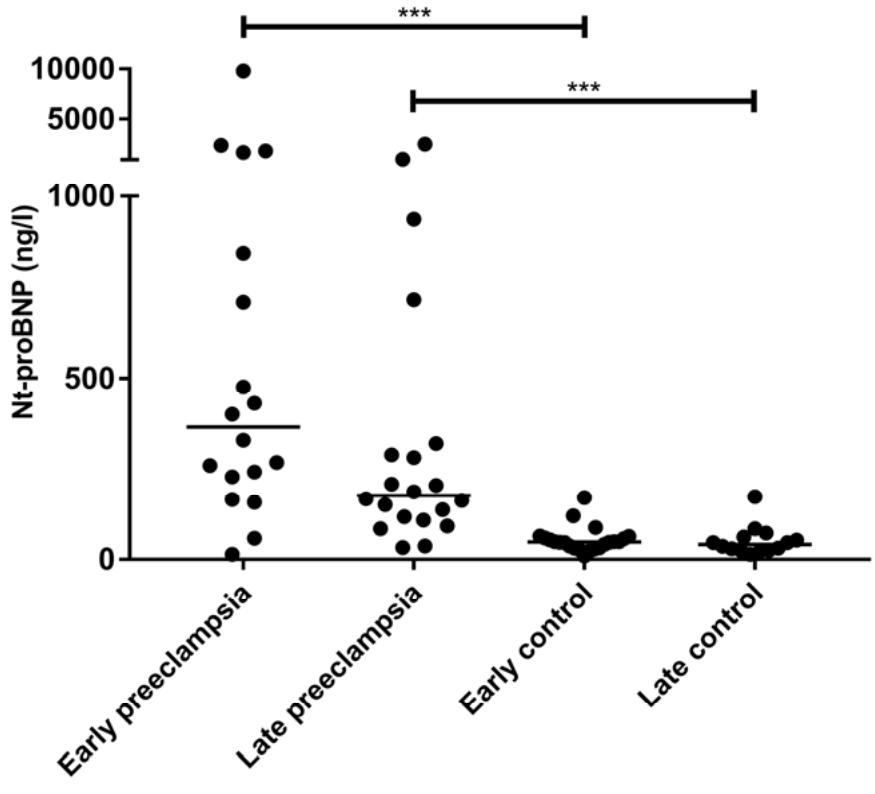

Figure 2. Maternal plasma levels of NT-proBNP in women with clinically evident preeclampsia and gestational-age-matched controls. Medians are marked with horizontal lines. ***$p < 0.001$.

The same study population used in the qRT-PCR experiments in Paper I was also used for qRT-PCR in Paper II (Table 2). Transcripts of *NPPB*, the gene encoding proBNP, were found in 20 out of 25 samples. There was no difference in *NPPB* mRNA levels between the groups.

Protein of proBNP/NT-proBNP was found in all placental samples examined by means of immunohistochemistry, both in women with preeclampsia and in women with uncomplicated pregnancy outcomes. Staining was especially profound in maternal arteries, but it was also visible in the syncytiotrophoblasts (Figure 3).

Placental samples from two women with uncomplicated pregnancy outcomes were used for explant culture. After 24 hours of culture, NT-proBNP levels in culture media were 7 ng/l and 6 ng/l. NT-proBNP was not detected in fresh culture medium.

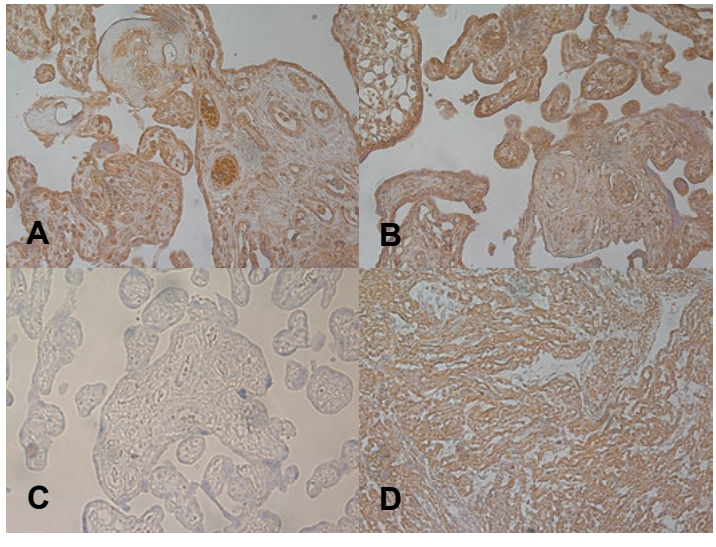

Figure 3. Representative immunohistochemical staining of A) BNP in placenta from early-onset preeclampsia, B) BNP in placenta from early control, C) negative control placenta, and D) BNP in human heart tissue (positive control).

Paper III

Maternal plasma levels of NT-proBNP were measured in the early second-trimester in 16 women that later developed early-onset preeclampsia and in 43 women with an uncomplicated pregnancy outcome. Background characteristics are presented in Table 6. Four of the women with early-onset preeclampsia had chronic hypertension. The median plasma levels of NT-proBNP did not differ between women who later developed early-onset preeclampsia (51.8 [26.1–131.9] ng/l) and women with an uncomplicated pregnancy outcome (53.0 [14.9–184.2] ng/l) (Figure 4).

