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Genetic Predisposition to Preeclampsia

Genetic Association Studies on Population-Based Cohorts and Transcriptional Studies on Decidua Basalis Tissue

Thesis for the degree of Philosophiae Doctor

Trondheim, February 2014

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Genetisk disposisjon for utvikling av svangerskapsforgiftning

Genetiske assosiasjonsstudier i populasjonsbaserte kohorter og genekspresjonsstudier i decidua basalis vev

Svangerskapsforgiftning (preeklampsi) kjennetegnes av høyt blodtrykk og protein i urinen etter 20. svangerskapsuke. Denne tilstanden oppstår i 2-8% av alle svangerskap, og er en av de viktigste årsakene til sykdom og død hos mor og foster. Til tross for intensiv forskning er årsakssammenhengene for utvikling av svangerskapsforgiftning fortsatt uklare. Vi vet at redusert blodgjennomstrømming i morkaken har en sentral rolle. Prosesser i decidua, som er den maternelle delen av morkaken og utgjør møtesonen mellom mor og foster, kan være avgjørende. Andre forhold virker også inn, som forhøyet kronisk inflammasjon hos mor. Forhøyet kronisk inflammasjon er også forbundet med overvekt, diabetes og forhøyet blodtrykk. Svangerskapsforgiftning kan ha konsekvenser på lengre sikt, blant annet er det en klar sammenheng mellom sykdommen og utvikling av hjerte-karsykdom senere i livet.

Det er økt forekomst av svangerskapsforgiftning i enkelte familier. Epidemiologiske studier har vist at genetiske faktorer utgjør omtrent 50% av risikoen for sykdomsutvikling. Formålet med denne studien var å kartlegge genetisk disposisjon for utvikling av svangerskapsforgiftning.

I studien ble det benyttet decidualt morkakevev fra kvinner med svangerskapsforgiftning, og/eller føtal veksthemming og kvinner med normale svangerskap samlet inn ved St. Olavs hospital og Haukeland universitetssykehus for å se på forskjell i genuttrykk mellom disse gruppene. RNA ble isolert, og en helgenomstranskripsjonsstudie med mer enn 48 000 transkripter fra alle kjente gener ble gjennomført. Deretter ble det foretatt nettverksanalyser for å se om de ulikt uttrykte genene påvirket hverandre. Videre har vi brukt data fra Helseundersøkelsen i Nord-Trøndelag (HUNT 2), Medisinsk fødselsregister og The Western Australian Pregnancy Cohort (Raine) Study til å sammenlikne gen-varianter mellom kvinner med svangerskapsforgiftning og normale svangerskap. Vi har også undersøkt om spesifikke gen-varianter på kromosom 2 kan disponere både for svangerskapsforgiftning og risikofaktorer for hjerte-karsykdom.

Analysene av decidualt morkakevev viste at 455 transkripter var ulikt uttrykt hos kvinner med svangerskapsforgiftning sammenliknet med kvinner med normale svangerskap. Disse transkriptene omfattet både nye kandidatgener, for eksempel *ARL5B* og *SLITRK4*, og gener som tidligere var assosiert med svangerskapsforgiftning, for eksempel *PLA2G7* og *HMOX1*. Sju signifikante reaksjonsveier som var assosiert med svangerskapsforgiftning ble identifisert. Disse var blant annet relatert til immunologiske mekanismer, utviklingen av blodkar, oksidativt stress, stress i endoplasmatisk retikulum (ER) og forstyrrelser i fettsyre-metabolismen. I videre analyser fant vi at stress i ER var særlig aktivert ved svangerskapsforgiftning med føtal vekstemming. Denne gruppen viste også ulikt uttrykk av *STOX2*, som kan være en medvirkende årsak til redusert blodgjennomstrømning i morkaken. Vi fant ut at fire gen-varianter på kromosom 2 som tidligere er assosiert med svangerskapsforgiftning også kan være assosiert med risiko-faktorer for hjerte-karsykdom.

Denne studien har bidratt til å underbygge og identifisere noen av de sentrale mekanismene som er knyttet til utviklingen av svangerskapsforgiftning, og vi forstår litt mer av sammenhengen til hjerte-karsykdom. Målet på lang sikt vil være å identifisere kvinner med økt risiko for svangerskapsforgiftning og hjerte-karsykdom, samt å kunne utvikle nye diagnostiske tester og behandlingsmåter.

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CONTENTS

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Mari Løset

ABBREVIATIONS

ACE	angiotensin I converting enzyme
ACOX2	acyl-coenzyme A oxidase 2
AGT	angiotensin
ANGPTL2	angiopoietin-like 2
ARE	antioxidant response element
ARL5B	ADP-ribosylation factor-like 5B
aRNA	antisense ribonucleic acid
ASA	American Society of Anesthesiologists
ATF6	activating transcription factor 6
BMI	body mass index
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CK7	cytokeratin 7
CNV	copy number variant
CS	caesarian section
CT	comparative threshold cycle
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CVD	cardiovascular disease
CYP2J2	CYP, family 2, subfamily J, polypeptide 2
DNA	deoxyribonucleic acid
dNK	decidual natural killer
DTX	deltex homolog 3
EDTA	ethylenediaminetetraacetic acid
EIF2α	eukaryotic translation initiation factor 2-alpha
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3
Eng	endoglin
ER	endoplasmic reticulum
ERAP2	endoplasmic reticulum aminopeptidase 2
EVT	extravillous trophoblast
F2	coagulation factor II
FV	coagulation factor V
FDR	false discovery rate
FGR	fetal growth restriction
FHL1	four and a half LIM domains 1
Flt-1	fms-related tyrosin kinase 1
FXR	farnesoid X receptor
FZD4	frizzled family receptor 4
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCA	grancalcin
GPR116	G protein-coupled receptor 116, transcript variant 1
GST3	glutathione s-transferase
GWAS	genome-wide association study
HELLP	hemolysis, elevated liver enzymes and low platelets
HES1	enhancer of split 1

HLA	human leukocyte antigen
HMOX1	heme oxygenase 1
HPSE	heparanase
HUNT2	the second Nord-Trøndelag Health Study
IDO	indoleamine 2,3-dioxygenase
IL6ST	interleukin 6 signal transducer, transcript variant 1
IL-8	interleukin-8
INHBB	inhibin beta-B
IPA	Ingenuity Pathway Analysis
IRE-1	inositol-requiring enzyme 1
ISSHP	International Society for the Study of Hypertension in Pregnancy
JAG1	jagged 1
KIR	killer immunoglobulin-like receptor
KREMEN1	kringle containing transmembrane protein 1, transcript variant 4
KYNU	kynureninase
LCT	lactase
LD	linkage disequilibrium
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LPL	lipoprotein lipase
LRP1B	low density lipoprotein receptor-related protein 1B
MAF	minor allele frequency
MAN1A2	mannosidase α, class 1A, member 2
MBRN	Medical Birth Registry of Norway
MIAME	Minimum Information About a Microarray Experiment
miRNA	micro ribonucleic acid
MR	magnetic resonance
mRNA	messenger ribonucleic acid
ND	nano drop
NGF	nerve growth factor
NO	nitrogen oxide
NOTCH3	notch homolog 3
NOTCH4	notch homolog 4
NRARP	notch-regulated ankyrin repeat protein
NRF	nuclear factor
NRF2	nuclear factor E2-related factor 2
OR	odds ratio
PCR	polymerase chain reaction
pEIF2α	phosphorylated eukaryotic translation initiation factor 2α
PERK	protein kinase ribonucleic acid-like endoplasmic reticulum kinase
PLA2G7	phospholipase A2, group VII
PlGF	placental growth factor
PPV	positive predictive value
qRT-PCR	quantitative real-time polymerase chain reaction
REN	renin
RIN	RNA integrity number
RNA	ribonucleic acid

RND3	rho family GTPase 3
ROAST	rotation gene set tests
ROBO4	roundabout homolog 4, magic roundabout
ROMER	rotation gene set enrichment analysis
ROS	reactive oxygen species
SD	standard deviation
sEng	soluble endoglin
SEPS1	selenoprotein S
SERPINE1	serpin peptidase inhibitor
sFlt-1	soluble fms-related tyrosine kinase 1
SGA	small for gestational age
SHANK3	SH3 and multiple ankyrin repeat domains 3, transcript variant 4
SLITRK4	SLIT and NTRK-like family, member 4
SNP	single nucleotide polymorphism
SOLAR	Sequential Oligogenic Linkage Analysis Routines
SRPRB	signal recognition particle receptor, B subunit
STOX1	storkhead box 1
STOX2	storkhead box 2
TBP	TATA box binding protein
Th	T helper
THBS2	thrombospondin 2
TMEM97	transmembrane protein 97
TNFSF13B	tumour necrosis factor superfamily, member 13b
Treg	regulatory T
UBASH3B	ubiquitin associated and SH3 domain containing B
UBE2K	ubiquitin-conjugating enzyme E2K
UPR	unfolded protein response
UTR	untranslated region
VEGF	vascular endothelial growth factor
VEGFA	vascular endothelial growth factor A
VEGFC	vascular endothelial growth factor C
XBP1	x-box binding protein 1
XBP1(S)	x-box binding protein 1 spliced
XBP1(U)	x-box binding protein 1 unspliced

LIST OF PAPERS

I Løset M*, Mundal SB*, Johnson MP, Fenstad MH, Freed KA, Lian IA, Eide IP, Bjørge L, Blangero J, Moses EK, Austgulen R. **A transcriptional profile of the decidua in preeclampsia.** American Journal of Obstetrics and Gynecology 2011;204(1):84 e1-27.
*Both authors contributed equally.

II Fenstad MH, Johnson MP, Løset M, Mundal SB, Roten LT, Eide IP, Bjørge L, Sande RK, Johansson ÅK, Dyer TD, Forsmo S, Blangero J, Moses EK, Austgulen R. ***STOX2*** **but not** ***STOX1*** **is differentially expressed in decidua from pre-eclamptic women: data from the Second Nord-Trøndelag Health Study.** Molecular Human Reproduction 2010;16(12):960-8.

III Lian IA, Løset M, Mundal SB, Fenstad MH, Johnson MP, Eide IP, Bjørge L, Freed KA, Moses EK, Austgulen R. **Increased endoplasmic reticulum stress in decidual tissue from pregnancies complicated by fetal growth restriction with and without pre-eclampsia.** Placenta 2011;32(11):823-9.

IV Løset M, Johnson MP, Melton PE, Ang W, Huang RC, Mori TA, Beilin LJ, Pennell C, Roten LT, Iversen AC, Austgulen R, East CE, Blangero J, Brennecke SP, Moses EK. **Preeclampsia and cardiovascular disease share genetic risk factors on chromosome 2q22.** Submitted to Pregnancy Hypertension, November 2013.

1. INTRODUCTION

1.1 Prevalence, definition and diagnosis

Preeclampsia

Preeclampsia affects 2-8% of all pregnancies and is a substantial burden on maternal and fetal health worldwide [1-3]. The rate of preeclampsia differs among nations, and women in developing countries and some ethnic groups (e.g., African-American and Filipino women) are at increased risk [2]. The rate of preeclampsia in the USA has been increased slightly during the past 30 years [4, 5]. This increase might, at least partially, be related to an increased prevalence of disorders with a predisposure towards preeclampsia, such as chronic hypertension, diabetes mellitus and maternal obesity [5, 6]. In Norway, the prevalence of preeclampsia increased from 2.1% in 1967 to 4.4% in 1999. From 2002 onwards, there has been a consistent decline, with an overall prevalence of 3.0% [7], and a similar trend has been reported in other countries, such as Sweden, Australia and Canada [8]. These reduced rates of preeclampsia have been observed despite the steadily increasing rates of the above mentioned predisposing disorders [7]. It has been speculated that elective delivery prior to the due date for women with high risk of preeclampsia and the use of interventions that reduce the risk of progression to preeclampsia are responsible for this trend [8].

The principal diagnostic clinical criteria for preeclampsia are new-onset hypertension and proteinuria after 20 weeks gestation [9]. The diagnostic criteria for preeclampsia in Norway are given by the Norwegian Association for Obstetrics and Gynecology [10] and primarily follow the recommendations of the (US) National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy [11]. According to these criteria, preeclampsia is defined as a pregnancy-specific syndrome that occurs after mid-gestation characterised by 1) *de novo* appearance of hypertension of ≥140/90 mmHg combined with 2) new-onset proteinuria of ≥0.3 g/L in a 24-hour urine sample. In addition, the Norwegian Association for Obstetrics and Gynecology requires two separate measurements of hypertension and proteinuria [10]. To provide accurate diagnosis, specific guidelines for blood pressure measurements and proteinuria are given. Two blood pressure measurements should be obtained at an interval of at

least four to six hours but not more than one week apart, and proteinuria should be measured on at least two occasions at an interval of four to six hours [12]. The gold standard for measuring proteinuria is a 24-hour urine sample, but when this is not feasible, a time measure corrected for creatinine excretion is recommended [13]. The dipstick test for urinary protein is simple and easy to perform. Excretion of ≥0.3 g protein/L in 24 hours usually corresponds to ≥30 mg/dl (≥1+) on a dipstick in a random urinary sample when there is no indication of urinary tract infection. On a clinical daily basis, this method is usually preferred for diagnostic purposes.

The clinical manifestations of preeclampsia are heterogeneous, and the symptoms range from mild to severe. Mild cases, with onset near term and only a small increase in the risk of an adverse pregnancy outcome, are clearly most common [12]. The differentiation of mild and severe preeclampsia remains a topic of discussion [14]. Severe preeclampsia is not well defined with specific characteristics but includes assessment of both maternal (severe hypertension, end organ manifestation and preterm disease) and fetal (intrauterine growth restriction, fetal movement assessment and oligohydramnios) phenotypes [11]. One severe form of preeclampsia is characterised by microangiopathic haemolytic anaemia and is termed HELLP (Haemolysis, Elevated Liver enzymes and Low Platelets) syndrome. Early-onset (<34 weeks of gestation) preeclampsia is generally considered a severe form of preeclampsia, but this opinion is also controversial [14]. Early-onset preeclampsia occurs in approximately 10% of preeclamptic cases [8] and is often associated with more severe outcomes for the mother and child. It has been speculated that early-onset and late-onset preeclampsia are different phenotypes with different aetiologies [15]. Preeclampsia occurs mainly in first pregnancies (approximately 70% of preeclamptic cases), and the majority do not experience preeclampsia in later gestations [16, 17]. Recurrent preeclampsia tends to have an early onset and to show a more severe phenotype compared to non-recurrent preeclampsia [16, 18]. This includes a higher risk of preterm labour, increased rates of fetal growth restriction (FGR) and higher risk of cardiovascular disease (CVD) in later life.

Fetal growth restriction

Inadequate fetal growth is a major concern in obstetric medicine, as fetal growth disorders are important causes of perinatal morbidity and mortality [19, 20]. In most pregnancies with inadequate fetal growth, the fetuses are physiologically normal and simply small for gestational age (SGA). This is in contrast to FGR, which is characterised by pathological small fetal size and failure to reach the genetically determined growth potential [21]. FGR affects 3-10% of all pregnancies [22] and is a substantial burden on perinatal morbidity and mortality [19, 20]. There is no universally accepted FGR definition, but international agreement has estimated that a fetal weight below the 10^{th} percentile of expected birth weight adjusted for gestational age should alert clinicians to potential small fetal size. By reducing the cut-off to less than the 5^{th} percentile or even lower, the specificity for true growth restricted infants might be increased, and help identify infants at increased risk of adverse outcome [21]. Clinical assessments during the pregnancy such as serial measurements of fundal height [23], serial ultrasound biometry [24], and Doppler ultrasound examination of umbilical arteries [25] are useful for diagnosis. However, as these methods are time consuming, measures of low birth weight (below a given percentile) as indicators of impaired fetal growth are often used for research purposes.

1.2 Maternal and fetal outcome of preeclampsia

In high-income countries, hypertensive disorders of pregnancy are responsible for 16% of maternal deaths [2]. Maternal mortality is even higher in low-income countries [2]. Furthermore, severe preeclampsia is a major cause of severe maternal morbidity (e.g., renal failure, hepatic infarction and rupture) and adverse perinatal outcomes such as poor growth and preterm delivery [3]. As preeclamptic symptoms resolve following delivery of the fetus and placenta, caesarean section (CS) performed due to a life-threatening condition for the mother makes the syndrome a major contributor to premature deliveries. In Norway, 15% of premature deliveries may be ascribed to preeclampsia [26].

Appropriate prenatal care is considered the most important factor in the management of preeclampsia [27] and reduces maternal and fetal mortality [28, 29]. In Norway,

antenatal care facilities and patient education are provided to pregnant women prenatally to ensure early detection of the clinical signs of preeclampsia. Special attention targeting new hypertension, new proteinuria, symptoms of headache, visual disturbance, epigastric pain, vomiting, reduced fetal movements and an infant that is SGA is emphasised [30]. Women with increased risk have frequent antenatal surveillance, including baseline and serial assessments of blood pressure, collection of urine samples, measurements of biochemical markers of renal function and liver function and of uric acid, and platelet counts. Unfortunately, these follow-ups are not performed in all parts of the world due to circumstances including lack of health care facilities, doctors, blood pressure measurement equipment and fetal monitoring, including Doppler ultrasounds and electrocardiograms. Consequently, the numbers of deaths from preeclampsia remains high globally [2]. The progression to generalised seizures of eclampsia is 10-30 times more common in developing countries than in high-income countries [3].

1.3 Risk factors for preeclampsia

Epidemiological studies have identified several risk factors for the development of preeclampsia. Some of these risk factors are listed in Table 1 (based on reviews by Dekker and Sibai [31] and Duckitt and Harrington [32]). The identification of women with an increased risk of preeclampsia is important to optimise the chances of prediction, prevention, and early diagnosis and to secure an appropriate management plan. The risk factors for preeclampsia may guide further research into its pathophysiological mechanisms. Genetic risk factors will be presented specifically in Section 1.6.

Table 1. Some known risk factors for preeclampsia.

Partner-related	Risk*
Nulliparity/primipaternity	4: 1
Limited sperm exposure	12: 1
Fathered a preeclamptic pregnancy in another woman	2: 1
Maternal-specific	
Age >40 years	2: 1
BMI >35 kg/m2	2: 1
Nulliparity/primiparity	3: 1
Preeclampsia in a previous pregnancy	7: 1
Family history of preeclampsia	3: 1
Pre-existing medical conditions	
Insulin dependent diabetes	4: 1
Chronic hypertension	4: 1
Renal disease	20: 1
Chronic autoimmune disease	7: 1
Antiphospholipid syndrome	10: 1
Pregnancy associated	
Multiple pregnancy	3: 1
Urinary tract infection	3: 2

* Published risk (odds ratio (OR) or relative risk), based on reviews by Dekker and Sibai [31] and Duckitt and Harrington [32].

1.4 Preeclampsia – a window to future cardiovascular health

CVD include disorders such as coronary heart disease, cerebrovascular disease, hypertension and peripheral artery disease. CVD is the leading cause of death in women in all major developed countries [33]. Female gender-specific factors and risk markers for CVD are well acknowledged, including menopause, hysterectomy and the use of exogenous hormones [34]. More recently, preeclampsia has been viewed as a separate risk factor for CVD in later life for both the women and offspring exposed to preeclampsia in utero [35-39]. A review and meta-analysis found that women with a history of preeclampsia have an approximately four-fold increased risk of chronic hypertension and a two-fold increased risk of coronary artery disease and stroke 10-15 years after pregnancy compared to unaffected women [40]. The offspring of women with preeclampsia have an almost two-fold greater risk of stroke in adulthood [38]. A ‘dose-response’ relationship between the severity of preeclampsia and future CVD has been proposed [35, 41]. This is supported by observations of a relative risk of 1.3-3.3 for CVD in later life among women with a history of preeclampsia, with a higher risk

range of 2.7-8.1 when the preeclampsia was considered severe [42]. However, a recent large review and meta-analysis did not support this relationship, as the risk of CVD did not appear to increase if preeclampsia was further complicated by a pre-term delivery [39].

The increased metabolic and vascular demands of a normal pregnancy may reveal a vulnerable maternal constitution, and pregnancy may represent a metabolic 'stress test' that unmasks an underlying risk of future CVD [43, 44]. Metabolic abnormalities might lead to preeclampsia and CVD at different times during a woman's life [43, 45]. The relationship between preeclampsia and CVD risk is illustrated in a model by Sattar and Greer (Figure 1) [43].

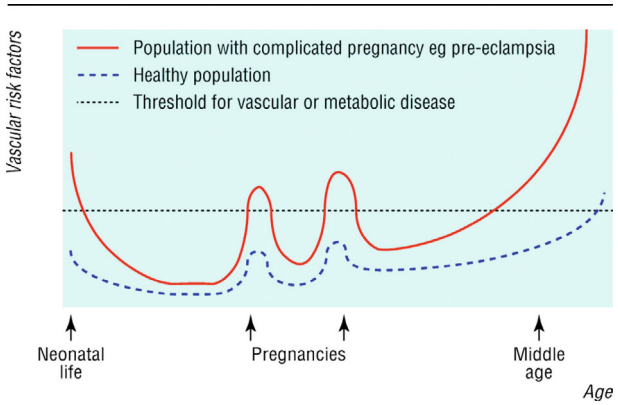

Figure 1. Risk factors for CVD throughout life (reproduced with permission [43]). Early in life, the cardiovascular risk is low. The risk increases steadily with increasing age and 'peaks' during pregnancy. After delivery, the risk decreases again but never returns to pre-pregnancy levels [43].

Preeclampsia and CVD share several risk factors for systemic inflammation (e.g., chronic hypertension, obesity, dyslipidaemia and insulin resistance) [46] as well as pathological features (endothelial dysfunction and inflammation) [45, 47] and tend to occur in the same families [48]. These common antecedents have drawn attention to the likelihood of a shared genetic susceptibility [49, 50]. This is supported by findings of Romundstad and co-workers [51]. They used the Medical Birth Registry of Norway (MBRN) to determine that the positive association between preeclampsia and CVD is

more dependent on shared pre-pregnancy risk factors than the influence of the hypertensive disorder in the pregnancy itself [51]. These similarities and associations have encouraged the search for genetic determinants common to both disorders, but only a few shared genetic risk factors have been identified [52-56].

1.5 Aetiology and pathogenesis of preeclampsia

Preeclampsia has been named the 'disease of theories', which reflects the wide range of potential causes, clinical signs and complications [57]. Its precise aetiology and underlying pathogenic mechanisms remain unknown, but the placenta has been acknowledged as a lead actor for a century. The role of the placenta is supported by the observation that preeclampsia occurs solely in the presence of placental tissue (i.e., in pregnancy or hydatidiform mole) [58] and remits dramatically in the postpartum period after the placenta is delivered. Abnormal fetal trophoblast invasion and impaired remodelling of maternal spiral arteries at 8-18 weeks' gestation are now widely accepted as primary causative factors [59]. In 1991, Redman described preeclampsia as a two-stage disorder, and this model has been a guiding framework for studies of the pathophysiology of preeclampsia (Figure 2) [60]. In the first stage, fetal trophoblasts fail to invade adequately into the decidua and the spiral arteries to achieve vessel remodelling, which is required for a proper increase in feto-placental blood flow. The second stage represents the maternal response to the first stage, in which systemic activation of the maternal endothelium is evoked by products released into the maternal circulation from the placenta [60].

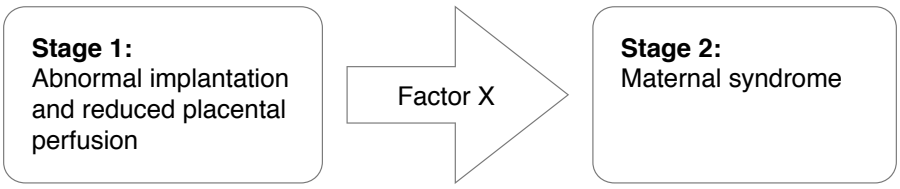

Figure 2. The two-stage model for the development of preeclampsia (modified from [60]). Stage 1 is reduced placental perfusion; stage 2 is the maternal syndrome. Several factors have been suggested as the 'factor X' that connects these stages.

However, not all cases of preeclampsia show abnormal placentation, and the complex interplay between the factors produced by the placenta and maternal adaptions to these factors is crucial for the development of disease. With increased knowledge of the complex aetiology and pathogenesis of preeclampsia, the two-stage model has been revised several times [61, 62]. Preeclampsia is now considered a multisystem disorder that is associated with pathological features including immune maladaptation, impaired placentation, angiogenic imbalance, placental stress, systemic inflammation and endothelial dysfunction [63]. These features will be presented in the following and further linked together in a step-wise model for the development of preeclampsia.

Immune maladaptation

Inadequate maternal tolerance of fetopaternal alloantigens has been suggested to be one of the primary dysfunctions in preeclampsia [64], leading to poor placentation and, consequently, reduced placental perfusion, increased oxidative stress and release of pro-inflammatory factors [65]. Epidemiological studies indicate that immunological mechanisms are central to the initial pathogenesis of preeclampsia, with risk factors related to limited exposure to paternal sperm and/or seminal plasma [31, 66]. Further, pregnancies achieved after oocyte donation (no genetic contribution from the mother), have been reported to have an approximately 30% risk of preeclampsia and/or gestational hypertension [67, 68]. This may be the most compelling evidence that there is an immunological basis to preeclampsia.

There are two main points of direct contact between the maternal immune system and the cells of the fetus: the local immune response in the decidua and the systemic immune response between maternal blood and syncytiotrophoblasts [69] (Figure 3). To ensure a successful pregnancy, an immunological truce must be made between the maternal and fetal cells at the local interface. In decidua, fetal trophoblasts are in direct contact with maternal immune cells in the decidua and in the intervillous space [70]. Syncytiotrophoblasts in the intervillous space do not express the classical human leukocyte antigen (HLA) class I or II molecules, and are relatively immunological inert. In contrast, extravillous trophoblasts (EVTs) in the decidua express polymorphic HLA-C, and non-polymorphic HLA-E, and HLA-G. In early pregnancy, nearly 40% of the

cells in the decidua are maternal immune cells, including decidual natural killer (dNK) cells, T cells, macrophages and dendritic cells [71, 72]. The interaction between EVTs and these maternal immune cells is crucial for successful trophoblast invasion, spiral artery remodelling and immunotolerance of the allogenic placenta and fetus [73]. The interaction of trophoblast HLA-C with killer immunoglobulin-like receptors (KIR) on dNK cells has been shown to promote beneficial production of cytokines and angiogenic factors [72]. Specific combinations of HLA-C and KIR isoforms are associated with preeclampsia [74].

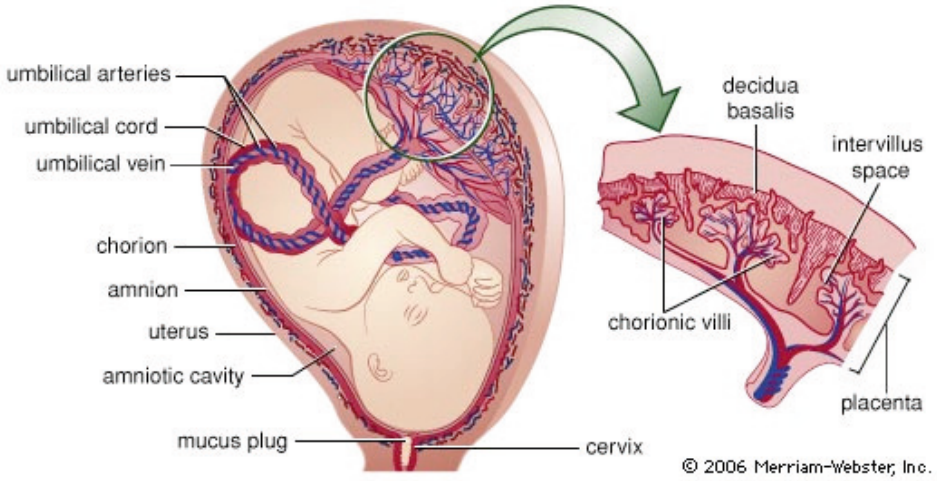

Figure 3. The placenta is composed of fetal tissue (villi) that lie in maternal vascular spaces (intervillous spaces) (reproduced with permission from Merriam-Webster Inc.)

Animal and human studies suggest that regulatory T cells (T_{reg} cells) are essential in immunoregulation at the maternal-fetal interface [75]. Pregnancy imprints the T_{reg} cells that sustain protective regulatory memory of the fetal antigen, leading to the proposal of antigen-specific 'memory' T_{reg} cells [76]. The presence of these memory cells could explain why rates of preeclampsia are reduced in secondary compared with primary pregnancy [16, 76].

Impaired placentation

Development of the placenta in the first half of pregnancy is essential to maintain a successful pregnancy. The placental bed underlies the fetal placenta and includes the decidua basalis, the maternal part of the placenta, and the underlying myometrium and contains the uterine spiral arteries (Figure 3). During normal early pregnancy, extensive vascular alterations occur in the spiral arteries to supply maternal blood to the placenta. These alterations involve two populations of EVTs: the interstitial cytotrophoblasts, which invade the decidual stroma and superficial myometrium, and the endovascular cytotrophoblasts, which invade the lumen of the spiral arteries [77, 78]. The EVTs invade the vessels of the maternal spiral arteries in the decidua and parts of the myometrium and modify the spiral arteries from low-flow, high-resistance vessels to high-flow, low-resistance vessels in order to reach the demands of the developing fetus and placenta [79-81] (Figure 4). Histopathological studies of placental bed biopsies show defective vascular changes in preeclamptic and FGR pregnancies [79, 81, 82], and inadequate blood flow is considered a key element in the development of these disorders (Figure 4), as supported by observations of increased resistance in the uterine artery by Doppler velocimetry [83, 84]. In preeclampsia and FGR, impaired trophoblast differentiation, limited migration and invasion of trophoblasts, reduced remodelling of spiral arteries and excessive apoptosis during placentation have been observed [79, 82] (Figure 4).

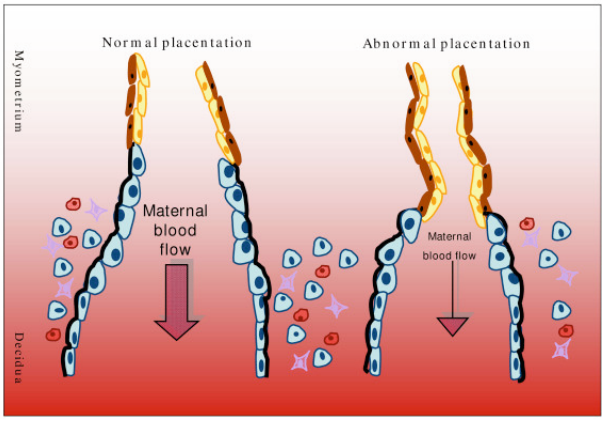

Figure 4. Spiral artery remodelling in normal and abnormal placentation (as occurs in some cases of preeclampsia and FGR) (reproduced with permission [85]).

Angiogenic imbalance

Angiogenesis is essential for placental vascular development, but knowledge about the details of the genetic program required to pattern this development is limited [86]. The release of several angiogenic factors by the placenta has been proposed, including vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and angiopoietins [87]. Increasing evidence suggests that an anti-angiogenic state plays an important role in the pathophysiology of preeclampsia [86]. Placental release of anti-angiogenic factors has been suggested to result from syncytial knots induced by placental hypoxia and/or disturbed placental development [86]. It has been speculated that the association between preeclampsia and CVD in later life could be due to a chronic anti-angiogenic state resulting from genetic variants within genes encoding angiogenic factors [86].

Placental stress

Impaired placentation leads to oxidative stress, endoplasmic reticulum (ER) stress and exaggerated local inflammation [63, 88]. There are close links between these three processes, with each being able to induce the others [88, 89]. Intermittent high uteroplacental velocity flow, with ischaemia-reperfusion insults, seems to explain why the oxidative stress arises better than chronic placental hypoxia [90]. Augmented oxidative stress and ischaemia-reperfusion insults have been well documented in placental tissue from pregnancies with preeclampsia and FGR compared to normotensive pregnancies [91-94]. This includes increased levels of reactive oxygen species (ROS), lipid peroxidation products and other oxidative stress markers [94-96]. The decidua basalis is probably one of the main sources of oxidative stress products in preeclampsia [92, 93]. Oxidative stress is an inflammatory stimulus and is highly related to chronic inflammation. Oxidative stress from other causes stimulates an inflammatory response, which generates oxidative stress; thus these phenomena are regarded as inseparable [97].

Hypoxia and ischaemia-reperfusion insults can induce ER stress [98], which may lead to a wide range of responses. This includes oxidative stress, inflammatory pathway activation, or, under severe or prolonged conditions of ER stress, apoptosis [98, 99].