Table 6. Background characteristics of the women in Paper III

	Early PE (*n* = 16)	**Controls (*n* = 43)**
Maternal age, years	30.5 (23-36)	31.0 (21-37)
BMI at first antenatal visit, kg/m^2	24.3 (16.6-32.2)	23.1 (18.6-31.6)
Nullipara	9 (56%)	23 (54%)
GA at sampling, days	124 ± 6	124 ± 8
Previous preeclampsia	2 (13%)	0 (0%)
SGA***	9 (56%)	0 (0%)
LGA	0 (0%)	3 (7%)
Female/male†	2 (12.5%)/14 (87.5%)	17 (40%)/26 (60%)
Chronic hypertension***	4 (25%)	0 (0%)
Pregnancy outcomes among women with early-onset preeclampsia		
GA at PE diagnosis, days	204 ± 26	
GA at delivery, days	218 ± 26	
Highest systolic BP, mmHg	174 ± 15	
Highest diastolic BP, mmHg	104 ± 8	
Highest MAP, mmHg	127 ± 7	
Antihypertensive treatment	14 (88%)	
Infant birth-weight, g	1519 ± 775	
IUGR	5 (31%)	

Data is presented as median (range), mean ± SD, or *n* (%). PE, preeclampsia; BMI, body mass index; GA, gestational age; SGA, small for gestational age; LGA, large for gestational age; BP, blood pressure; MAP, mean arterial blood pressure; IUGR, intrauterine growth restriction.
$^{***}p < 0.001$; $^{\dagger}p = 0.048$

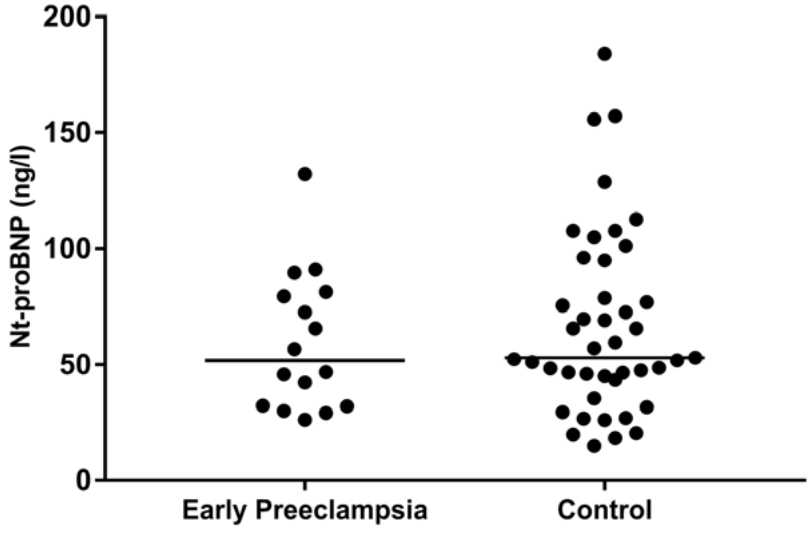

Figure 4. Maternal plasma levels of NT-proBNP in the early second-trimester in women who later developed early-onset preeclampsia and controls. Medians are marked with horizontal lines. Levels of NT-proBNP were similar in the two groups.

Paper IV

Out of ten isolations, primary term trophoblasts were successfully isolated from six placentas. Of the six isolations that were included in the immunopurification step, another two had to be excluded from the final analyses as a result of failed viability test with no increase in hCG levels during cell culture. This resulted in only four studied isolations and no statistical analyses were carried out because of the low sample size.

NT-proBNP secretion by primary trophoblasts from first-trimester and term pregnancy was investigated. In first-trimester trophoblasts, NT-proBNP was detected in the supernatant of only one sample and at a very low concentration (5.12 ng/l). This sample contained trophoblasts from gestational week seven and was cultured for 48 h in 12 % O_2. In term trophoblasts, raw values of NT-proBNP were reported in all the supernatants tested, but the levels were low in several of them. Mean values of the replicates having NT-proBNP levels > 8 ng/l (the clinically used cut-off for this method) are presented in Figure 5.

mRNA levels of *NPPB*, *NPR1* and *NPR2* were measured in primary term trophoblasts. In all studied samples, mRNA of the three genes was found in both study groups (Figure 6).

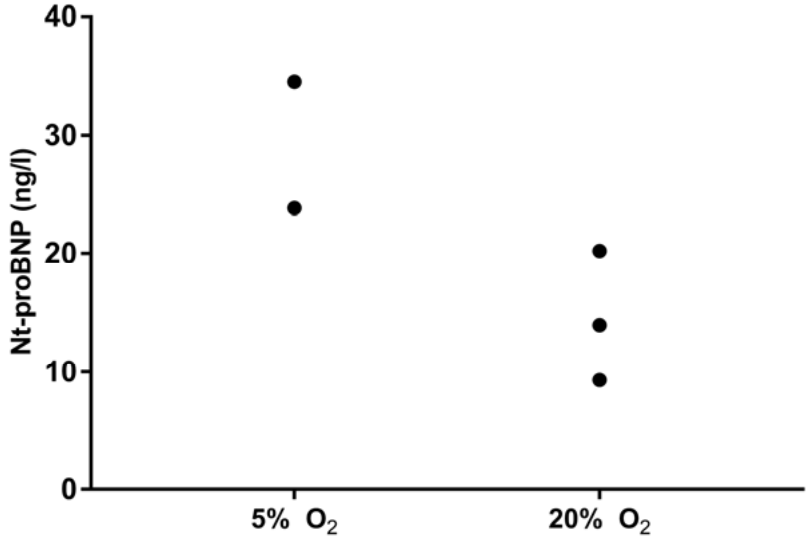

Figure 5. Replicate mean values of NT-proBNP in supernatants from primary term trophoblasts cultured for 48 h in 5% or 20% O_2. Three different experiments, including trophoblasts from four different placentas were carried out after isolations of poor quality had been removed. The cut-off point is normally set to 8 ng/l when reporting results from the chosen analysis method, and therefore only values of > 8ng/l were used when calculating the mean.