Correspondingly, ER stress has been implicated in a wide range of human disorders, including diabetes mellitus, obesity and atherosclerosis [100, 101]. ER stress results in activation of a coordinated adaptive program referred to as the unfolded protein response (UPR). The UPR consists of three signalling pathways, which are activated by the proximal ER transmembrane sensors inositol-requiring enzyme 1 (IRE-1), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6) [99]. The aim of the UPR is to re-establish homeostasis in the ER by decreased protein synthesis, decreased cell proliferation and cell cycle arrest. If these attempts fail then the apoptotic machinery is activated [102]. Molecular evidence confirms high levels of ER stress and activation of the UPR in placentas from preeclamptic women [102-104]. Further, our Australian/New Zealand and Norwegian cohorts have revealed that variations in the selenoprotein S (*SEPS1*) and endoplasmic reticulum aminopeptidase 2 (*ERAP2*) genes, which are involved in ER stress, are associated with preeclampsia [105, 106].

Preeclampsia is characterized by an increased placental inflammation [69]. It has been proposed that the early and local inflammation is due to increased production of pro-inflammatory factors. This production may result from several processes, including inadequate tolerance between maternal immune cells and fetal trophoblasts [69], and as earlier mentioned, placental oxidative and ER stress [63, 88]. It is hypothesised that inflammatory mechanisms generate the atherosclerotic-like lesions termed acute atherosis [62], which are observed in the decidual spiral arteries of 20-40% of preeclamptic women [107, 108]. Acute atherosis is characterised by the accumulation of lipid-filled foam cells, fibrinoid necrosis and leukocyte infiltration [109]. These lesions are similar to those occurring in the early atherosclerotic changes frequently observed in coronary and other large arteries in the middle aged and elderly. Acute atherosis may reduce the spiral artery calibre and likely impairs intervillous blood flow and contributes to placental dysfunction [110]. Decidual oxidative stress may arise by peroxidation of these lipid depositions. Correspondingly, increased contents of lipid peroxides in decidual tissue from preeclamptic pregnancies have been reported, and it is proposed that release of such oxidative lipid derivates could include endothelial dysfunction when reaching the maternal circulation [92].

Placental stress is likely to arise through interplay between oxidative stress, ER stress, and local inflammation. These processes generate the release of 'danger signals', subsequently affecting placental function and leading to endothelial dysfunction and systemic inflammation in the mother at later gestation [111].

Systemic inflammation and endothelial dysfunction

Pregnancy is a natural inflammatory state, which represents the body's physiological adaption to pregnancy [112]. Inflammatory responses cause metabolic adaptions to ensure fetal growth and development [113], including induction of insulin resistance [114] and hyperlipidaemia [115]. In preeclampsia, all the inflammatory changes of normal pregnancy are exaggerated, and become a too large burden to the maternal system. In this manner, preeclampsia could be recognised as the extreme end of a continuum of maternal systemic inflammatory responses caused by pregnancy itself [116]. The elevated inflammation in preeclampsia might be triggered by placental debris shed into the maternal circulation (e.g., syncytiotrophoblast microparticles, soluble fms-related tyrosine kinase 1 (sFlt-1), soluble endoglin (sEng), activin-A, leptin, and PlGF), products of oxidative stress/ER stress, thrombin, pro-inflammatory cytokines, fetal haemoglobin and free fetal deoxyribonucleic acid (DNA). The levels of these activators are significantly increased in preeclampsia [63, 117-119].

Endothelial cells are a part of the inflammatory network and possess receptors that activate innate immune responders, present antigens to T cells after stimulation, produce pro-inflammatory cytokines and stimulate and are stimulated by inflammatory leukocytes [120]. Endothelial dysfunction or inappropriate endothelial activation is a hallmark of the development of preeclampsia and cardiovascular disorders. In preeclampsia, an excessive amount of activated neutrophils adhere to the endothelium and release proteases and ROS. Endothelial cell damage in preeclampsia leads to increased capillary permeability, platelet thrombosis and increased vascular resistance, preceding onset of symptomatic clinical disease [121].

A step-wise model for the development of preeclampsia

Since the presentation of the two-stage model for the development of preeclampsia (Figure 2) [60], researchers have been looking for the 'factor X' that connects inadequate placental perfusion with overt preeclampsia, which remains an area of intense investigation. However, poor uteroplacental arterial remodelling is not a pathognomonic finding for preeclampsia, and clearly preeclampsia can be caused by factors other than inadequate placental perfusion. Thus, the concepts of 'placental preeclampsia' and 'maternal preeclampsia' have been proposed [122]. 'Placental preeclampsia' has been proposed to be the outcome of implantation problems and immunological issues at the maternal-fetal interface. 'Maternal preeclampsia', which mainly presents as a late-onset disease, does not necessarily feature abnormal placentation but occurs when the mother is predisposed to systemic inflammation (e.g., chronic hypertension, obesity and insulin resistance). In the maternal presentation, the maternal vessels react abnormally to the stress of even a normal pregnancy. However, many cases are a mix of both the maternal and placental presentations and are not easy to separate clinically. A step-wise model for the development of preeclampsia is shown in Figure 5.

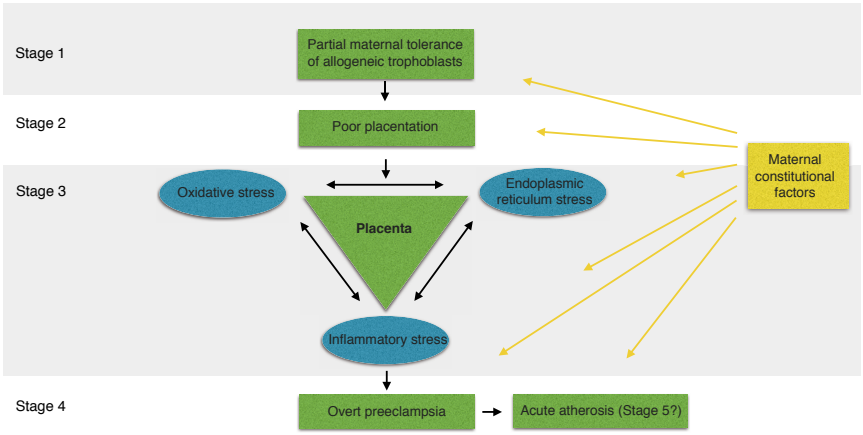

Figure 5. A step-wise model for the development of preeclampsia (modified from [60-63]). Stage 1 is poor immune regulation and/or vascular inflammation, while Stage 2 is poor placentation. The three-way interaction between oxidative stress, ER stress and inflammatory responses in the placenta give rise to the inflammation that drives preeclampsia (stage 3). Stages 1, 2 and 3 lead to Stage 4, a systemic inflammatory disorder and overt preeclampsia. Most recently, Staff et al. included acute atherosis as a possible stage 5 in some pregnancies [62].

1.6 Genetics of preeclampsia

Preeclampsia shows a strong familial aggregation, both within sibships and over generations, suggesting the importance of genetic factors [50, 123, 124]. Studies of families have indicated a 3- to 5-fold increased risk for a mother to develop preeclampsia if a first-degree relative (mother, sister or both) has experienced preeclampsia [125-127]. If the mother's partner was born after a preeclamptic pregnancy, the risk increases 1.5- to 2-fold [127, 128], suggesting that susceptibility can also be transmitted via paternal genes, presumably acting through the fetus. From studies of monozygotic and dizygotic twin pairs, the heritability of preeclampsia is estimated to be greater than 50% [129]. A large population-based Swedish cohort study including 244,564 sib pairs with a total of 701,488 pregnancies reported that 35% of the variance in the risk of preeclampsia was attributable to maternal genetic effects, 20% to fetal genetic effects (with an equal contribution from maternal and paternal genes), 13% to the liability of a specific couple, <1% to shared sib environment, and 32% to undetermined factors [130].

Candidate gene studies

A large number of candidate gene studies have been performed to elucidate the genetic background of preeclampsia. Candidate genes involved in the haemodynamic changes that occur during pregnancy (e.g., renin (*REN*), angiotensin (*AGT*) and nitrogen oxide (NO) synthetase), thrombophilia, oxidative stress and immune genetics [50, 123] have been proposed. However, most of these studies have yielded inconsistent and/or conflicting results. This lack of reproducibility may be due to inadequate sample sizes, population diversity, different environmental factors, false results in the original study and an unclear definition of the phenotype, among other factors [50, 131]. A recent review and meta-analysis of individual genetic variants associated with preeclampsia identified associations between preeclampsia and genetic variants in or near the genes angiotensin I converting enzyme (*ACE*), cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*), coagulation factor II (thrombin) (*F2*), coagulation factor V (*FV*), lipoprotein lipase (*LPL*) and serpin peptidase inhibitor (*SERPINE1*) [132]. On the basis of the identification of these individual genetic variants, the authors proposed that the following systems play a role in the pathogenesis of preeclampsia: the renin-angiotensin

system, coagulation and fibrinolysis, lipid metabolism and inflammation [132]. Notably, many of these variants are also risk factors for CVD, supporting a shared genetic susceptibility for preeclampsia and CVD [132].

Genome-wide studies

Given the conflicting results of studies of candidate genes in preeclampsia, genome-wide studies have been initiated to avoid focusing on a single gene. Genome-wide linkage studies in preeclampsia families have identified 'hot spots' on chromosomal regions 2p13 and 2q23 [133], 2p25, 4q32 and 9p13 [134], 2q22 [135], 4q34 [136], 5q and 13q [137], 10q, 12q and 22q [138] and 11q23–q24 [139]. Unfortunately, the findings of these studies have been inconsistent, illustrating the genetic complexity of preeclampsia and reflecting the possibility that different genetic factors might be involved in different populations.

Advances in genotyping technology have allowed rapid genome-wide screening of common variants in large populations. To date, only a few genome-wide association studies (GWAS) of preeclampsia have been published [140-142]. Johnson et al. identified two significant single nucleotide polymorphisms (SNPs) both in the chromosome 2q14 region located downstream of the inhibin beta-B (*INHBB*) gene [142]. Neither SNP appeared to be causative, and thus the authors hypothesised that they were in linkage disequilibrium (LD) (the co-occurrence of an allele at two or more loci at a frequency greater than that expected by chance) with proximal causal variants. Six genes were identified in the region +/- 500 kB of the GWAS SNPs and their promoters, untranslated region of genes (UTRs) and exons are now targeted for sequencing with the aim of identifying any candidate causal variants in those regions (E. K. Moses, personal communication, July, 2013). In the other two published GWAS [140, 141], none of the detected SNPs reached genome-wide significance, most likely because these studies lacked power to detect effects of the size expected in preeclampsia. The InterPregGen research study is currently conducting a GWAS analysis of maternal and fetal genes in 13,000 preeclamptic pregnancies with the aim of examining maternal-fetal gene interactions and individual effects [143].

1.7 Gene expression studies of preeclampsia

Placental and decidual tissues are central in the pathogenesis of preeclampsia and are both considered most relevant sites for studies of the pathogenesis of preeclampsia. Microarray-based transcriptional profiling can be a powerful strategy for the identification of disease-related genes and pathways [144], and placental/decidual ribonucleic acid (RNA) may serve as novel biomarkers for the prediction of preeclampsia. Several genome-wide expression studies have been performed in placental tissue from preeclamptic pregnancies [145]. Placental tissue is relatively easy to obtain from deliveries, and thus the placenta is a convenient source of gestational tissue for gene expression studies. A variety of differentially expressed genes have been reported, including genes associated with obesity [146], hypoxia [147], HLA-G genotype [148] and angiogenesis [149, 150]. A few micro-RNA (miRNA) expression studies have been performed in which gene expression in preeclamptic and non-preeclamptic placental tissue was compared [151, 152]. miRNAs regulate gene expression and are considered a regulatory layer for silencing gene expression, predominantly by degradation of the target messenger RNAs (mRNAs) [153]. A recent review and meta-analysis of array-based gene-expression studies identified a placental meta-signature comprising 40 annotated gene transcripts and 17 miRNAs related to preeclampsia [154]. Among these transcripts, up-regulation of the well-known and validated transcripts fms-related tyrosine kinase 1 (Flt-1), endoglin (Eng), vascular endothelial growth factor A (VEGFA) and leptin was observed [154].

To our knowledge, only three decidual genome-wide studies have been published [135, 155, 156]. Moses et al. focused on genes residing within a region previously shown to be in significant linkage with preeclampsia on chromosome 2q, and 17 transcripts within this region showed at least a 2.5-fold change in expression in association with preeclampsia [135]. Herse et al. observed dysregulation of the circulating and tissue-based renin-angiotensin systems in decidual samples from preeclamptic pregnancies compared to controls [155]. Winn et al. identified 55 transcripts that were differentially expressed, which included transcripts previously associated with preeclampsia (e.g., Flt-1 and leptin) and novel preeclampsia-associated genes (e.g., sialic acid binding Ig-like lectin 6) [156].

2. AIMS

Epidemiological research has led to an understanding of the genetic susceptibility and risk factors for preeclampsia. The understanding of preeclampsia pathophysiology has increased immensely in the last two decades. However, the association between genetic determinants of susceptibility and the complex causal pathways underlying the disease aetiology remain largely unknown and represent the current direction of preeclampsia research. The overarching objective of this thesis was to investigate genetic variations influencing preeclampsia susceptibility. To explore this theme, genetic association studies using population-based cohorts and transcriptional studies on decidua basalis tissue were performed. This thesis addresses the following specific aims:

Paper I: To investigate how gene expression in the decidua basalis contributes to the pathogenesis of preeclampsia. This was assessed via genome-wide transcriptional profiling (measuring ≥48,000 transcripts from all known genes) of decidua basalis tissue from preeclamptic and normal pregnancies. We further aimed to identify the genetic pathways and gene-gene interaction networks represented by the differentially expressed genes using pathway analytical tools.

Paper II: To evaluate the hypothesis that genetic variation in the storkhead box 1 (*STOX1*) gene residing within the 10q22 preeclampsia susceptibility locus is a causal factor for developing severe preeclampsia. This was assessed by genetic studies of SNPs in *STOX1* using a Norwegian preeclampsia population cohort. We further aimed to investigate the expression levels of the *STOX1* gene and related transcripts in decidua basalis tissue from women with preeclampsia and/or FGR and those with uncomplicated pregnancies.

Paper III: To investigate if the degree of ER stress and activation of the ER stress pathways differ in pregnancies complicated by preeclampsia and/or FGR. This was assessed by comparing the gene expression and protein levels of key mediators of each branch of the ER stress response in decidua basalis tissue from pregnancies complicated by preeclampsia and/or FGR and to that of uncomplicated pregnancies.

Paper IV: To replicate the association between our recently reported 2q22 preeclampsia risk variants and cardiovascular-related traits in an independent Australian population-based cohort. This was assessed by genotyping four independent SNPs from four genes, lactase (*LCT*, rs2322659), low density lipoprotein receptor-related protein 1B (*LRP1B*, rs35821928), rho family GTPase 3 (*RND3*, rs115015150) and grancalcin (*GCA*, rs17783344), and further association analysis of these genotypes, preeclampsia and cardiovascular risk factors for mothers and adolescents.

3. MATERIALS

The papers included in this thesis are based on data from different sources. Papers I-III use data from the Biobank for pregnancy complications (the PE/FGR Biobank). In addition, Paper II also makes use of data from the second Nord-Trøndelag Health Study (HUNT2) and the MBRN. Paper IV uses data from The Western Australian Pregnancy Cohort (Raine) Study. These data sources are briefly summarised in the following sections.

3.1 Biobank for pregnancy complications (the PE/FGR Biobank)

Decidual tissue from women with pregnancies complicated by preeclampsia and/or FGR and those with non-preeclamptic pregnancies was collected at St. Olav's University Hospital (Trondheim, Norway) and Haukeland University Hospital (Bergen, Norway) from 2002 to 2006. Preeclampsia was defined as persistent hypertension (blood pressure of ≥140/90 mmHg) plus proteinuria (≥0.3 g/L or ≥1+ on a dipstick) developing after 20 weeks of pregnancy [11]. FGR was defined by birth weight ≤2 standard deviations (SD) below the expected birth weight according to gestational age and sex, corresponding to the 2.5^{th} percentile in a Scandinavian normogram [157]. In addition, restricted fetal growth was confirmed by at least one of the following additional criteria; 1) reduced fundal height in serial measurements, 2) serial ultrasound biometry identifying a failure to grow along a consistent percentile, or 3) abnormal umbilical artery waveform. For the purpose of Paper III, cases with a debut of preeclamptic symptoms or FGR before gestational week 34 were classified as early-onset. Preeclampsia was considered severe in accordance with the classification recommended by Sibai et al. [27], including severe hypertension (>160 mmHg systolic and/or 110 mmHg diastolic) associated with proteinuria or severe proteinuria (≥5g per day) associated with hypertension and/or multiorgan involvement (pulmonary oedema, seizures, oliguria, thrombocytopenia, abnormal liver enzymes, etc.). Severe FGR was defined as birth weight $<1.7^{th}$ percentile [19].

Due to tissue sampling procedures, only women with no labour activity who delivered by CS were included. Women with preeclamptic pregnancies underwent CS for medical

indications (indicating a severe manifestation of the disease), whereas women with normal pregnancies underwent CS for reasons considered irrelevant to the aims of the studies (e.g., breech presentation, cephalopelvic disproportion in previous delivery and fear of vaginal delivery). Only healthy women with no history of preeclampsia were accepted in the non-preeclamptic group. Multiple pregnancies and pregnancies with chromosomal aberrations, fetal and placental structural abnormalities or suspected perinatal infections were not included.

Decidua basalis tissue was obtained by vacuum suction of the placental bed, a procedure which allows the collection of tissue from the entire placental bed [158]. The procedure is described in detail elsewhere [92, 158]. Briefly, the placenta was located by manual palpation after delivery and allowed to separate spontaneously. Collected samples were flushed with saline solution to remove excessive blood. The decidual tissue was immediately submerged into RNA-later (Ambion, Austin, Texas) (for microarray analyses), 10% neutral-buffered formalin and paraffin embedded (for immunohistochemical analyses), or snap-frozen in liquid nitrogen (for Western blot analyses) within 15 min of tissue collection. The quality of the decidual material was assessed by immunohistochemistry as previously described [159].

3.2 The second Nord-Trøndelag Health Study (HUNT2)

HUNT2 is a multipurpose population-based health survey conducted in Nord-Trøndelag County in Norway between 1995 and 1997 [160]. This population is ethnically homogeneous (>97% Caucasians) with low and stable immigration and emigration rates (net out migration of 0.3% per year). All residents of Nord-Trøndelag County aged 20 years and older were invited to participate, and 75.5% of women (n = 35,280) accepted the invitation. They attended a standardised clinical examination including standardised measurements of height, weight, waist circumference, blood pressure, and non-fasting measurements of serum lipids and glucose, and they completed comprehensive questionnaires. A large biobank was established [160]. Further details about the study are described elsewhere [160].

To obtain extensive obstetrical data, all participating women in HUNT2 were linked to MBRN. Preeclampsia was defined in accordance with the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy criteria [11], using diagnoses reported to MBRN (ICD-8 until 1998, ICD-10 after 1998). DNA samples were available for 1,139 women registered with preeclampsia and 2,269 non-preeclamptic women. Of the available women registered with preeclampsia, 1003 women were registered with one and 136 women were registered with more than one (recurrent) preeclamptic pregnancy. The mean follow-up time from diagnosis in the MBRN to inclusion in the study was 25 ± 10 years. Two controls per case were identified at random among parous women in the HUNT2 cohort with no registered preeclamptic pregnancy in the MBRN.

3.3 The Medical Birth Registry of Norway (MBRN)

Since 1967, all deliveries (2.6 million births) from the 16th week of gestation (12th week since 2002) in Norway have been recorded in MBRN [161]. For each birth, the unique national identification numbers of the child and parents are registered, and midwives or doctors at the delivery units complete a compulsory standardised form including information on maternal health before and during pregnancy, previous reproductive history, if there have been any complications during pregnancy and perinatal data of the newborn. Preeclampsia is entered as a specified diagnosis and is defined as an increase in blood pressure to at least 140 mmHg systolic or 90 mmHg diastolic (or an increase in diastolic blood pressure ≥15 mmHg from the level measured before 20th gestational week), combined with proteinuria (protein excretion of ≥0.3g/24 h or ≥1+ on a dipstick) [11]. Cross-linking of the HUNT2 and MBRN data was performed at MBRN, resulting in the identification of 1,179 women registered with preeclampsia [105]. A total of 2,358 women with no preeclampsia were selected randomly on the basis of the next two normal pregnancies in the HUNT2 cohort (non pair-wise matching).

3.4 The Western Australian Pregnancy Cohort (Raine) Study

The Western Australian Pregnancy Cohort (Raine) Study is a pregnancy cohort that was recruited prior to 18 weeks' gestation from the public antenatal clinic at King Edward

Memorial Hospital or surrounding private clinics in Perth, Western Australia (WA). The study has been described in detail previously [162]. Pregnant women (*n* = 2,979) were enrolled between August 1989 and April 1992. From the original cohort of women, 2,868 of their children have been followed over the last two decades with detailed assessments performed every 2-3 years. At each follow-up, the participants attended a standardised clinical examination including standardised measurements of height, weight, waist circumference, blood pressure, and skinfolds. They also completed comprehensive questionnaires, and fasting venous blood samples were obtained for DNA and biochemical analyses [163, 164]. For this study, data from the pregnant women, the neonates, and the 8-, 14- and 17-year cohort follow-ups were assessed.

The presence of preeclampsia and history of preeclampsia were collected from the mother at antenatal visits at the delivery units and later assessed from the medical records. The medical records were reviewed by obstetricians and research midwives to confirm a standardised diagnosis of preeclampsia as a pregnancy-induced increase in systolic blood pressure ≥140 mmHg and/or a diastolic blood pressure ≥90 mmHg in women who were normotensive before the 24th week of pregnancy, combined with significant new onset proteinuria (≥ 0.3 g/l in a 24-hours specimen) [165]. The maternal data for the present study were obtained from a simplified examination and blood sample collection at their children's age 8 follow-up and included 1,685 mothers. The adolescent data were obtained from age 14 and 17 follow-ups with a cardiovascular risk assessment and included 1,094 [163] and 1,053 participants [164], respectively. Only subjects that had two Caucasian parents, were biologically unrelated, and who had no congenital deformities, were included in the study.

4. METHODS

The papers included in this thesis used different methods to assess gene expression (Papers I-III) and genetic association (Papers II and IV) data. These methods will be briefly summarised in the following sections.

4.1 Gene expression data

Total RNA extraction and quality control

Decidual tissue from the PE/FGR Biobank was used to obtain RNA (Papers I-III). Total RNA was isolated using a TRIzol (Invitrogen, Carlsbad, CA) extraction protocol with chloroform interphase separation, isopropanol precipitation, and ethanol wash steps. The isolated total RNA was purified with an RNeasy Mini Kit using spin technology (Qiagen, Valencia, CA, USA). The precipitated total RNA was resuspended in RNase-free water and purified with an RNeasy Mini Kit using spin technology (Qiagen, Valencia, CA). A Nano-Drop ND-1000 (Thermo Scientific, Wilmington, DE) was used to evaluate the presence of impurities and low RNA concentrations after isolation. Samples with concentrations too low for accurate measurement or high levels of impurities (phenol, proteins, etc.), were excluded from further analysis. Total RNA quality was measured using an RNA 6000 Nano Series II Kit on a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA). This system reports an RNA integrity number (RIN) that is based on 10 parameters, all indicative of RNA integrity [166]. In the performed studies (Papers I-III), seven samples were excluded from the microarray analysis after assessment of the RIN value (<5) and visual evaluation of digital gel electrophoresis images.

Microarray gene expression data

Microarrays consist of small, solid surfaces onto which short oligonucleotide sequences (probes) are attached at fixed locations. Each probe represents an mRNA transcript. The Illumina technology used in the current studies (Papers I-III) is a 1-channel system that uses probe-covered beads to detect specific mRNA types. Each array contains up to 48,000 probes covering RefSeq and UniGene annotated genes. For decidual gene expression (Papers I-III), purified antisense RNA (aRNA) was hybridised to Illumina's

HumanWG-6 v2 Expression BeadChip (Illumina Inc. San Diego, CA). Washing, blocking and transcript signal detection (streptavidin-Cy3) was performed using Illumina's 6x2 BeadChip protocol. Samples were scanned on the Illumina BeadArray 500GX Reader using Illumina BeadScan image data acquisition software (version 2.3.0.13). Illumina's BeadStudio Gene Expression software module (version 3.2.7) was used to subtract background noise signals and generate an output file for statistical analysis.

Quantitative RT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) is based on PCR, a primer-mediated enzymatic amplification of specific DNA sequences that can be used to quantify mRNA transcript abundance. To control for technical bias (Paper I), we verified the results of the microarray experiment using qRT-PCR of six of the most differentially expressed transcripts: phospholipase A2, group VII (PLA2G7), angiopoietin-like 2 (ANGPTL2), mannosidase α, class 1A, member 2 (MAN1A2), SLIT and NTRK-like family, member 4 (SLITRK4), frizzled family receptor 4 (FZD4) and ADP-ribosylation factor-like 5B (ARL5B). These transcripts were prioritised for qRT-PCR based on beta values (a measure of distance between group means, expressed in SD units), false discovery rate (FDR) [167] *p*-values and manual literature searches. The same samples and RNA isolation methods were used as for the microarray experiment. A 7900HT Fast Real-Time PCR instrument (Applied Biosystems, Foster City, CA) was used. qRT-PCR was performed with 93 samples; two of the total collection of 95 samples were excluded due to a shortage of biological material. Pre-optimised TaqMan Gene Expression Assays (Applied Biosystems) were performed in triplicate to measure mRNA expression levels relative to the reference genes, TATA box binding protein (*TBP*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Reverse transcription and PCR amplification were performed in a two-step procedure according to the Applied Biosystems High-Capacity complementary DNA (cDNA) ReverseTranscription Kit Protocol and TaqMan Gene Expression Master Mix Protocol. Negative controls were performed in triplicate without RT enzyme or cDNA template.

4.2 Western blot analyses

Western blotting is used to detect and quantify proteins in a given tissue or cell sample. Denatured proteins are separated based on polypeptide length on a gel by electrophoresis. The proteins are then transferred to a membrane for detection and visualisation using antibodies specific to the target protein [168, 169]. Western blotting was used to detect and quantify the protein levels of phosphorylated eukaryotic translation initiation factor 2α (pEIF2α), eukaryotic translation initiation factor 2-alpha (EIF2α), ATF6, x-box binding protein 1 unspliced XBP1(U), and x-box binding protein 1 spliced XBP1(S) in decidua basalis tissue (Paper III) using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Cambridge, UK). To ensure true positive staining and minimise background staining, this method is highly dependent on the sensitivity and specificity of the antibodies used. We selected antibodies by searching for previous studies that utilised the same antibodies. For the Western blot analysis, a subset of cases was chosen on the basis of clinical characteristics suggesting vascular malperfusion (i.e., abnormal uterine artery Doppler and/or early onset, severe preeclampsia and FGR). A total of 30 samples (preeclampsia, n=7; FGR, n = 7; preeclampsia and FGR, n = 8; and controls, n = 8) were included.

4.3 Immunohistochemical analyses

Immunohistochemistry is a method for localising proteins in tissue sections based on the principle of an antigen-antibody reaction. The reaction can be visualised by immunoenzymatic staining utilising antibodies conjugated to an enzyme than generates a coloured product [170]. Alternatively, in immunofluorescent techniques, the antibody can be labelled with a fluorophore, such as fluorescein, to indicate the occurrence of a specific antigen-antibody reaction [171]. Immunofluorescence analyses were used to assess cellular localisation and expression of ATF6 and x-box binding protein 1 (XBP1) in decidual tissue (Paper III). The same antibodies against XBP1 and ATF6 that were used in the Western blot experiments were used in the immunohistochemical analyses, along with antibodies against cytokeratin 7 (CK7) to detect EVTs, vimentin and prolactin to detect decidual cells, and CD68 to detect macrophages. Sixteen decidual samples that were randomly selected from the Western blot study population were

included in these analyses (preeclampsia, *n* = 4; FGR, *n* = 4; preeclampsia and FGR, *n* = 4; and controls, *n* = 4).

4.4 Genetic association data

DNA extraction

For the HUNT2 subjects (Paper II), DNA for genotyping was extracted from blood samples obtained each time the participants attended HUNT2. DNA was extracted at the HUNT biobank as described elsewhere [105, 160]. In brief, DNA was extracted from peripheral blood leukocytes from blood clots or from ethylenediaminetetraacetic acid (EDTA)-treated blood using PUREGENE chemistry (Gentra Systems Inc., Minneapolis, MN) for large volumes (clots and EDTA-treated blood greater than 1 ml) or the MagAttract DNA Blood M48 kit (Qiagen, Hilden, Germany) for low volumes (<400 μl). For the Western Australian Pregnancy Cohort (Raine) Study subjects (Paper IV), DNA for genotyping was extracted from blood samples obtained from the mothers and adolescents during the 14- or 17-year cohort follow-ups as described elsewhere [172]. In brief, DNA was extracted from 4 ml EDTA-treated blood using Qiagen PureGene chemistry (Qiagen, Hilden, Germany).

Genetic association studies

Genetic association studies aim to identify associations between one or more genetic polymorphisms and a trait, which may be a quantitative characteristic or a discrete attribute or disease. Candidate-gene approaches were used to select six SNPs within *STOX1* (Paper II) and four SNPs within four genes on chromosome 2q22: *LCT*-rs2322659, *LRP1B*- rs35821928, *RND3*-rs115015150 and *GCA*-rs17783344. In Paper II, the SNPs were genotyped using Applied Biosystems' SNPlex^{TM} Genotyping System, and sample genotypes were interrogated using Applied Biosystem's GeneMapper (version 4.0) software. In Paper IV, the SNPs within *LCT* and *GCA* had already been genotyped in a previously performed GWAS of adolescents [173]. The genotyping and quality control performed in this GWAS have been described in detail elsewhere [173]. In brief, Raine adolescent samples were genotyped on the Illumina Human 660W-Quad SNP Chip at the Centre for Applied Genomics (Toronto, Ontario, Canada). For the

other two SNPs for mothers and adolescents, genotyping was commercially performed by KBioscience (now LGC Genomics), (Hertfordshire, UK) using their proprietary fluorescence-based competitive allele-specific PCR genotyping assay, KASP^{TM}.

4.5 Ethical approval

Informed written consent was obtained from all participants included in the studies (Papers I-IV). The studies using the PE/FGR Biobank (Papers I-III) were all approved by the Regional Committee for Medical Research Ethics, REK Central Norway. Ethical approval for total RNA processing and decidual expression analysis was obtained from the institutional review board at the University of Texas Health Science Center in San Antonio (Papers I-III). The study using HUNT2 and MBRN (Paper II) was approved by the Regional Committee for Medical Research Ethics, REK Central Norway, the National Data Inspectorate and The Directorate of Health and Social Welfare, Norway. Ethical approval for genotyping (Paper II) was obtained from the institutional review board at the University of Texas Health Science Center in San Antonio. For the study using the Western Australian Pregnancy Cohort (Raine) Study (Paper IV), ethical approval was obtained for all protocols from the Human Ethics Committees of King Edward Memorial Hospital and Princess Margaret Hospital Ethics Committee, Perth, Western Australia.

4.6 Statistical analyses

Clinical characterisation

For Papers I-III, SPSS (*v*16.0 for Papers I and II and *v*15.0 for Paper III) (SPSS, Chicago, IL) was used to compute descriptive statistics such as means and SDs. *P* values were computed based on *t*-test statistics for normally distributed variables. Non-parametric methods (χ^2) were used for categorical variables. For Papers II and III, subgroups of cases were analysed separately. In Paper II, multivariate logistic regression was used to model preeclampsia as the (dichotomous) dependent variable against maternal age. A threshold of $\alpha = 0.05$ was set for statistical significance of all computed analyses. For Paper IV, the software package R (www.r-project.org) was used to compute descriptive statistics, means and 95% confidence intervals (CIs).

Microarray gene expression analyses

Microarray expression analyses (Papers I-III) were performed in close collaboration with the AT&T Genomics Computing Center, Texas Biomedical Research Institute, San Antonio, Texas. The choices of methods were guided by their extensive experience with such analyses. The expression analyses were mainly performed using the Sequential Oliogenic Linkage Analysis Routines (SOLAR) statistical analysis software program [174], as previously described [175]. Briefly, transcript data for each sample were log transformed and quantile normalised prior to analyses. To evaluate the magnitude of differential gene expression the displacement of each detected transcript's mean expression value was measured between the groups. A standard regression analysis was performed to determine if the mean transcript level differed between the groups. The microarray data were reported to Array Express (www.ebi.ac.uk/arrayexpress/) in accordance with the Minimum Information About a Microarray Experiment (MIAME) guidelines under accession number E-TABM-682.

Quantitative RT-PCR analyses

For qRT-PCR data (Paper I), the mRNA expression levels were calculated by the comparative threshold cycle (CT) method as described elsewhere [176]. For each target gene, the mean CT value for each sample was used for analysis, after exclusion of outliers. Outliers were determined as values >2 SDs from the mean. Delta CT (ΔCT) values were computed as the difference between the given mean value for a target gene and the mean of the CT values for the two reference genes [177]. Fold-change values were calculated based on the differences in the ΔCT values between tissue from preeclamptic and non-preeclamptic pregnant women (2-ΔΔCT) [176]. A *t*-test statistic using SPSS *v*16.0 (SPSS, Chicago, IL) was used to evaluate differences between the ΔCT values of the preeclamptic pregnancies and those of the normal pregnancy group. Analysing the two reference genes separately did not change the results.