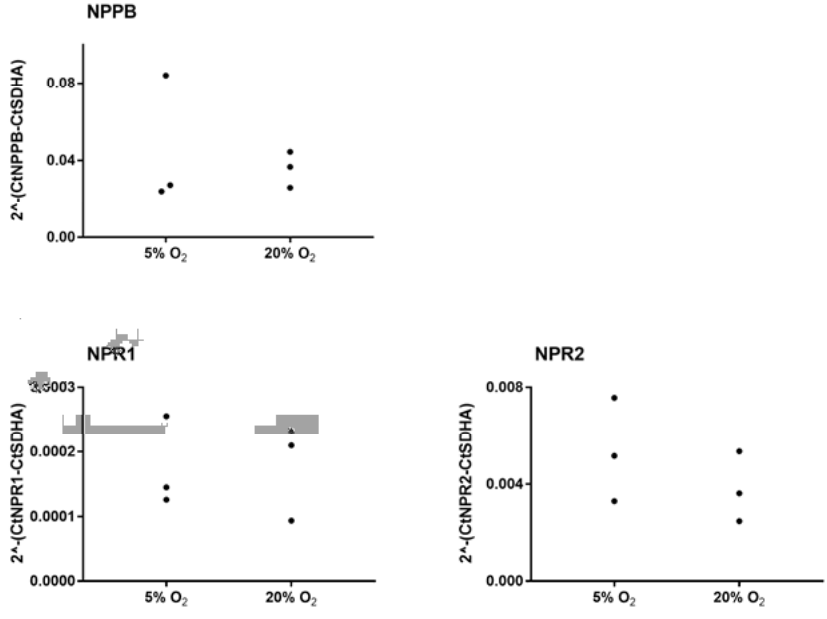

Figure 6. Normalized mRNA levels in primary term trophoblasts cultured for 48 h in 5% or 20% O_2. Trophoblasts from four different placentas remained after isolations of poor quality had been removed. Experiments with pooled cells from two different placentas were carried out in duplicates or triplicates and the mean values of these are presented in the figure.

Discussion

General discussion

Placental transcription in early- and late-onset preeclampsia

Several genes were differentially expressed in early- and late-onset preeclampsia (Table III). Verification of the microarray data by qRT-PCR showed similar results for the four selected genes, implying that the microarray data are reliable (Figure 1).

During data processing, spots of low quality were removed in several steps. Only transcripts that were reported in all 15 arrays after this process were included when the transcription profiles of early- and late-onset preeclampsia were compared. *NPPB*, the gene encoding proBNP, was not present in all arrays and was therefore not included in this final analysis.

Placental genome-wide transcription in early- and late-onset preeclampsia has been studied before[106, 107] and after[108, 109] Paper I was published. Distinct differences between early- and late-onset preeclampsia have been observed in all these studies, including the one in this thesis. These findings support the concept of different pathophysiological pathways in early- and late-onset preeclampsia. There is generally a low degree of overlap in reported transcripts between the studies. This could be due to the multifactorial nature of preeclampsia, or to methodological differences between the studies.

Pathway analysis of the altered transcripts in Paper I indicated involvement of the *Alzheimer's disease pathway*. This pathway has been reported to be involved in preeclampsia before, and, interestingly, it also seems to be involved in CVD.[110] It has also been proposed that preeclampsia and Alzheimer's disease share a conserved pathway involving the preeclampsia-susceptibility gene *STOX1*.[111] In fact, one of the most accepted preeclampsia animal models is developed based on this gene.[112]

Using biological function analysis, differences in the expression of transcripts associated with *angiogenesis* were found. Levels of the angiogenesis-related transcripts *ACVRL1* and *EGFL7* were lower in early-onset preeclampsia than in both late-onset preeclampsia and gestational-age-matched controls. Angiogenesis is well-known to be associated with preeclampsia.[95, 98, 113-115] The novel discovery that *ACVRL1* and *EGFL7* were differently expressed in early-onset preeclampsia thus strengthens earlier findings.

The gene *ACVRL1* encodes ALK1, a co-receptor in the transforming growth factor (TGF)-β signaling pathway. Other factors in this pathway are ACVR2A and endoglin (ENG). *ACVR2A* is a preeclampsia-susceptibility gene, which binds Activin A.[116, 117] Levels of activin A are elevated in the maternal circulation of some women developing preeclampsia.[118, 119] Levels of both ENG and its soluble form sENG (an antagonist to TGF-β) are elevated in preeclampsia,[120] especially in early-onset disease.[121] Soluble ENG seems to be regulated in a similar way as sFlt1 and levels are elevated weeks before the onset of preeclampsia.[114] Since Paper I was published, a genetic variation in the ENG pathway has been investigated and this is associated with preeclampsia. Small nucleotide polymorphisms (SNPs) in *ACVRL1* were not associated with preeclampsia in an American Caucasian cohort,[122] or in a Norwegian cohort.[123] However, in a Latin cohort from southern California, there was an association between the *ACVRL1* SNP rs706819 and preeclampsia.[123]

The protein EGFL7 is important for angiogenesis during embryonic vascular development. It is associated with a wide range of cancers and functions by mediating the Notch, Integrin and EGF signaling pathways.[124] The result in Paper 1 is comparable with that in a genome-wide transcription study of decidua from early-onset preeclampsia and controls, where *EGFL7* was one of five altered transcripts.[125] Since then, EGFL7 has been shown to be expressed by human trophoblast cells and to be down-regulated in preeclamptic placentas.[126]

BNP and NT-proBNP in preeclampsia

In papers II and III, plasma NT-proBNP and placental BNP in women with preeclampsia were studied. In Paper IV, BNP was studied in primary trophoblasts from normotensive pregnancies.

In Paper II higher levels of plasma NT-proBNP were found in women with both early- and late-onset preeclampsia than in their respective gestational-age-matched controls. It is possible that the high levels of NT-proBNP observed in women with preeclampsia correlate with symptoms of cardiac strain, for example, dyspnea or edema. However, no obvious cardiac symptoms were evident from the medical charts. No difference in NT-proBNP was found between the two preeclampsia subtypes but there was a tendency towards a difference that led us to suspect that the study was underpowered to detect this. These findings are in line with that in the available literature.[81-87] This generated the hypothesis that NT-proBNP is a placenta-derived factor, and that the placenta is partly responsible for the high levels of NT-proBNP in the circulation of women with preeclampsia. This hypothesis was put forward in a context where preeclampsia was most often considered a placental disorder. Since then, the heart in relation to preeclampsia has been brought into focus and NT-proBNP is proposed to reflect the strain on the heart observed in preeclampsia.[81, 85, 87, 127]

In Paper III, we hypothesized that plasma levels of NT-proBNP were elevated in the early second-trimester in women developing preeclampsia. However, the NT-proBNP levels were similar in these women and in women with an uncomplicated pregnancy outcome. This finding indicates that BNP is not part of a pathophysiological pathway leading to early-onset preeclampsia, but is elevated as a result of secondary organ involvement.