Western blot analyses

Band intensities were determined from two or three scans and quantified by densitometric analysis using Odyssey imaging software *v*3.0 (Li-Cor Biosciences) (Paper III). Band intensities were normalised relative to the loading control. Differences

in protein levels between study groups were tested using the Mann-Whitney U test in SPSS *v*15.0 (SPSS, Chicago, IL).

Immunohistochemical analyses

The proportions of XBP1- and ATF6-immunopositive EVTs, decidual cells and macrophages were counted in five randomly selected fields on each slide and calculated as the number of positive cells among the total number of CK7-, vimentin/prolactin- or CD68-positive cells, respectively (Paper III). Differences in the proportions of XBP1- and ATF6-immunopositive cells between study groups were tested using the Mann-Whitney U test. Differences in the staining intensities of XBP1 and ATF6 in the different cells types between study groups were also tested using the Mann-Whitney U test. Both analyses were performed using SPSS *v*15.0 (SPSS, Chicago, IL).

Genetic association analyses

For Paper II, association analyses were performed for the total case/control cohort using Pearson's χ^2 statistics in SPSS *v*16.0 (SPSS, Chicago, IL). Multivariate logistic regression was used to model preeclampsia as the dependent variable against maternal age. ORs with 95% CIs were calculated. Concordance with Hardy-Weinberg proportions ($p > 0.05$) was tested using a χ^2 goodness-of-fit statistic. A threshold of $\alpha = 0.05$ was set for statistical significance of all computed analyses.

For Paper IV, measured genotype association analyses were conducted for all phenotypes by applying variance-component methods with implementation in SOLAR [174]. Because variance components are sensitive to kurtosis, all quantitative phenotypes were transformed using SOLAR's inverse normalisation procedure. Genetic association was tested for each SNP under an additive genetic model allowing the mean phenotype value to vary by minor allele. This model was compared with the null model of no difference in mean phenotype value by SNP genotype using a likelihood ratio test. Twice the difference in the log-likelihoods of these models was distributed as a χ^2 random variable with 1 degree of freedom. Concordance with Hardy-Weinberg

proportions was tested using a χ^2 goodness-of-fit statistic. A threshold of $\alpha = 0.05$ was set for statistical significance of all computed analyses.

4.7 Pathway analyses

Differentially expressed transcripts in the preeclamptic group ($p < 0.05$, FDR $p < 0.1$) were imported into Ingenuity Pathways Analysis (IPA) *v*7.5 (Ingenuity Systems, Redwood City, CA) (Papers I and III). The transcripts gene identifiers were mapped to their corresponding gene object in the Ingenuity Pathways Knowledge Base. IPA was used to bioinformatically identify functional (i.e., cellular, physiological and disease processes) categories, pathways and gene-gene interaction networks (which diagrammatically represent molecular relationships between gene-gene products) potentially involved in preeclampsia within our data set. The significance of the association between the pathway and the dataset was calculated using Fisher's exact test.

To further explore the role of ER stress in the pathogenesis of both preeclampsia and FGR (Paper III), we performed pathway analyses on a group of 20 ER stress-related transcripts (as annotated by IPA) in cases and controls. Pathway analyses were performed using Rotation Gene Set Tests (ROAST) [178] and Rotation Gene Set Enrichment Analysis using rotation (ROMER) [179], implemented in the *limma* package [180]. ROAST was used to determine if any of the transcripts in the ER stress pathway were differentially expressed, and ROMER was used to determine if the subset of transcripts in the pathway was more differentially expressed than any other subset of transcripts in the total dataset. For both methods, transcripts were ranked by moderated *t*-statistics, and a rotation test [181], which is a smoothed version of permutation suitable for linear models, was used to assess significance. Both methods are available via the Bioconductor Project (Fred Hutchinson Cancer Research Center, Seattle, WA) [180].

4.8 Correction for multiple testing

Correction for multiple testing was required for both the microarray gene expression analyses (Papers I-III) and the genetic association analyses (Papers II and IV). Multiple testing is considered a major problem in whole genome analyses (Paper I), in which the number of statistical inferences far outnumbers the number of samples and the number of false positives using unmodified *p*-values will be unacceptable. The Illumina HumanWG-6 *v*2 whole genome expression bead chip used in Paper I allows us to explore the expression of 48,000 transcripts simultaneously, which will give 2,400 expected false-positive transcripts with the use of a *p*-value of 0.05. To correct for this statistical source of error, Benjamini-Hochberg correction for multiple testing [167] was applied to the results. This model aims to control the FDR in the list of transcripts by modifying *p*-values to a level reflecting the acceptable number of false positives in the final list of transcripts. The factor used to modify the *p*-value is based on the rank of the original *p*-value, with a more stringent correction of low-ranking *p*-values. However, even with the use of an FDR *p*-value of 0.1, 10% of the 455 significantly differentially expressed transcripts are expected to be false positives. The Benjamini-Hochberg correction is less stringent than a traditional Bonferroni correction, and was used in Paper I because a strict correction for multiple testing would exclude true positive transcripts (increase the number of false negatives). These limitations are generally accepted and considered as weaknesses associated with microarray technology [182]. In Paper IV, correction for multiple hypothesis testing was performed according to a Bonferroni correction (α/(total number of SNPs x total number of phenotypic traits)).

5. MAIN RESULTS

Paper I: A transcriptional profile of the decidua in preeclampsia

Whole-genome microarray analysis revealed 455 differentially expressed transcripts when decidual basalis tissue from preeclamptic and normal pregnancies was compared. The technical replicate of the microarray experiment using qRT-PCR analysis of six of the most differentially expressed transcripts showed a high grade of correlation with the microarray data. Pathway analysis of the differentially expressed transcripts identified seven significant pathways, including tryptophan metabolism, notch signalling, endoplasmic reticulum stress, nuclear factor E2-related factor 2 (NRF2)-mediated oxidative stress response and pathways related to the metabolism of fatty acids (linoleic acid metabolism, fatty acid metabolism and arachidonic acid metabolism). Network analysis showed that 64 of the preeclampsia-associated transcripts could be connected into a single network of gene-gene product interactions, including a cluster of 15 up-regulated genes associated with ER stress and oxidative stress.

Paper II: *STOX2* but not *STOX1* is differentially expressed in decidua from pre-eclamptic women: data from the Second Nord-Trøndelag Health Study

Genetic association analysis of candidate SNPs in *STOX1* could not confirm an association with preeclampsia. However, the recurrent group showed a tendency towards a higher frequency of the C genotypes for the previously reported *STOX1A*-Y153H variation. Women experiencing recurrent preeclampsia showed an increased risk of complications and comorbidity (preterm birth, reduced birth weight and development of metabolic syndrome) compared to women experiencing preeclampsia once. Gene expression levels of the *STOX1* paralogue storkhead box 2 (*STOX2*) were significantly lower in decidual tissue from pregnancies complicated with both preeclampsia and FGR compared to the other study groups. No differential gene expression of *STOX1* was observed in any of the case groups. Furthermore, there was a strong correlation between transcriptional alterations reported previously in cultured trophoblast (JEG-3) cells overexpressing *STOX1A* and alterations observed in decidual tissue from pregnancies complicated with preeclampsia and FGR.

Paper III: Increased endoplasmic reticulum stress in decidual tissue from pregnancies complicated by fetal growth restriction with and without pre-eclampsia

Pathway analyses of decidual tissue transcriptional profiles revealed up-regulation of the ER stress pathway in pregnancies complicated by preeclampsia with FGR and by FGR. In pregnancies complicated by preeclampsia alone, only a trend towards up-regulation was observed. Targeted transcriptional and protein analyses of the three ER stress signalling branches showed up-regulation of the PERK-pEIF2α and ATF6 branches in preeclampsia with FGR and FGR. In preeclampsia alone, none of these branches were significantly altered; however, a marked increase in XBP1(U) was observed. Both nuclear and cytoplasmic immunoreactivity for XBP1 and ATF6 were observed in most (>80%) EVTs, decidual cells and macrophages. No differences in the proportion of XBP1- or ATF6-immunopositive cells or staining patterns were observed between any of the study groups. Correlation analyses demonstrated a negative correlation between pEIF2α/EIF2α and placental weight.

Paper IV: Preeclampsia and cardiovascular disease share genetic risk factors on chromosome 2q22

Genetic association analysis of four independent SNPs in four genes on chromosome 2q22 (*LCT*-rs2322659, *LRP1B*-rs35821928, *RND3*-rs115015150 and *GCA*-rs17783344) previously associated with preeclampsia susceptibility and cardiovascular risk factors showed associations between all four SNPs and cardiovascular risk traits, for both mothers and adolescents in an Australian population cohort. Carrying the A allele of *LCT* rs2322659 was associated with decreased levels of the adolescent's blood glucose in both mothers and adolescents and decreased maternal height in mothers. Carrying the T allele of *LRP1B* rs35821928 was associated with increased maternal height in both mothers and adolescents and decreased maternal weight in mothers. An association between the A allele of *RND3* rs115015150 and decreased adolescent's waist-hip ratio was observed in mothers, whereas this SNP was associated with decreased level of adolescent's triglycerides in adolescents. In mothers, carrying the C allele of *GCA* rs17783344 was associated with increased adolescent's height, whereas in adolescents,

carrying the C allele was associated with a lower risk of being born of a preeclamptic pregnancy and a lower risk of having a mother who had previously experienced a preeclamptic pregnancy. Our collective findings support the hypothesis that genetic mechanisms for preeclampsia and CVD are, at least in part, shared, but this conclusion requires some caution as a Bonferroni correction for multiple testing adjusted the statistical significance threshold.

6. DISCUSSION

6.1 Discussion of materials and methods

The accuracy of findings in a research study depends on the precision and validity of estimation in the study design. The estimation process is a combination of planning, conducting and analysing the data of the studies. The aim of this section is to summarise the methodological limitations and strengths of the studies included in this thesis, and to discuss the extent to which these limitations might have influenced the results.

6.1.1 'Hypothesis-driven' versus 'hypothesis-generating'

There are two main categories of genetic studies: 'hypothesis-driven' (e.g., candidate-gene approaches) and 'hypothesis-generating' (e.g., genome-wide approaches). Candidate SNPs (Papers II and IV) may be used in candidate gene analyses because they are hypothesised to be causal variants or because they are in LD with other SNPs in that genomic region and can therefore act as proxies for multiple variants. The latter are often referred to as marker SNPs, and their use allows a high degree of variation to be captured while typing a minimal number of SNPs. Therefore, identifying associations between SNPs and disease traits enables the ascertainment of potentially causative variants or variants in strong LD with potentially causative variants. By contrast, genome-wide hypothesis-generating approaches (Paper I) represent a (relatively) unbiased option that can be attempted even in the absence of convincing evidence regarding the function or location of the causal genes. With the use of genome-wide transcriptional profiling (Paper I), the expression of thousands of genes can be assayed simultaneously, reducing cost, time and biological material requirements. This method offers an opportunity to identify novel candidate genes and pathways for disease and could also facilitate the prioritisation of genes for further studies, e.g., sequencing after GWAS [135]. However, genome-wide studies are performed on commercial arrays, which are based on a number of assumptions. Further, assumptions about biology are necessary in order to narrow the question inquiry. The differentially expressed transcripts were translated into knowledge based on available information about biological pathways (Papers I and III). To prioritise findings for further examination (Paper III), we used previously obtained biological knowledge.

6.1.2 Diagnostic criteria and phenotypes

Validity of the preeclampsia diagnosis

The specificity and sensitivity of the preeclampsia diagnosis is of major importance for the power to detect true associations. Preeclampsia is a syndrome for which diagnostic criteria have arisen from consensus and not an understanding of the pathogenesis. The variation in clinical definitions of preeclampsia complicates comparisons between different studies. For research purposes, continuous measurements of blood pressure and proteinuria are forced into stringent definitions of preeclampsia by the use of cut-off values, which might mask the phenotypic diversity observed in clinical practice [50] and moreover, camouflage the underlying mechanisms of genetic susceptibility.

A strength of the preeclampsia diagnosis in the PE/FGR Biobank (Papers I-III) and in the Western Australian Pregnancy Cohort (Raine) Study (Paper IV) is that preeclampsia was diagnosed by an obstetrician following the prevailing guidelines at a given period of time. These guidelines include thorough monitoring, including multiple blood pressure and proteinuria measurements, prenatal ultrasound measurements and fetal birth weight estimations. In addition, the women with preeclampsia in the PE/FGR Biobank were hospitalised and delivered by CS due to maternal and/or fetal medical indications, thereby decreasing the probability of a false-positive diagnosis. These women likely had more severe forms of preeclampsia.

In the HUNT2 cohort (Paper II), preeclampsia diagnosis is based on cut-off values of hypertension and proteinuria from registrations in MBRN but is also a clinical diagnosis based on symptoms (e.g., epigastric pain, persistent headache or other cerebral or visual disturbances). The registration in MBRN is based on the information in the scheme completed by the midwives/obstetricians at the delivery ward. As diagnostic criteria for preeclampsia have changed over time, the diagnosis reported by midwives and obstetricians to MBRN may be based on individual interpretations of the total clinical presentation rather than the criteria set by MBRN. Moreover, with the use of large computerised systems such as MBRN, some registration errors are inevitable. An evaluation of the validity of the preeclampsia diagnoses reported by MBRN was recently published [183]. Medical doctors and/or study nurses at the obstetric

departments in which the women of the HUNT2 preeclampsia cohort gave birth compared the reported MBRN diagnoses to clinical findings documented in hospital records. The diagnosis was confirmed in 88.3% of the pregnancies [183]. Similar results have been reported for the Medical Birth Registries of Denmark [184] and Sweden [185]. The high positive predictive value (PPV) in the Norwegian validation study [183] indicates that MBRN is a reliable source of high-quality data for research purposes. Causes of exclusion from diagnosis were unfortunately not noted during the examination of the journals of the women in the HUNT2 preeclampsia cohort. To speculate, mild preeclampsia might have been misclassified as gestational hypertension (and vice versa) in MBRN.

Validity of the FGR diagnosis

In the PE/FGR Biobank (Papers I-III), the FGR diagnosis was assessed by stringent criteria by an obstetrician (as described in Section 3.1). Medical doctors and/or study nurses confirmed the FGR diagnosis at the obstetric departments in which the women of the PE/FGR Biobank gave birth. This confirmation was made by comparing the registry data of the biobank to clinical findings documented in hospital records (unpublished data). Validation of diagnosis was performed before the studies included in this thesis were undertaken, and only subjects with confirmed clinical diagnosis were included. Unfortunately, the PPV of these studies are not known.

In the HUNT2 cohort (Paper II), we used a stringent SGA definition as a proxy for a definition of FGR. This method has proven to be more likely to identify true FGR cases within the SGA group [186, 187]. Growth estimations were based on ultrasonically estimated fetal weights appropriate for the population and considering the sex of the fetus [157]. Thus, we believe that SGA is a good approximation to FGR in this study.

Validity of the control group

In the studies included in this thesis, the preeclamptic cases were compared to non-preeclamptic controls. The same women from the PE/FGR Biobank (Papers I-III) served as controls in the three studies. The control group was termed ‘normal pregnancies’ (Paper I) and ‘uncomplicated pregnancies’ (Papers II and III), both of

witch are rather unspecific terms. For the PE/FGR Biobank, it is specified that 'Exclusively healthy women with no prior pregnancy complications were included as controls.' However, there was no scientific basis to the term 'healthy'. A stringent definition of 'healthy' might have been beneficial, such as the one provided by the American Society of Anesthesiologists (ASA) Classification I or II, which includes women with no significant medical or surgical illness, non-smokers and unmedicated women.

In the cohort studies (Papers II and IV), the controls were termed 'non-preeclamptic'. We do not know if these women had other medical health problems and/or were medicated. For the HUNT2 cohort (Paper II), the controls were identified at random among parous women in the cohort. The only criterion was that they had no registered preeclamptic pregnancy in the MBRN, and women with a multiple pregnancy (e.g., twins) were excluded. For the Western Australian Pregnancy Cohort (Raine) Study, the controls were mothers included in the study who were registered without a history of preeclampsia.

In conclusion, the uncertainties about the women serving as controls in these papers may add bias to the results. Due to incomplete registrations and misclassification, we cannot rule out the possibility that false-positives and false-negative controls were included in the studies and that their inclusion may have affected our results.