There are other studies of early pregnancy plasma levels of BNP/NT-proBNP in women who later develop preeclampsia. The finding in Paper III is consistent with that in a study by Uyar et al. Circulating levels of NT-proBNP at gestational weeks 21–24 were measured in women with bilateral notch together with a uterine artery mean pulsatile index above the 90th centile and in women with normal Doppler findings. There was no difference between the groups. Levels of NT-proBNP were also similar in women who later developed preeclampsia and the rest of the cohort.[128] In another study, NT-proBNP was studied throughout pregnancy in women with chronic hypertension. The levels of NT-proBNP were higher at gestational week 16 in women with chronic hypertension than in normotensive women. Among the women in the cohort who later developed superimposed preeclampsia NT-proBNP levels increased every month from 16 weeks of gestation until term. Levels of NT-proBNP in women with chronic hypertension who did not develop preeclampsia increased initially until gestational weeks 20–24, with no further elevation after that. In contrast, the levels of NT-proBNP among normotensive women slowly decreased from gestational week 16 until term.[129]

BNP and the placenta

The hypothesis of BNP being a placenta- or trophoblast-derived factor was tested. In Paper II, mRNA and protein of BNP were detected in placental tissue from both women with preeclampsia and controls. Transcripts of *NPPB*, the gene encoding BNP, were found in 20 out of 25 placental samples. All four study groups were represented in the five placental samples with no *NPPB* mRNA. In Paper IV, there were measurable amounts of NT-proBNP in the supernatants from primary term trophoblasts, indicating release from these cells.

It is not known if BNP has an effect on the placenta. Therefore, mRNA levels of the two natriuretic peptide receptors *NPR1* and *NPR2* in primary term trophoblasts were studied in Paper IV, where low levels of both *NPR1* and *NPR2* mRNA were found. Caution must be taken when interpreting these results, as the protein levels of the receptors were not studied. The findings are consistent with those in two previous studies indicating that biologically that biologically active natriuretic peptide receptors are present in human placental tissue.[90, 130] In a recent study, the effects of ANP and BNP were evaluated in a trophoblast cell line.[131] Carvajal and co-authors found that BNP, but not

ANP, increased wound healing, and there was a tendency towards increased trophoblast migration in BNP-treated cells.[131]

Origins of preeclampsia – a role of BNP?

The findings of normal plasma levels of NT-proBNP in early second-trimester in women who later developed early-onset preeclampsia and the absence of detectable NT-proBNP in supernatants from cultured first trimester trophoblasts, indicate that BNP is not a trophoblast-derived factor contributing to the second-stage of the modified two-stage model of preeclampsia. Various different factors are dysregulated in women presenting with preeclampsia. In agreement with the two-stage model of preeclampsia, it is possible that increased levels of NT-proBNP are a result of cardiac strain secondary to placental dysfunction.

However, increased levels of NT-proBNP precedes the clinical stage of preeclampsia, as evident from a study analyzing NT-proBNP (median gestational week 35) in women who later developed the condition.[127] As mentioned in the introduction, pregnancy is associated with cardiovascular adaptations, and these processes are impaired in women with preeclampsia. In fact, the maladaptation precedes the clinical stage of preeclampsia, and it has been proposed that impaired cardiovascular adaptation in pregnancy actually causes placental dysfunction.[132]

Pregnancy places a high burden on the cardiovascular system and it appears that for some women, the demands of the growing fetus and placenta on the maternal circulation cannot be met. A parallel can be drawn to cases of heart failure, where the heart is unable to pump sufficiently to meet the need of the body. This cardiovascular maladaptation to pregnancy may lead to inadequate placental perfusion, and consequential syncytiotrophoblasts-stress and the clinical stage of preeclampsia.

In heart failure, the cardiac ventricles release BNP as a response to cardiac strain.[78] It is plausible that the same mechanism takes place in women with cardiac maladaptation to pregnancy, as our and others findings of increased levels of NT-proBNP in women with preeclampsia indicate. The effects of BNP include, but are not restricted to, vasodilation and decreased plasma volume. Effects that possibly could contribute to an impaired placental perfusion and thereby to the development of preeclampsia.

How BNP affects the pregnant woman, however, is not fully elucidated. The findings in the current work of BNP mRNA and protein in placenta, trophoblast-derived NT-proBNP, and presence of NPR mRNA in trophoblasts suggest that BNP may also have a direct effect on the placenta. The excess of BNP present in the circulation of women with preeclampsia may therefore cause an imbalance in the hypothetical downstream effects of placental BNP-signaling and thereby contribute to placental dysfunction.

The increased risk of CVD later in life in women who have experienced preeclampsia is well-documented.[6] It has been proposed that sFlt1 induces permanent cardiovascular changes that lead to the future cardiovascular risk in women with previous preeclampsia.[133] However, the association between preeclampsia and post-pregnancy cardiovascular risk appears to be a result of shared pre-pregnancy risk factors rather than preeclampsia itself.[134] Supporting this concept are the facts that cardiovascular dysfunction precedes preeclampsia and symptomatic cardiovascular impairment is sometimes evident long after preeclampsia has resolved.[132] The findings in the current work, i.e. normal levels of NT-proBNP in early second-trimester in women who later developed early-onset preeclampsia and elevated levels of NT-proBNP in the clinical stage of preeclampsia, are also consistent with this concept. Altogether, this point towards a cardiovascular origin of preeclampsia.[67, 132]

Methodological considerations

Study design and study populations

As mentioned, the view of preeclampsia not as a single disease but rather as a group of preeclampsia subtypes with partly different and partly overlapping pathophysiological pathways is becoming increasingly established. To study preeclampsia subtypes, it is important to use well-characterized cases and homogenous groups.[36, 46] In this work, two subtypes – early- and late-onset preeclampsia were studied.

At the time the projects for Papers I and II were initiated, there was no consensus on how to define early-and late-onset preeclampsia. In these papers, early-onset preeclampsia was defined as preeclampsia diagnosed before or at 32 weeks' gestation and late-onset preeclampsia was defined as preeclampsia diagnosed in gestational week 35 or later. By excluding women diagnosed with preeclampsia between these weeks, we opted for more homogeneous study groups. By the time the project for Paper III was planned, the ISSHP had defined early-onset preeclampsia as manifesting before or at 34 weeks' gestation.[12] To contribute to a more unified definition and to enable comparisons between different studies, this definition was used in Paper III.

In the microarray experiments in Paper I, no control groups were included. During normal placental development mRNA levels vary.[105] The altered GO groups in Paper I do not match gene ontology groups previously reported to differ between second-trimester and term placentas.[105] However, the findings obtained when comparing samples from early- and late-onset preeclampsia may be due to the difference in gestational age between these two groups. Therefore, control placentas were included in the qRT-PCR experiments. It is difficult to select early control placentas, as by definition they come from preterm deliveries and therefore cannot be considered normal. In Papers I and II,

the early control placentas were from normotensive preterm deliveries, without signs of infection. Preterm deliveries are associated with poor placentation.[45] Data obtained from early control placentas may therefore not reflect normal gene expression at this gestational age. In addition, these samples were difficult to obtain and the median gestational age in the early control group was about a month shorter than in the early-preeclampsia group (Table 2). These limitations need to be considered when interpreting the results.

In Paper III, women with preeclampsia superimposed on chronic hypertension were included (n = 4). This phenotype may be considered as a particular subtype and by including these samples some heterogeneity was introduced to the study group. Women who later develop preeclampsia superimposed on chronic hypertension are reported to have higher levels of NT-proBNP at gestational week 16 than women whose pregnancies continue to be normotensive.[129] By including these women in the study population, a higher median NT-proBNP level in the preeclampsia group could have been expected. However, no difference was noted between the groups and in a secondary analysis, excluding women with chronic hypertension, the result was similar (data not shown).

Sample size

If the sample size is too small, there is less chance of detecting a true effect but also a higher risk of over-interpreting a significant result. When planning a study, the effect size may not be known in advance, and therefore a power calculation cannot be done. Another obstacle in obtaining an adequate sample size, for example, can be a low incidence of the condition of interest.

The sample sizes in the studies included in this work are generally small. Microarray methodology, especially at the time the experiments were done, is costly, limiting the samples that can be studied. In Paper I, the sample size is comparable to those in similar studies.[108, 135]

The samples in Paper II had already been collected and used in other studies.[95-98] The higher NT-proBNP plasma levels in both early- and late-onset preeclampsia *vs.* respective control groups, and the similar findings by others [81-84] indicate that the sample size was large enough to detect this difference. There was a tendency towards a difference between NT-proBNP levels in early- and late-onset preeclampsia. In line with this, higher levels of NT-proBNP in early- than in late-onset preeclampsia have been reported elsewhere.[85-87] Together, this points towards a power-related false-negative finding in Paper II.

In Paper III, all cases of early-onset preeclampsia in the biobank at the time were included. No power calculation was done a priori as a result of limited earlier data on expected effect size. To increase the sample size, women with preeclampsia superimposed on chronic hypertension were included, rendering a less homogeneous study group. In this study, there was no difference in

plasma NT-proBNP levels in women developing preeclampsia and gestational-age-matched controls. There is a possibility that this finding could be a false negative (type II error) and that a larger sample size may have resulted in a different conclusion. However, there was not even the slightest tendency towards a difference between the two groups (Figure 4). Also, similar results have been reported elsewhere.[128]

In Paper IV several of the samples had to be excluded as a result of experimental difficulties and poor quality. The small final sample size made it impossible to carry out any statistical analyses, and therefore no firm conclusions could be drawn from these experiments. We plan to continue these experiments and include more samples. As in Paper III, this is a novel study and a power calculation has not been done.

Experimental methods and considerations

Sampling

The placenta is a heterogeneous organ made up by different cell types including, trophoblasts, mesenchymal cells, fibroblasts, macrophages (Hofbauer cells), vascular smooth muscle cells, and endothelial cells.[38] The inter-individual differences that exist between different placentas are not taken into account when analyzing whole tissue, as in Paper I. Gene expression can also vary in different parts of the placenta.[136, 137] The early and late placenta samples in Papers I and II were collected at two different clinics, with slightly different protocols for sample collection. This difference in handling may have affected gene expression. However, care was always taken to collect samples from a central part of the placenta and to avoid macroscopic areas containing infarctions and calcifications.

There are various techniques to isolate specific cells before analysis. These techniques can be based on physiological properties of the cells of interest such as size or density. Other techniques are laser microdissection, fluorescence activated cell sorting, and magnetic activated cell sorting.[138] These kinds of techniques have been used in transcriptional microarray experiments to study cultured trophoblasts from women with preeclampsia,[139] and to study trophoblast subpopulations in severe preeclampsia.[140] In Paper IV a combination of filtration based on size and magnetic sorting was used to isolate trophoblasts, which increased the probability that we had the cells of interest and that the cell suspension was free of other types of cell.