Studies of phenotypic subgroups

As presented in Section 1.1, preeclampsia is a heterogeneous disorder, and the pathogenic mechanisms underlying the different clinical manifestations may differ. Consequently, a logical assumption would be that preeclampsia is more than a single disorder. Thus, it has been suggested that studies of preeclampsia should divide preeclampsia into phenotypically homogenous subgroups, such as with or without FGR [188] and early- versus late-onset of disease [104]. We included the preeclamptic subgroups 'preeclampsia with FGR' (Papers II and III), 'recurrent preeclampsia' (Papers II and III) and 'severe preeclampsia' (Paper III). These subgroups may represent an extreme preeclampsia phenotype, which might help clarify disease

mechanisms and add power to genetic association studies [189]. In Papers I and IV, all women with preeclampsia, irrespective of the presence of FGR, recurrence of preeclampsia and/or gestational age at onset of disease, were included in the case group to increase sample size in order to increase the statistical power. Accordingly, these preeclamptic cases could be rather heterogeneous, which must be considered when interpreting the results and comparing our results with other studies. However, the preeclamptic women included in Paper I were all hospitalised and delivered by CS due to maternal and/or fetal medical indications, indicating severe/serious manifestation of the disease. Whole-genome profiling was performed using a large sample size (95 samples) in Paper I, which may at least partially compensate for inter-individual variations. However, an increase in the number of samples would be beneficial and enable for subgroup analysis in future research. We are currently enrolling more women into the PE/FGR Biobank to enable such analyses.

6.1.3 Confounding

Confounding is a mixing or confusion of effects between the target effect and the effect(s) of a third factor(s). A confounder is a variable that is not the focus of the study and that is causative for the phenotype while at the same time being associated with the variable under investigation. Confounding raises the possibility of both false findings (positive confounding) and obscuring true associations (negative confounding), thus reducing the power to detect an effect.

Population stratification

If the confounder is ethnic affiliation of the individuals in the study, the effect is called population stratification, also termed confounding by ethnicity. This can occur if the cases and controls come from different ethnic populations and if the investigated marker alleles are unequally distributed in these populations. As an example (adapted from Hamer and Sirota [190]), if you are investigating the genetic basis for eating with chopsticks and collect your cases in Trondheim, Norway, it is most likely that the cases who eat with chopsticks are primarily of Asian origin, whereas the controls who do not eat with chopsticks will be mostly Caucasians. If you find a significant association of the case-control status to a marker locus, this could be nothing more than the HLA

locus, which is distributed differently in Asian and Caucasian individuals. This illustrates the importance of planning a case-control study to know the extent of population differentiation that can be expected in the area from which cases and controls are to be drawn. Genetically homogeneous populations are preferred to populations with high heterogeneity to avoid positive and negative confounding.

In the PE/FGR Biobank (Papers I-III), we excluded mothers of non-Caucasian ethnicity (self-reported). However, we did not have any information about the ethnicity of the father. In the HUNT2 cohort (Paper II), we used a population considered ethnically homogeneous, with less than 3% non-Caucasians [160]. With the exception of selecting cases and controls from the same population, we did not perform any further approaches to correct for population stratification (such as genomic control or direct estimation of admixture with ancestry-informative markers). In the Western Australian Pregnancy Cohort (Raine) Study, only mothers and offspring of Caucasian ethnicity were included (self-reported). In the studies in which ethnicity was assessed, identification was based on self-reported ancestry, which has been determined to be a reasonable proxy for genetic ancestry [191]. However, this can be undermined by factors of cultural versus genetic specification and the occurrence of more hidden relatedness among the cases than among the controls simply because they share a genetically determined disorder. These factors may have biased our results.

Covariates

Measuring the known confounders and including them as covariates in multivariate analyses could control for confounding and was performed to a limited extent in all studies. In particular, differences in gestational age between the preeclamptic cases and non-preeclamptic controls could affect the results in Papers I-III. This is discussed in detail in Section 6.1.4. For the transcriptional data analyses, we did not match the case and control groups nor adjust for possible confounders, including age, body mass index (BMI), parity and medication. However, we reported some clinical data for the women included and were aware of these potential pitfalls when interpreting the results. In Paper IV, we only adjusted for maternal age and sex as confounding factors in the analyses. We did not adjust for other known confounding factors for CVD, including

socioeconomic status (e.g., education, receiving social security benefits) and smoking status, which may have affected our results.

6.1.4 Gene expression studies

Decidual samples

Tissue was obtained by vacuum suction of the placental bed during CS [92, 158]. This procedure is considered safe, with no observed complications [158], and provides representative material for studies of feto-maternal interactions in the decidua basalis [92, 158]. A high success rate of obtaining spiral arteries has been demonstrated with the use of this method [158, 159]. This procedure ensures collection of decidual tissue from the entire placental bed, and the collected samples are more representative for the placental bed as a whole than are traditional placental bed biopsies [158]. However, the uncertain topographic localisation of tissue fragments is a disadvantage of this method. Moreover, with the use of the vacuum suction method, there is a risk of collecting decidua parietalis together with decidua basalis. To ensure that decidua basalis was collected, paraffin-embedded slides were stained for CK7 to demonstrate the presence of extravillous trophoblasts (which are not present in the decidua parietalis) [159]. Only samples with extravillous trophoblasts (96.4%) were included for further study.

Microarray analysis of complex tissue

We performed microarray expression analyses on whole sample (decidua) homogenates (Papers I-III). Compared to *in vitro* studies, analysing gene expression in natural tissues reflects processes occurring in the undisturbed *in vivo* situation at the time of collection. However, natural tissues consist of a number of cell types that likely have varying expression patterns [192]. Decidua basalis material consists of approximately 40% maternal leukocytes, 20% extravillous trophoblasts and 30% decidual stromal cells [193]. The various cell types constituting the decidua will naturally affect our results, which must be considered when analysing the data. In Paper III, we followed up the microarray results for the ER stress pathway using immunohistochemical analysis to identify the cells expressing the genes in question. It was not feasible to perform this follow-up analysis for all differentially expressed transcripts. However, our results were

interpreted in relation to and are consistent with earlier observations in decidual and placental tissue as well as in trophoblast cell lines [194-198].

Gestational age - a possible confounding factor

In the PE/FGR Biobank (Papers I-III), a lower gestational age was found in the preeclamptic group (average, 32 weeks; range, 28-36 weeks) as compared to the non-preeclamptic group (average, 39 weeks; range, 38-40 weeks). This is not unexpected due to the need for early delivery in patients with severe preeclampsia. Because gene expression levels in utero-placental tissues may be influenced by gestational age [199-201], it cannot be excluded that some of the differences observed between the preeclamptic and non-preeclamptic groups are, in fact, gestational age-related.

Lian et al. evaluated the relative effect of gestational age and preeclampsia on gene expression [201]. The authors pooled our transcriptional data set (Paper I) with two other genome-wide transcription studies of tissue from the maternal-fetal interface [201]. A total of 22 transcripts were associated with preeclampsia, whereas 92 transcripts were associated with gestational age [201]. This indicates that a large proportion of the variation in transcript levels can be attributed to gestational age. Pathway analysis of the transcripts associated with preeclampsia revealed an over-representation of genes associated with metabolic disease [201]. However, their study was performed on samples collected with different sampling techniques and two profiling platforms (Illumina and Affymetrix). These differences restricted the number of possible comparisons, and the authors were only able to target 14,678 genes shared by both technological platforms.

In our own comparison of possible gestational age related differentially expressed transcripts (Paper I), we compared our results to the data of Winn et al [199]. Of the 455 transcripts found as differentially expressed (Paper I), 368 genes demonstrated no gestational age influenced changes [199]. This strengthens the assumption that the differential expression of these 368 genes may be related to disease mechanisms at play in preeclampsia. Fourteen of our differentially expressed genes (*TEMEM97, KIAA1598, FHL1, PLA2G7, SHANK3, NOTCH4, UBASH3B, ROBO4, NRARP, GPR116, IL6ST,*

LDLR, SRPRB, KREMEN1) were possibly related to differences in gestational age between preeclamptic cases and controls [199], and our conclusions might have been affected by this fact.

Microarray technology - multiple testing and pathway analysis

The possible pitfalls related to performing multiple tests when using whole genome analysis were presented in Section 4.8. In genome-wide transcriptional profiling (Paper I), the analysis of *groups* of genes is a strategy to increase power and reduce the dimensionality of the underlying statistical problem following multiple testing [202]. Furthermore, it may be advantageous to focus on functional categories, pathways and networks instead of single genes when the aim is to obtain insight into the pathophysiology of complex diseases such as preeclampsia. The high interconnectivity of focus genes with other correlated genes within a biological network may imply the functional and biological importance of these genes [203, 204]. To assess this interconnectivity in a comprehensive manner, we increased the FDR cut-off from 0.05 to 0.1 and, consequently, the number of genes included in the analysis. An increased FDR *p*-value cut-off may be associated with the reporting of more false-positive, individual, and differentially expressed transcripts (genes). However, numerous genes, perhaps a family or network of genes, contribute to complex disease susceptibility. Although one gene alone may not depict significant differential expression patterns, this gene may be part of a greater network of genes or pathway(s) that are significantly associated with complex disease susceptibility. Hence, individual genes with small, non-significant, changes in gene expression may act in an additive or multiplicative manner with other networked genes displaying small changes in gene expression.

Technical replication

The key advantage of microarray technology versus qRT-PCR is the high throughput. However, microarrays tend to have a low dynamic range, leading to small but significant under-representation of fold changes in gene expression. qRT-PCR has a higher dynamic range and is used to validate observed trends rather than to duplicate the absolute values obtained by microarray chip experiments [146]. The described overall concordance of trends between the two techniques is 40-80% [205-207]. A technical

replication between these platforms was performed in Paper I. The trends towards up- and down-regulation for the six transcripts tested by microarray analysis were consistent with those detected by qRT-PCR, confirming a good correlation.

Causality

Women with a pregnancy complicated by preeclampsia are in a state of exaggerated oxidative stress and inflammation. This state itself is expected to result in changes in the expression of genes in the placenta in these women. Consequently, discriminating between causal factors and secondary responses is challenging. An alteration of the expression level of one gene may influence the transcription of others included in the network. Thus, it is difficult to precisely identify the genes with primary roles in perpetuating preeclampsia from our data set. Consequently, we focused on pathways and networks rather than single transcripts, with the aim of confirming the biological processes that are perturbed in preeclampsia.

6.1.5 Genetic association studies

A major source of frustration in genetic association studies has been the difficulty of obtaining robust replication of initial positive findings. Much of this inconsistency can be attributed to inadequacies in study design, implementation and interpretation, with the use of inadequately powered sample groups as a major concern. Some of these issues will be discussed in the following sections.

Study sample size and effect sizes

Study sample size is recognised as a key factor in the determination of quality in an association study. Meta-analyses have indicated that common causal variants of complex disorders may, when evaluated individually, have rather small effects, and thus large studies will be necessary to detect these effects [208]. This effectively means sample sizes in the thousands [209]. This phenomenon is most likely also relevant for preeclampsia due to its complex nature. Therefore, power and sample size calculations should be performed prior to conducting a genetic association study to determine if the study design is appropriate to answer the question of the study.

A priori power calculations ad modum Lalouel and Rohrwasser [210] were performed for the SNPs rs1341667 (*STOX1A* Y153H) (Paper II) and rs2322659 (*LCT*), rs35821928 (*LRP1B*), rs115015150 (*RND3*) and rs17783344 (*GCA*) (Paper IV). The calculations included information on the number of subjects in the study groups and the minor allele frequencies (MAFs) of the individual SNPs. The MAFs were obtained from publically available databases. Effect sizes (OR) with according power were assessed. The SNP rs1341667 (*STOX1A* Y153H) demonstrated 90% power to detect an OR difference of 1.3 for the total HUNT2 preeclampsia/non-preeclampsia cohort and 1.9 for the recurrent preeclampsia group (Paper I). The SNPs rs2322659 (*LCT*), rs35821928 (*LRP1B*), rs115015150 (*RND3*) and rs17783344 (*GCA*) demonstrated power to detect an OR difference of 1.5 for the total preeclampsia/non-preeclampsia cohort of 27%, 11%, 3% and 30% respectively, for both mothers and adolescents (Paper IV). The low power demonstrated by the latter calculations is typical of genetic variants with low MAF and the use of cohort studies. Unless the disease is very common, the study samples that cohort studies generate will be highly unbalanced, with far fewer individuals with the disease than without. Consequently, the nested case-control samples that emerge are often relatively modest in size.

Accuracy of genotyping

A genotyping error occurs if the observed marker genotype deviates from the true marker genotype. Genotype errors can have a marked effect on the outcome of an association study. They can increase the estimated recombination fraction between a marker and a disease locus and reduce the power to detect true case-control differences. Genotyping errors depend on many factors, including both human and technological factors. Most association studies are based on the assumption that the genotypes obtained are 100% correct, but this is seldom or never the case. Reduction of the impact of genotyping error on association studies require advances in genotyping platforms and the use of methods that establish and monitor the performance of each assay and reduce the possible sources of error and bias [211].

To avoid batch-related differences in genotyping performance, case and control samples were mixed on the well plates (Papers II and IV). Further, statistical calculations,

including accordance with Hardy Weinberg proportions among the controls and controls for gender inconsistencies, levels of heterozygosity and inter-sample relatedness, were used to predict genotype error.

6.1.6 Preeclampsia – a complex disorder

Preeclampsia is a complex disorder that does not involve simple Mendelian monogenic inheritance but rather is likely to involve interactions between variation in many genes and environmental factors [123]. As with other complex disorders, it is likely that each individual genetic locus contributes only modestly to preeclampsia, preeclampsia commonly occurs in the general population, does not follow a specific known pattern of inheritance and does not segregate within families with a clear pattern of inheritance. This makes genetic study design challenging, and international collaborations with large sample sizes to detect variants with small to modest effects are needed. This knowledge has encouraged collaborative approaches between research groups such as the InterPregGen research study (www.interpreggen.org). This is the largest international research project studying genetic susceptibility for preeclampsia, and includes participating centres from the UK, Norway, Finland, Iceland and two countries in Central Asia (Uzbekistan and Kazakhstan). Data from 10,400 maternal cases and 7,800 affected babies will be assessed to identify preeclampsia susceptibility genes (www.interpreggen.org).

Investigations into the aetiological effect of genetic variants continue to evolve with advances in technology and biological knowledge [212]. SNPs have usually been studied for common complex disorders, but the involvement of gene copy number variants (CNVs), deletions, insertions, miRNAs and the complex interplay between environmental exposure and polygenic susceptibility, are now gaining acceptance as possible major contributors to disease susceptibility. There is a growing body of evidence that non-coding genetic variation is involved in the heritability of many common complex disorders. SNPs account for approximately 90% of all known sequence variation, which is mostly located in non-coding regions of the genome [213]. Thus, many functional SNPs are likely located outside coding regions and affect the regulation of gene expression [214]. Recent estimates based on data from the 1000

Genomes Project indicate that the numbers of non-coding and coding functional variants are similar [213]. Thus, the genetic studies performed in this thesis have the potential to discover only some of the genetic causes of preeclampsia.

The genome is apparently highly dynamic, and epigenetic mechanisms (changes in gene expression or cellular phenotype that are caused by mechanisms other than changes in the underlying DNA sequence) have been suggested to be involved in preeclampsia [215, 216]. Genomic imprinting is an epigenetic phenomenon in which certain genes are expressed in a parent-of-origin-specific manner. Imprinted alleles are silenced such that the genes are either expressed only from the non-imprinted allele inherited from the mother or from the non-imprinted allele inherited from the father. Up to now, about 80 imprinted genes are known in humans (www.otago.ac.nz/IGC), but the actual number is expected to be much higher [217]. Evidence for a maternal effect in one of the preeclampsia susceptibility loci has been observed for a region of genes on chromosome 10q22 [215]. However, our studies were not able to assess epigenetic effects, which likely decreased our power to detect true associations.

6.2 Discussion of biological observations

Figure 6 places the most important results of this thesis in the context of a step-wise model for the development of preeclampsia (modified from [60-63]). These results will be discussed in detail in the following sections.

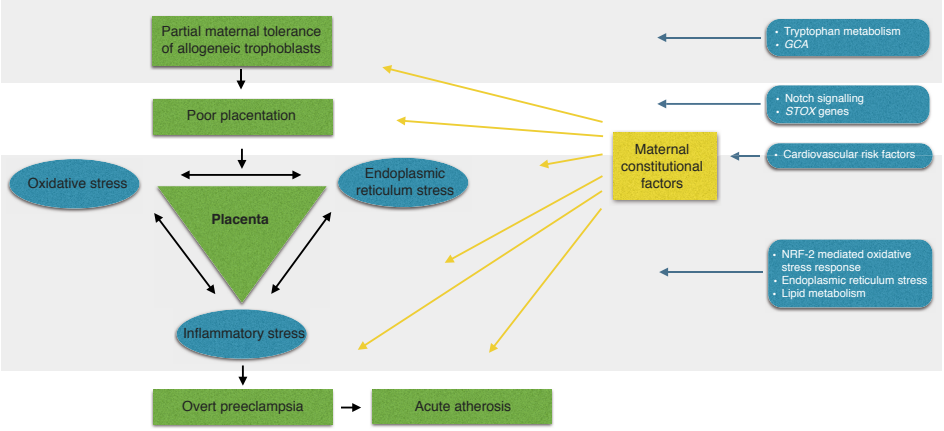

Figure 6. Summary of the major findings of this thesis in the context of a step-wise model for the development of preeclampsia (modified from [60-63]). Tryptophan metabolism (Paper I) and *GCA* (Paper IV) are known factors involved in immune maladaptation. Notch signalling (Paper I), *STOX* genes (Papers I and II) may be implicated in impaired placentation. The oxidative stress, ER stress and inflammatory response pathways (Papers I and III) in the placenta give rise to the inflammatory drive of preeclampsia. Acute atherosis is recently suggested as possible last stage of development of preeclampsia in some cases [62], and is suggested as a link to later life CVD (Papers I and IV). Constitutional risk factors for preeclampsia are similar to cardiovascular risk factors (Paper IV), and could possibly affect all the above-mentioned stages.

Immune maladaptation

Pathway analysis revealed that tryptophan metabolism was the most significant pathway among the differentially expressed transcripts (Paper I). Tryptophan metabolism, including the enzyme indoleamine 2,3-dioxygenase (IDO), is important for promoting immune tolerance at the maternal-fetal interface [218]. In preeclampsia, placental tryptophan degrading activity and placental IDO expression are reduced [219, 220]. In the absence of IDO, T_{reg} cells are reprogrammed to resemble the phenotype of the more

pro-inflammatory T helper (Th) cells Th17 [221], which are increased in preeclampsia [222]. Other tryptophan catabolites can directly modulate the functions of the immune system [223-226] and might therefore also be important in the immunoregulation of pregnancy and the development of preeclampsia. Correspondingly, decreased levels of L-kynurenine have been observed in preeclampsia [219]. We observed an increased expression of the transcript encoding the enzyme kynureninase (KYNU). KYNU metabolises L-kynurenine, which suppress proliferation of T- and NK cells and influences immunotolerance to foreign antigens. We speculate that increased levels of KYNU could contribute to decreased levels of L-kynurenine, which may impair the necessary immune modulation during pregnancy in preeclamptic women. This could potentially lead to deficient placentation, as mentioned in Section 1.5.

Previous findings from our collaborators' Australian/New Zealand cohorts have demonstrated that variations in the genes tumour necrosis factor superfamily, member 13b (*TNFSF13B*) and *GCA*, which both are involved in functions of the immune system, are associated with preeclampsia [227, 228]. *GCA* impacts the neutrophil adhesive properties to fibronectin [229], which promotes cytokines such as interleukin-8 (IL-8) to exert their chemotactic effects. This could contribute to the pronounced abundance of neutrophils in the maternal systemic vasculature of both preeclamptic [230] and obese [231] women. In Paper IV, we showed nominal associations between *GCA* and lower risk in adolescents born of a preeclamptic pregnancy and those having a mother with a prior preeclamptic pregnancy. Together with the results of Johnson et al. [228], this finding supports the emerging evidence of an immune component to preeclampsia pathophysiology [232]. However, our results were not significant after correction for multiple testing, and must be interpreted with some caution.

Impaired placentation

Notch signalling was one of the top significant pathways among the differentially expressed transcripts (Paper I) and included the four transcripts deltex homolog 3 (DTX3), hairy and enhancer of split 1 (HES1), notch homolog 3 (NOTCH3) and notch homolog 4 (NOTCH4). The involvement of Notch signalling has also been demonstrated in two global gene expression studies of placental tissue from

preeclamptic and non-preeclamptic women [233, 234]. The Notch signalling pathway is a conserved family of four transmembrane receptors (NOTCH1-4) and five ligands (DLL1/3/4 and JAG1/2). The pathway is important for embryonic development and normal development of the human placenta, and, as will be discussed in the next section, angiogenesis [196, 235, 236]. Both cytotrophoblasts and syncytiotrophoblasts have been shown to express NOTCH3 and NOTCH4, suggesting an involvement in the trophoblast differentiation program and invasion process of EVTs [196]. Moreover, protein analysis of placentas from preeclamptic women has indicated decreased levels of the Notch pathway members in all compartments of the placental villi [237]. We observed a down-regulation of both NOTCH3 and NOTCH4 in the preeclamptic cases compared to the controls, suggesting that both transcripts may be involved in the impaired trophoblast invasion observed in preeclampsia. However, NOTCH4 is reported to change expression with gestational age [199], and this might have affected our results.

STOX1 is believed to play a role in trophoblast dysfunction underlying a familial form of early-onset preeclampsia with FGR [238]. The preeclampsia susceptibility linkage locus for *STOX1* originally identified on chromosome 10q22 showed matrilineal inheritance [215]. Our genetic association analysis of candidate SNPs within *STOX1* (Paper II) could not confirm an association with preeclampsia, nor was differential gene expression of STOX1 observed in any of the case groups. We were not able to assess the proposed maternal imprinting effect on *STOX1*, which might have affected our results. In addition, the *STOX1* genetic association was observed in Dutch families. The variants identified in preeclampsia families, which by definition have a higher penetrance, may not be replicated in population-based studies. However, we observed differential expression of STOX2 in preeclamptic pregnancies complicated with FGR. Information on the biological role of *STOX2* is limited, and its plausibility as a candidate gene for preeclampsia rests largely on research done on *STOX1*, of which *STOX2* is a paralogue (and is expected to be involved in some of the same biological processes) [239, 240], and that *STOX2* resides close to or under a replicated preeclampsia susceptibility locus (4q31-q32) [134, 136, 139]. Thus, further molecular genetic studies are needed to evaluate the biological importance of our finding.

Angiogenic imbalance

Immunostaining of endothelial cells of human placentas has revealed intense immune-positivity of Notch family members [197], suggesting a role for the Notch signalling pathway in the placental angiogenesis process. In placentas from women with preeclampsia, the immunoreactivity of Notch proteins is decreased compared to that in placentas from non-preeclamptic women [237]. Consistently, all the four differentially expressed transcripts in the Notch signalling pathway were down-regulated in preeclamptic cases (Paper I). Hunkapiller et al. observed a correlation between absent jagged 1 (JAG1) expression in cytotrophoblasts and failed vascular remodelling in preeclampsia [241]. This is suggestive of an important functional role for Notch signalling in the remodelling of human spiral arterioles [241]. However, many questions remain to be answered concerning this possible relationship to determine the biological implications for preeclampsia. This includes an evaluation of possible causality, e.g., if decreased expression of Notch receptors and ligands in preeclampsia are prerequisites or by-products of failed endovascular invasion [241], as well as the clarification of up-stream signalling pathways or factors controlling the expression of Notch family members in human placental trophoblasts and blood vessels, which are largely unknown [196].

Cytochrome P450 (CYP)-dependent eicosanoids regulate vascular function, inflammation, and angiogenesis, all which are important factors in the pathogenesis of preeclampsia. We observed increased expression of CYP, family 2, subfamily J, polypeptide 2 (CYP2J2) in decidual tissue from preeclamptic cases compared to controls (Paper I). This is in accordance with findings in placental and decidual tissues from preeclamptic women published by Herse et al. [198]. These authors further localised CYP2J2 in trophoblastic villi and decidua at 12 weeks and term and observed that CYP2J2 metabolites were elevated in preeclamptic women compared to controls [198]. Furthermore, rat models have indicated reduced uterine perfusion related to increased levels of CYP2J2 protein expression [198]. Taken together, these results indicate that increased uteroplacental expression CYP2J2 could contribute to the disturbed spiral artery remodelling observed in preeclampsia.

Placental stress

There is substantial evidence of decidual oxidative stress in preeclamptic women [91-93, 107]. Pathway analysis demonstrated that the NRF2-mediated oxidative stress response was on the list of top pathways among the differentially expressed transcripts (Paper I). Our results suggest a possible role of NFR2 in preeclampsia, and this hypothesis is strengthened by similar findings of others [242, 243]. NRF2 plays an essential role in the defence against oxidative stress [244] by regulating the expression of antioxidant response elements (AREs) [89]. During excessive oxidative stress, ROS, NO and pro-inflammatory cytokines activate NRF2, which is translocated to the nucleus, where it binds to ARE sequences, leading to transcriptional activation of antioxidant genes, e.g., glutathione and heme oxygenase 1 (*HMOX1*) [245]. Several enzymes metabolise ROS to exportable compounds, and in our study, the antioxidant enzymes glutathione S-transferase (GST3), HMOX1 and ubiquitin-conjugating enzyme E2K (UBE2K) were up-regulated. HMOX1 has previously been suggested to have a role in the pathogenesis of preeclampsia [246, 247]. Using a subset of the decidua basalis samples in the PE/FGR Biobank (used in Papers I-III), Eide et al. demonstrated that HMOX1 protein expression was significantly increased in preeclamptic cases compared to controls [247]. In addition, these authors found elevated maternal serum HMOX1 levels in these preeclamptic women and a positive link between serum HMOX1 protein levels and the severity of maternal hypertension [247]. Taken together, the overall up-regulation (seven transcripts up-regulated, two transcripts down-regulated) of the transcripts within the NRF2-mediated oxidative stress response pathway might indicate a defensive response to increased oxidative stress in the decidua basalis observed in preeclampsia.

Our transcriptional data and pathway analyses suggest a central role of ER stress in the pathogenesis of preeclampsia (Paper I), preeclampsia with FGR and isolated FGR (Paper III). We observed up-regulation of key molecules in each of the signalling pathways of the UPR, including PERK (EIF2AK3), ATF6 and XBP1. Activation of the UPR has previously been associated with preeclampsia with FGR and isolated FGR in placental tissue [104], but our findings are novel because they were reported from decidua basalis tissue. Moreover, we identified increased activation of the UPR in

decidual tissue from preeclamptic women compared to controls at the protein level (Paper III). Yung et al. proposed ER stress as a mechanism responsible for the small placental phenotype observed in some cases of preeclampsia with FGR and FGR [102, 104]. Our data support this hypothesis as we observed a negative correlation between placental weight and protein levels of ER stress markers (Paper III). Recently, the nuclear factor (NRF)-family proteins were suggested to play a central role in activating UPR transcription [248]. Additionally, the authors showed that URP and oxidative stress responses were integrated through NRF [248]. These findings support our observations of direct relationships between genes involved in the ER stress- and NRF2-mediated oxidative stress response pathways in the network of preeclampsia-correlated genes (Paper I).

Acute atherosis was observed more frequently in our decidual tissue from cases (preeclampsia and/or FGR) compared to controls [159]. The mechanisms underlying the development of acute atherosis are largely unknown. However, a similarity with early atherosclerotic changes has led to the suggestion that acute atherosis is the final manifestation of several inflammatory processes [62]. Our transcriptional analyses (Papers I and III) indicate an integrated role of decidual ER stress, oxidative stress, and altered lipid metabolism in the generation of inflammation and the development of acute atherosis, which are observed in both preeclampsia and CVD. ER stress appears to have negative effects on endothelial cell stability, and it has been speculated that this may in part account for plaque susceptibility to rupture through ER-stress mediated apoptosis, promoting loss of the endothelial layer covering the plaque [249]. Statins are anti-atherosclerotic and stabilises vulnerable plaques, and have been speculated to potentially ameliorate acute atherosis [62]. Research in this field might lead to preventive drugs for the development of preeclampsia and later CVD in subsets of women with preeclampsia. Currently, the StAmP (Trial of provaStatin to Ameliorate early onset Pre-eclampsia) trial is testing statins for the treatment of early-onset preeclampsia [250].

Preeclampsia and CVD

The results of this thesis support the hypothesis that genetic mechanisms for preeclampsia and CVD are, at least in part, shared (Papers I, III and IV). In Paper I, the network of differentially expressed transcripts included 30 genes that were previously shown to be involved in CVD. Furthermore, the functional category 'CVD' included 21 genes involved in, e.g., hypertension (*HMOX1, NGF, REN*), atherosclerosis (*LDLR, PLA2G7*) and invasion of endothelial cells (*HPSE, THBS2, VEGFC*), according to the Ingenuity Pathways Knowledge Base (Ingenuity Systems, Redwood City, CA). In Paper IV, we observed a shared genetic nominal association between the four SNPs rs2322659 (*LCT*), rs35821928 (*LRP1B*), rs115015150 (*RND3*) and rs17783344 (*GCA*) and several cardiovascular risk factors. However, none of these association results satisfied our stringent correction for multiple testing, and consequently, the results must be interpreted with caution.

Preeclampsia and CVD share several metabolic abnormalities, such as an increase in small, dense low density lipoprotein (LDL) and triglycerides [251-253]. Thus, dyslipidaemia serves as a link between maternal constitutional risk factors (insulin resistance, pre-pregnancy obesity) for preeclampsia and the development of CVD later in life. Three of the pathways identified in Paper I represent lipid metabolism: linoleic acid metabolism, fatty acid metabolism, and arachidonic acid metabolism. Johansson et al. combined our gene expression data (Paper I) with genome-wide SNP genotyping and cardiovascular-related lipid phenotypic data and identified the down-regulation of acyl-Coenzyme A oxidase 2 (ACOX2) in our material as a common risk factor for preeclampsia and CVD [254]. ACOX2 is involved in the degradation of long branched fatty acids and the production of bile acids, and insufficient levels of ACOX2 may increase the fraction of bile acid intermediates relative to the fraction of mature bile acids [254]. Mature bile acids activate the farnesoid X receptor (FXR), leading to reduced levels of triglycerides [255], suggesting that FXR plays a critical role in lipid metabolism. The authors proposed that their observation of an inverse correlation between the transcript levels of ACOX2 and triglyceride levels implicates ACOX2 as a possible regulator of triglyceride levels through the FXR [254].

Pregnancy is a unique opportunity to identify women and offspring at increased risk of premature CVD [43], and clinical risk assessments and preventive programmes are under development [256]. However, the optimal follow-up regimen of women after a preeclamptic pregnancy remains uncertain and was discussed without any clear answers at a panel debate of invited clinicians at the most recent European Congress of the International Society for the Study of Hypertension in Pregnancy (ISSHP) (12-14 June 2013, Tromsø, Norway). In Norway, national guidelines for general practitioners recommend the evaluation of the risk for CVD 3-6 months post partum, with assessment of regular cardiovascular risk factors including measurements of blood pressure and weight and encouraging optimal weight range through diet, exercise and smoking cessation. A recent study indicated that lifestyle interventions after preeclampsia were likely to decrease future cardiovascular risk [257]. Nevertheless, the Norwegian national guideline for the primary prevention of CVD, NORRISK [258], does not address the association between preeclampsia and CVD in any form, which is similar to guidelines used in other European counties [259]. A few studies have investigated awareness and knowledge among physicians of future health risks (including CVD) after preeclampsia, long-term treatment after preeclampsia and the knowledge of current national guidelines [260-262]. The results of these studies are conflicting, ranging from good to limited knowledge among physicians, with a range of 50-80% of responders aware of this association [260-262]. These results indicate the need for further academic education and increased flow of information between the different medical specialities, including obstetricians, cardiologists and general practitioners. Hopefully, research results in the near future will provide a basis for sound guidelines for the follow-up of women with a history of preeclampsia. In the long term, genetic studies will ideally increase the understanding of the pathophysiology of preeclampsia and CVD, thereby improving the prediction, prevention and management of both disorders.

7. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The results of this thesis suggest that perturbed gene expression at the maternal-fetal interface and genetic polymorphisms are likely to explain some of the mechanisms involved in the pathogenesis of preeclampsia. We have demonstrated that immunological perturbations in preeclampsia might be linked to disturbed tryptophan metabolism. We have also confirmed that decidua basalis tissue from pregnancies complicated with preeclampsia is characterised by the co-occurrence of increased oxidative, ER, and inflammatory stress. The investigations in this thesis generated evidence for the involvement of metabolic pathways in preeclampsia, particularly the involvement of cardiovascular risk factors. Our findings support the hypothesis that preeclampsia shares some underlying genetic risk factors with CVD.

Some of these results are hypothesis generating, and further replication studies are required to elucidate the exact pathophysiological mechanisms involved in preeclampsia. Findings from gene expression studies must be verified, and protein analysis may confirm that the differences in mRNA expression between cases and controls are correlated with differences in protein levels. Genotype-level and functional studies (cell culture, animal models) may also be needed to confirm the involvement of the suspected genes/mechanisms in the pathogenesis of the disease [263]. Our results underpin preeclampsia as a complex disorder that involves different biological processes. We have identified possible preeclampsia susceptibility genes, each of which likely have a modest effect on the development of the disorder. Replication is needed to improve the statistical significance of the findings, decrease errors and biases, and affirm that the associations uncovered reflect processes that are biologically interesting, rather than methodological inadequacies [264].