Advantages and limitations of gene transcription studies

Large-scale gene transcription profiling became possible with the introduction of microarray technology in the 1990s. In a single experiment, expression of the whole genome could be analyzed. Since then, the field of genome-wide

transcriptional studies has developed rapidly and sequencing-based technologies, such as RNA Seq, are gradually taking over. Sequencing-based methods have several advantages over microarray-based methods, improved reliability in measuring absolute levels, a greater dynamic range, and the capacity to measure novel transcripts being some.[141, 142]

There are several factors apart from the condition studied, gestational age, the tissue, and the method of sampling (as discussed above), that may affect placental gene transcription. Diet, parity, stress, medication, and mode of delivery are some.[143]

Bias can also be introduced by the microarray method itself. To some extent this can be compensated for in the analysis. One example of this is the Lowess normalization used in Paper I, which compensates for variation in labeling.

In Paper I, genes of interest were verified by qRT-PCR. In the early control group, mRNA was extracted by using a different method than in the three other groups. This may have affected the result. Moreover, all the verified genes were downregulated in early-onset preeclampsia and this may also have introduced a bias.

All women with early-onset preeclampsia in Paper I delivered by cesarean section. This differed from both the women with late-onset preeclampsia and the early control group (Table 2). Most studies report that mode of delivery affects placental transcripts.[137, 144-146] In contrast, Sitras et al. concluded that gene transcription in near-term placentas was not altered by labor. However, a relatively strict level of significance (> 2.5-fold change and a P-value of < 0.01) was used, which may explain their result.[147] It is possible that that mode of delivery may have had an impact on our results and further studies are needed to truly elucidate this.

Microarray studies have been conducted to identify placental genes involved in the pathophysiology of preeclampsia and to generate new hypotheses of its etiology. These studies display a multiple picture of transcription profiles, and have highlighted the heterogeneous pathophysiology of preeclampsia.[110] Genes associated with angiogenesis and the immune response are differently expressed in placentas from pregnancies complicated by preeclampsia, compared with placentas from normal pregnancies.[148] RNA sequencing has been used to characterize placentas from cases of early- and late-onset preeclampsia,[109] and decidua from women with severe early-onset and severe late-onset preeclampsia.[149] In most microarray studies, preeclampsia subtypes have not been studied. Differences in placental gene expression between early- and late-onset preeclampsia are, however, evident in two studies.[106, 107] The genome-wide transcriptome of maternal whole blood from early- and late-onset preeclampsia has also been studied.[150]

Placental *in vitro* models

In vitro models are used to study mechanisms, test the release of biomarkers, and evaluate the effect of possible therapeutic treatments.

Trophoblast cell cultures can be carried out in cell lines such as the widely used BeWo cell line originating from choriocarcinoma, or in primary trophoblasts isolated from placentas. The most important advantage of a primary cell culture over a cell line is that it reflects the *in vivo* situation to a greater extent. Trophoblast cell lines, however, are easier to work with, can be maintained for a longer time, and the method is less costly than using primary trophoblasts.

When isolating a single cell type the effect on that specific type can be studied. However, cells *in vivo* interact with nearby cells of different sorts, and it is not possible to study this interaction by using an isolated cell type. In explants, small pieces of villous placenta tissue are cultured intact, enabling the study of cell-to-cell interactions between different cell types.

Summary

The different transcription profiles of the two preeclampsia subtypes strengthen the concept that early- and late-onset preeclampsia have partly different pathophysiological pathways.

We found no evidence that the high levels of BNP/NT-proBNP in preeclampsia are placenta-derived. Recent literature rather suggests that the high levels in preeclampsia are secondary to cardiac strain. However, some evidence of BNP being synthesized and released by the placenta was found. The data also implies that BNP may have a receptor-mediated effect on the placenta. If and how the elevated BNP levels in preeclampsia affect the placenta and the development of preeclampsia are matters for future studies.

Conclusions

I Altered mRNA levels were noted for 196 genes when investigating placentas from early- *vs*. late-onset preeclampsia. mRNA levels of the angiogenesis-related genes *EGFL7* and *ACVRL1* were confirmed to be lower in early- onset preeclampsia than in both early controls and late-onset preeclampsia. Differences in the transcription profiles between early- and late-onset preeclampsia support the concept that they are at least partly two different entities.

II Plasma levels of NT-proBNP were higher in both early- and late-onset preeclampsia than in gestational-age-matched controls. There was evidence of both BNP mRNA and protein in placental tissue suggesting possible placental synthesis and release of BNP.

III Early second-trimester plasma levels of NT-proBNP were similar in women who later developed early-onset preeclampsia and women with uncomplicated pregnancy outcomes. Early second-trimester levels of NT-proBNP cannot be used as a predictive biomarker of early-onset preeclampsia.

IV NT-proBNP could be measured in the supernatant of cultured term but not first-trimester primary trophoblasts, indicating synthesis and release by term trophoblasts.

Future perspectives

A better understanding of the pathophysiology of preeclampsia is important for the development of new strategies to predict, prevent and possibly treat the condition. Such strategies would not only reduce the suffering from maternal and fetal morbidity and mortality but also be economically beneficial for society. The complexity of preeclampsia pathophysiology, where the maternal and fetal genome is interacting, places high demands on researchers. Lately, the importance of studying well-defined subtypes of preeclampsia, which might have different pathophysiological pathways, has been highlighted.[8, 9, 12, 20, 36] If there are several pathways to preeclampsia, it follows that no single test will predict all types of the disorder and no single treatment will prevent all types of preeclampsia, as is the case for low-dose aspirin treatment for women with a high risk of developing preterm preeclampsia.[31, 32] A better classification of the different preeclampsia subtypes forms a basis for future research on prevention, prediction, diagnosis and treatment of the disorder.

To be able to divide preeclampsia cases into different subtypes, large study populations are needed. The Uppsala Biobank for Pregnant Women now contains samples from about 400 women who developed preeclampsia later in their pregnancies. The samples were collected in the early second-trimester. Using a proteomic approach, these samples will be analyzed using a panel containing 92 proteins. The main aim is to identify biomarker candidates for prediction of different preeclampsia subtypes. Such biomarkers could strengthen the prediction model that already exists for preterm preeclampsia or be of use to predict other subtypes. Potential biomarkers can also be of use to define possible subtypes of preeclampsia based on the pathological mechanisms involved. Perhaps preeclampsia subtypes can be defined on the basis of the involvement of inflammatory, antiangiogenic, oxidative stress, or cardiovascular biomarkers.

In Paper IV, we found that NT-proBNP was released into the culture medium of primary term trophoblasts that had been cultured for 48 h in either 5% or 20% oxygen. We will continue the experiments with more samples from uncomplicated pregnancies and also include women with preeclampsia. Moreover, our results indicate a role of BNP in the physiology of pregnancy. Therefore, it would be interesting to study the effect of BNP on cultured primary trophoblast cells.

Sammanfattning på svenska

Havandeskapsförgiftning (preeklampsi) är en potentiellt allvarlig sjukdom som drabbar cirka 3 – 5% av alla gravida kvinnor. I svåra fall kan sjukdomen leda till att den blivande modern får exempelvis njursvikt, leversvikt, lungödem eller eklampsi (svåra kramper). Även fostret kan påverkas och barn till mammor med havandeskapsförgiftning är till exempel oftare tillväxthämmade jämfört med barn till friska mammor. Variationen är dock stor och många gånger har den gravida kvinnan inte några, eller mycket milda, symtom. I dessa fall upptäcks sjukdomen oftast vid ett rutinbesök på mödravården. Diagnosen ställs om den gravida kvinnan har nytillkommet högt blodtryck tillsammans med protein i urinen efter graviditetsvecka 20. Ett milt sjukdomstillstånd kan mycket snabbt utveckla sig till ett svårt fall med allvarliga komplikationer och därför är det av stor vikt att kvinnor med havandeskapsförgiftning följs av sjukvården. Det enda sättet att stoppa progressen av ett svårt fall är att förlösa kvinnan. Detta leder till att ett stort antal barn blir för tidigt födda till följd av sjukdomen. I länder med bristande tillgång till sjukvård, där inte sjukdomen upptäcks i tid, leder havandeskapsförgiftning ofta till att både mamman och barnet dör. I ett globalt perspektiv är havandeskapsförgiftning den näst vanligast orsaken till mödradödlighet.

Vad som orsakar havandeskapsförgiftning är inte helt känt, men moderkakan verkar vara central i sjukdomsförloppet. På grund av den stora variationen i symtom och även i objektiva fynd hos kvinnor med havandeskapsförgiftning tror man att det kan finnas flera olika mekanismer som leder till sjukdomen. För att studera dessa mekanismer närmare behöver man dela in sjukdomen i olika varianter. Ett sätt är att skilja mellan tidig (debut före graviditetsvecka 34) och sen (debut i graviditetsvecka 34 eller senare) havandeskapsförgiftning som verkar ha delvis olika underliggande sjukdomsmekanismer. Tidig havandeskapsförgiftning har ofta ett svårare förlopp och är oftare förknippat med att barnet är tillväxthämmat.

Framförallt tidig havandeskapsförgiftning är förknippat med en avvikelse i den viktiga process där moderkakan fästs till livmoderväggen och dess blodkärl. Detta verkar leda till ett försämrat blodflöde i moderkakan som i sin tur leder till oxidativ stress. Detta kan leda till att olika proteiner och partiklar frisätts från moderkakan till moderns blodomlopp, vilket i sin tur påverkar moderns organ och leder till havandeskapsförgiftning. För att förstå sjukdomsmekanismerna bättre är det viktigt att identifiera vilka faktorer som fri-

sätts från moderkakan och ger upphov till sjukdomen. I sen havandeskapsförgiftning är det inte lika vanligt med en försämrad infästning av moderkakan. Det verkar snarare som att det är andra faktorer som leder till moderkakans försämrade funktion i dessa fall.

Vid överbelastning av hjärtat frisätts hormonet natriuretisk peptid B (BNP) från hjärtats kammare vilket leder till ökad urinproduktion, ökad utsöndring av natrium via urinen samt till minskad spänning i blodkärlens väggar. Förenklat kan man säga att detta tillsammans leder till ett lägre blodtryck. BNP bildas genom att prohormonet proBNP klyvs till de två peptiderna NT-proBNP och BNP. NT-proBNP har inga kända funktioner i kroppen men det är mer stabilt och därför lättare att mäta, till exempel för diagnos av hjärtsvikt. Kvinnor med havandeskapsförgiftning har högre nivåer av NT-proBNP i blodet jämfört med friska gravida kvinnor. Vår hypotes var att BNP/NT-proBNP kan frisättas från moderkakan och att de höga nivåerna i blodet hos kvinnor med havandeskapsförgiftning delvis kommer från moderkakan.

Det övergripande syftet med det här arbetet var att öka kunskapen om de underliggande sjukdomsmekanismer som leder till havandeskapsförgiftnings genom att studera de två varianterna tidig och sen havandeskapsförgiftning, samt peptiderna BNP och NT-proBNP. En ökad kunskap om de sjukdomsmekanismerna som leder till havandeskapsförgiftning är en grundförutsättning för att utveckla metoder för att förutsäga, diagnostisera och behandla sjukdomen.

I delarbete I jämfördes genuttrycket i moderkakor från kvinnor med tidig och sen havandeskapsförgiftning. Nivåerna av mRNA för 196 gener skiljde sig åt mellan moderkakor från kvinnor med tidig och sen havandeskapsförgiftning. mRNA nivåerna för två gener som är förknippade med kärlnybildning, *EGFL7* och *ACVRL1*, bekräftades vara lägre i tidig havandeskapsförgiftning än i både tidiga kontroller och sen havandeskapsförgiftning. Skillnaderna i genuttryck mellan tidig och sen havandeskapsförgiftning stärker hypotesen att de har delvis olika sjukdomsmekanismer.

I delarbete II jämfördes koncentrationen av NT-proBNP i blodprover från kvinnor med tidig och sen havandeskapsförgiftning med koncentrationerna hos friska gravida kvinnor vid motsvarande graviditetslängd. Vi undersökte även om det fanns BNP mRNA och protein i moderkakor från kvinnor med tidig och sen havandeskapsförgiftning samt friska kontroller. Koncentrationen av NT-proBNP i tidig och sen havandeskapsförgiftning var högre än i respektive kontrollgrupp med friska kvinnor. BNP protein och mRNA fanns i moderkakan från både kvinnor med havandeskapsförgiftning och friska kontroller vilket skulle kunna betyda att moderkakan bildar och frisätter BNP.

Eftersom att nivåerna av NT-proBNP är förhöjda hos kvinnor med havandeskapsförgiftning vid diagnos, ville vi undersöka om nivåerna var förhöjda redan tidigare under graviditeten. I delarbete III, fann vi att koncentrationen av NT-proBNP i blodprover tagna i graviditetsvecka 18 var lika hög hos kvin-

nor som senare under sin graviditet diagnostiserades med tidig havandeskapsförgiftning jämfört med kvinnor vars graviditeter fortsatte utan komplikationer. Koncentrationen av NT-proBNP i blodprover tagna i graviditetsvecka 18 kan därför inte användas för att förutsäga vilka kvinnor som kommer drabbas av tidig havandeskapsförgiftning.

För att vidare undersöka om moderkakan bildar och frisätter BNP studerades celler från moderkakan (trofoblaster). I delarbete IV, isolerades och odlades trofoblaster från tidig graviditet och från fullgången graviditet. Nivåerna av NT-proBNP mättes sedan i vätskan som cellerna odlats i. Vi fann att trofoblaster från fullgången, men inte från tidig, graviditet verkar frisätta BNP. Vilken biologisk funktion detta har är inte känt.

Sammanfattningsvis stödjer resultaten från detta arbete idéen om att tidig och sen havandeskapsförgiftning har delvis olika underliggande orsaker. Det verkar som att moderkakan bildar små mängder av NT-proBNP men vi fann inget som stödjer hypotesen att de mycket höga koncentrationerna av NT-proBNP som återfinns hos kvinnor med havandeskapsförgiftning kommer därifrån. Vilken betydelse BNP har i moderkakan är en fråga för framtida studier.

Acknowledgements

This work was carried out at the department of Women's and Children's health, Uppsala University. I would like to express my sincere gratitude to all the people who made it possible.

First and foremost, all participating women, without you, none of this could have been done.

Matts Olovsson, my main supervisor. Thank you for allowing me such freedom in conducting this work and for guiding me ever so patiently. I always feel uplifted after our meetings.

Anna-Karin Wikström, co-supervisor, encouraging, enthusiastic and supportive. Thank you for always taking your time. I'm looking forward to future collaborations.

Stefan Hansson, co-supervisor, creative and challenging. Thank you for introducing me to the world of research in general and to the intriguing world of preeclampsia and the placenta in particular.

Agneta Skoog Svanberg, Jan Gustafsson, and Ove Axelsson present and former heads of the Department, thank you for the possibility to conduct my thesis at the department.

Magnus Centlow, co-writer. Thank you for sharing all your knowledge, and for all the fun times. I will never forget digging the really loud classical music in the car that early morning on the ferry from Germany.

The placenta-lab in Graz. Gernot Desoye for inviting me. Alejandro Majali-Martinez, co-writer, for sharing your samples with me. Ate, for teaching me how to grow those trophies. And to everybody else at the lab, what a hit it was to spend time there at the Fasching.

Theodora Kunovac Kallak. Co-writer. You just can't stop helping other people. Not even when in active labor! Thanks for your dedication. But most of all, thank you for being my friend.

Christina Asplund, Erik Salaneck and Niclas Abrahamsson, for the opportunity to do “Forskar-AT”, and for being so unbelievable flexible, allowing me to change my “blockplan” numerous times and giving me time off to finish this work, it is much appreciated.

All my present and former fellow PhD-students, for creating such a pleasant work-environment. Elin and Malin H, for making me feel at home from the start. My latest roomies for the invaluable support those last months: Charlotte, for sharply putting things in perspective, I’m so glad we’re related now. Åsa, for being so quick-witted, it is always fun to go to work when you are there. And to Karin, Helena, Hanna H, Emma B, Cecilia, and all others, for entertaining and interesting discussions.

Everyone else at the department. Anneli, Alkistis, and Inger, for being model scientists and for organizing trips, parties and whatnot. Lena and Eva for all the fun times and for practical and emotional support. Susanne, for sharing my fascination of the placenta. I’m so glad you’ve joined the lab! Martin, Malin G, Hans, and Hanna T for much appreciated administrative help.

Everybody at the clinic, I miss working with you!

My colleagues from my time in Lund. Iréne, Karen, Tina, and Zuzana, for pleasant times in and outside the lab.

My friends who became researchers. Lina, Mia, Ebba, and Martin. You all are an inspiration to me!

My other friends, for travels, dinners, coffee, wine, talks, laughs and tears. You are the source of true knowledge.

“Världens bästa fantastiska supermammagrupp (för att uttrycka sig ödmjukt)”, for being funny, smart and sincere, you kept me sane this winter.

My mom, for awaking my interest in science to begin with.

My dad, for unconditional love.

Boel, for always being the one most interested in what I’m up to.

Mira, min allra käraste syster, for always inspiring me to be my best self. And thanks to you and Gustaf for all the enjoyable moments, and for sharing your lives with me.

Staffan, my loving, encouraging, patient, funny, and intelligent best friend and partner in crime. Thank you for taking an interest in my work, knowing way too much about trophoblast invasion. And Irma. You two bring balance, meaning, and joy to my life!

Finally, an extra special thanks to my own “thesis rescue-team”, Theo, Charlotte, and “mammas Martin” who helped me with the final “spik i kistan”.

This work was supported by the General Maternity Hospital Foundation, the Uppsala-Örebro Regional Research Council, the Swedish Research Council, the Erik, Karin and Gösta Selanders Foundation, the Swedish Society for Medical Research, and Födelsefonden.

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Editor: The Dean of the Faculty of Medicine

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