The technological developments of the last decade have been striking, and new and powerful tools such as GWAS, exome sequencing and genome-wide sequencing are now being used to identify genetic variants, gene-gene and gene-environment interactions and underlying epigenetic mechanisms. A GWAS on a large (>5,000 cases), population-based cohort may further elicit genetic variations associated with

preeclampsia. Such studies require huge national and international investment. Consequently, co-authors of the manuscripts comprising this thesis are currently collaborating through the InterPregGen research study, which plans to use genome-wide approaches including HUNT2 samples from preeclamptic and non-preeclamptic women. These sample sizes might also enable subgroup analysis of preeclampsia phenotypes. The InterPregGen research study further plans to analyse maternal-fetal interactions, as investigations of both maternal and fetal genotypes are essential to increase our understanding of the genetics of preeclampsia. We investigated both maternal and fetal genotypes in Paper IV; however, we did not assess mother-offspring pairs, which could be performed in further studies. Combinations of SNPs that may explain the variation in preeclampsia could also be investigated in larger samples that are powered to detect such associations. The recent technological advances and efficiencies in high-throughput next-generation DNA/RNA sequencing have enabled deep gene sequencing. We plan to investigate some of the genes investigated in this thesis by re-sequencing unrelated preeclampsia and control women from Australian, Finnish and Norwegian populations. The steady decrease in cost will soon enable increased use of genome-wide sequencing, which might become the state-of-the-science for genetic dissection of complex traits. The use of this technology will also require the development of analytical tools to interpret the results; the statistical methods and ability to process data continue to lag far behind the ability to produce huge amounts of genomic data.

The understanding of preeclampsia pathophysiology has increased immensely in the last two decades, but only very modest changes to prevention, prediction and management have been presented [265]. To date, preeclampsia still lacks a reliable, early diagnostic marker and safe and effective treatment. Prediction of preeclampsia at an early stage would enable close surveillance and preventive strategies, which could have a great impact on maternal and fetal health. Biochemical markers have been tested throughout pregnancy for the prediction and diagnosis of preeclampsia, but no compelling evidence for the usefulness of these predictors has been reported [266, 267]. However, recent studies have yielded promising results [268, 269], and combinations of markers may help clinicians to identify women who should be followed more closely. Metabolomics

technology is a promising technique to characterise and identify biomarkers for preeclampsia [268]. Our research group is currently establishing metabolic profiles of body fluids (urine and serum) from women with and without preeclampsia by magnetic resonance (MR) metabolomics to identify potential clues to preeclampsia aetiology and pathogenesis, with promising results [270]. The identification of causal genetic factors underlying preeclampsia might enable fundamental insights into its pathophysiology and potentially lead to the development of better and more specific prediction and interventions to reduce the impact of preeclampsia on the community. A vital question is the cost-effective translation of genomic findings into improved health outcomes. This has proven difficult, and for most diseases, we are still far from personalised medicine informed by pharmacogenomics [271]. Current applications of genetic epidemiology have begun to lay the foundation for understanding the complex pathways that result in preeclampsia, but much work remains to obtain a complete picture. This thesis has presented novel investigations that have contributed to the current body of knowledge regarding preeclampsia and outlined potential directions for future research.

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Paper I

GENETICS

A transcriptional profile of the decidua in preeclampsia

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OBJECTIVE: We sought to obtain insight into possible mechanisms underlying preeclampsia using genomewide transcriptional profiling in decidua basalis.

STUDY DESIGN: Genomewide transcriptional profiling was performed on decidua basalis tissue from preeclamptic (n = 37) and normal (n = 58) pregnancies. Differentially expressed genes were identified and merged into canonical pathways and networks.

RESULTS: Of the 26,504 expressed transcripts detected, 455 were differentially expressed ($P < .05$; false discovery rate, $P < .1$). Both novel (ARL5B, SLITRK4) and previously reported preeclampsia-associated (PLA2G7, HMOX1) genes were identified. Pathway analysis revealed that tryptophan metabolism, endoplasmic reticulum stress, linoleic acid metabolism, notch signaling, fatty acid metabolism, arachidonic acid metabolism, and NRF2-mediated oxidative stress response were overrepresented canonical pathways.

CONCLUSION: In the present study single genes, canonical pathways, and gene-gene networks that are likely to play an important role in the pathogenesis of preeclampsia have been identified. Future functional studies are needed to accomplish a greater understanding of the mechanisms involved.

Key words: decidua, genomewide gene expression, microarray, preeclampsia

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The etiology of preeclampsia is not fully understood, but a number of observations suggest that divergent abnormalities may be involved (immunological, inflammatory, vascular/ischemic).[1] In a normal pregnancy extravillous trophoblasts (of fetal origin) invade decidua basalis and modify the spiral arteries. In preeclampsia, this pregnancy-associated adaption of spiral arteries may fail, with a hypoperfused placenta as a result. Oxidative stress is suggested to play a central role in the pathogenesis of preeclampsia,[2] and may be generated in the decidua basalis.[3,4] Heritability of the disease has been estimated to be >50%,[5,6] with both maternal and fetal (paternal) contributions.[7]

Microarray-based transcriptional profiling can be a powerful strategy for identification of disease-related genes and pathways,[8] and this approach has been used for analysis of placental[9] as well as decidual[6,10,11] tissues from preeclamptic pregnancies. However, the data obtained have been inconsistent. In the case of the 3 decidual studies reported, the diverging results may be due to the relatively small number of samples analyzed (≤12 preeclamptic samples included).[6,10,11] In the current study, we have applied genomewide transcriptional profiling (measuring ≥48,000 transcripts from all known genes) on a large collection of decidual samples (from 37 preeclamptic and 58 normal pregnancies) to comprehensively investigate how gene expression at the maternal-fetal interface may be contributing to the pathogenesis of preeclampsia. We further aimed to identify the genetic canonical pathways and gene-gene interaction networks represented by the differently expressed genes using contemporary bioinformatic approaches.

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The first 2 authors contributed equally to this work.

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Materials and Methods

Human subjects

Women with pregnancies complicated by preeclampsia (n = 43) and women with normal pregnancies (n = 59) were recruited at St. Olav's University Hospital (Trondheim, Norway) and Haukeland University Hospital (Bergen, Norway) from 2002 through 2006. Preeclampsia was defined as persistent hypertension (blood pressure of ≥140/90 mm Hg) plus

proteinuria (≥0.3 g/L or ≥1+ by dipstick) developing >20 weeks of pregnancy.[12] Due to tissue sampling procedures, only pregnancies delivered by cesarean section were included. Women with preeclamptic pregnancies had cesarean section performed for medical indications, whereas women with normal pregnancies underwent cesarean section for reasons considered irrelevant to the aim of the study (eg, breech presentation, cephalopelvic disproportion in previous delivery, and fear of vaginal delivery). None of the included mothers were in labor prior to cesarean section. Exclusively healthy women with no history of preeclampsia were accepted in the normal pregnancy group. Multiple pregnancies, pregnancies with chromosomal aberrations, fetal and placental structural abnormalities, or suspected perinatal infections were excluded from both study groups. The study was approved by the Norwegian Regional Committee for Medical Research Ethics. Informed consent was obtained from all participants prior to collection of decidual samples.

Decidual tissue collection

Samples of decidua basalis tissue were obtained by vacuum suction of the placental bed, a procedure that allows the collection of tissue from the whole placental bed.[13] Collected samples were flushed with saline solution to remove excessive blood. The decidual tissue was immediately submerged in RNA-later (Ambion, Austin, TX).

Total RNA isolation

Total RNA was isolated using a TRIzol (Invitrogen, Carlsbad, CA) extraction protocol with chloroform interphase separation, isopropanol precipitation, and ethanol wash steps. Precipitated total RNA was resuspended in Rnase-free water and purified with an RNeasy Mini Kit using spin technology (Qiagen, Valencia, CA). Spectrophotometric determination of purified total RNA yield (μg) was performed using the NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE). Total RNA quality was measured using RNA 6000 Nano Series II Kit on a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA). Ethical approval for total RNA processing and decidua expression analysis was obtained from the institutional review board at the University of Texas Health Science Center in San Antonio.

Synthesis, amplification, and purification of antisense RNA

Antisense RNA (aRNA) was synthesized, amplified, and purified using the Illumina TotalPrep RNA Amplification Kit according to manufacturer's instructions (Ambion, Austin, TX). Synthesis of aRNA was performed using a T7 Oligo(dT) primer, and the amplification underwent in vitro transcription with a T7 RNA polymerase to generate multiple copies of biotinylated aRNA from a double-stranded complementary DNA (cDNA) template. Purified aRNA yield was determined spectrophotometrically using the NanoDrop ND-1000.

Microarray data

Purified aRNA was hybridized to Illumina's HumanWG-6 v2 Expression BeadChip (Illumina Inc, San Diego, CA). Washing, blocking, and transcript signal detection (streptavidin-Cy3) was performed using Illumina's 6 × 2 BeadChip protocol. Samples were scanned on the Illumina BeadArray 500GX Reader using Illumina BeadScan image data acquisition software (version 2.3.0.13). Illumina's BeadStudio Gene Expression software module (version 3.2.7) was used to subtract background noise signals and generate an output file for statistical analysis.

Real-time quantitative polymerase chain reaction

We performed a verification of the microarray experiment with quantitative real-time (RT)-polymerase chain reaction (PCR) on 6 of the most differentially expressed transcripts using a 7900HT Fast RT-PCR instrument (Applied Biosystems, Foster City, CA). The 6 genes were prioritized for RT-PCR based on beta values, false discovery rate (FDR) *P* values, and manual literature searches. RT quantitative PCR was run with 93 samples. Two of the total collection of 95 samples were excluded due to shortage of biological material. Preoptimized TaqMan Gene Expression Assays (Applied Biosystems) were run, in triplicate, to measure messenger RNA expression levels relative to the reference genes, TATA box binding protein and glyceraldehyde-3-phosphate dehydrogenase. Reverse transcription and PCR amplification was performed in a 2-step procedure, following Applied Biosystems High-Capacity cDNA ReverseTranscription Kit Protocol and TaqMan Gene Expression Master Mix Protocol. Negative controls were run, in triplicate, without RT enzyme or no cDNA template.

Statistical analysis

Transcript data for each sample were preprocessed and analyzed using our Sequential Oligogenic Linkage Analysis Routines (SOLAR) statistical analysis software program,[14] as previously described.[15] To evaluate the magnitude of differential gene expression the displacement of each detected transcript's mean expression value was measured between the 2 groups. A standard regression analysis was performed on the preeclamptic group to test whether the mean transcription level differed from that of the normal pregnancy group.

The messenger RNA expression levels were calculated by the Comparative threshold cycle (CT) method, as described elsewhere.[16] For each target gene, the mean CT value for each sample was used for analysis, after exclusion of outliers. Outliers were determined as values >2SD from the mean. Delta CT (ΔCT) values were computed as the difference between the given mean value for a target gene and the mean of the CT values for the 2 reference genes.[17] Fold change values were calculated, based on the differences in ΔCT values between tissue from preeclamptic women and women with normal pregnancy ($2^{-\Delta\Delta CT}$).[16] A *t* test statistic (SPSS, version 16; SPSS, Inc, Chicago, IL) evaluated the difference between the ΔCT values of the preeclamptic pregnancies, compared with the normal pregnancy group. Analyzing for the 2 reference genes separately did not change the results.

Canonical pathway and network identification

Differentially expressed transcripts in the preeclamptic group ($P < .05$; FDR,[18]

$P < .1$) were imported into Ingenuity Pathways Analysis (IPA, v7.5; Ingenuity Systems, Redwood City, CA). Transcripts' gene identifiers were mapped to their corresponding gene object in the Ingenuity Pathways Knowledge Base. IPA was used to bioinformatically identify canonical (ie, cell signaling and metabolic) pathways and gene-gene interaction networks potentially involved in preeclampsia within our dataset. IPA gene-gene networks were constructed from the published literature, and they diagrammatically represent molecular relationships between gene-gene products.

Significant IPA pathways were further analyzed with Rotation Gene Set Enrichment Analysis (ROMER; Fred Hutchinson Cancer Research Center, Seattle, WA) pathway analysis, using the *limma* package, available via the Bioconductor Project (Fred Hutchinson Cancer Research Center).[19]

RESULTS

Human subjects

The clinical information of women/pregnancies enrolled is presented in Table 1. Only those samples of sufficient RNA quality for gene expression analysis have been included. In the preeclamptic pregnancies, both mean gestational age and birthweight were lower than in the normal pregnancies (Table 1). As expected, the mean blood pressure was higher among preeclamptic than normal pregnancies (Table 1).

Decidual genomewide transcriptional profiling

In total, 43 women with pregnancies complicated by preeclampsia and 59 women with normal pregnancies were included in the study. Six samples from preeclamptic pregnancies and 1 sample from a normal pregnancy were excluded from gene expression analyses due to low RNA quality. The 95 samples with good RNA quality were hybridized onto Illumina's HumanWG-6 v2 genomewide Expression BeadChip.

The nonnormalized decidua basalis transcriptional profile data (n = 48,095) may be found at ArrayExpress (European Molecular Biology Laboratory-European Bioinformatics Institute, Hinxton, UK) (accession code E-TABM-682). We detected 26,504 significantly expressed transcripts (55.1%), of which 455 were differentially expressed after FDR correction ($P < .05$; FDR, $P < .1$); 285 were down-regulated and 170 were up-regulated. The significant differentially expressed transcripts are presented in Table 2, together with the corresponding *P* values (raw and FDR adjusted) and preeclampsia-correlated expression. The RT quantitative PCR for the 6 genes (PLA2G7, ANGPTL2, MAN1A2, SLITRK4, FZD4, and ARL5B) tested showed a high grade of correlation with the microarray data (Table 3).

TABLE 1
Clinical characteristics of study groups

Variable	Preeclamptic pregnancies[a] (n = 37)	Normal pregnancies[a] (n = 58)
Gestational age, wk	32 ± 4[b]	39 ± 1
Systolic blood pressure, mm Hg	152 ± 16[b]	116 ± 10
Diastolic blood pressure, mm Hg	96 ± 10[b]	70 ± 9.0
Birthweight, g	1555 ± 769[b]	3619 ± 469
Body mass index, kg/m^2[c]	27.7 ± 6.2	25.3 ± 5.7

Values are means ± SD.

[a] In all, 43 women with pregnancies complicated by preeclampsia and 59 women with normal pregnancies were included in the study. Six samples from preeclamptic pregnancies and 1 sample from normal pregnancy were excluded from gene expression analysis due to low RNA quality; [b] $P < .001$ obtained with *t* test statistics with software (SPSS, version 16; SPSS, Inc, Chicago, IL); [c] Body mass index was measured at first antenatal care visit.

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Canonical pathways and network

The 455 differentially expressed transcripts were analyzed using IPA. The significant canonical pathways ($P < .01$) are shown in Table 4, along with the included genes and *P* values. They included tryptophan metabolism, endoplasmic reticulum (ER) stress, linoleic acid metabolism, notch signaling, fatty acid metabolism, arachidonic acid metabolism, and NRF2-mediated oxidative stress response. All the canonical pathways identified in IPA were also found to be significant ($P < .01$) using ROMER (Table 4), with the exception of the NRF2-mediated oxidative stress response canonical pathway (IPA, $P = .009$; ROMER, $P = .067$).

Using network analysis in IPA, 59 of the preeclampsia-associated genes could be connected into a single network of gene-gene product interactions (Figure). The genes in this network were among others involved in the function of ER, oxidative stress, notch signaling, and cell migration. The network included a cluster of 15 up-regulated genes (ATP2A2, TRAM1, FKBP2, HMOX1, SPCS2, ATF6, DNAJC3, EIF2AK3, PIGA, SEC23B, SEC24D, DNAJB9, SRPRB, DNAJB11, and X-box binding protein 1 [XBP1]) associated with ER stress and oxidative stress (Figure). All these genes were in a direct relationship to XBP1. Epidermal growth factor receptor (EGFR) was another focus molecule with a direct relationship to 7 other genes (PLCG1, NGF, MET, LRIG1, SLN, ATP2A2, and SHC2) in the network.

COMMENT

In this study, 455 differentially expressed transcripts were found when decidua basalis tissue from preeclamptic and normal pregnancies was compared. Some transcripts were novel findings (ie, ARL5B and SLITRK4), whereas others, such as PLA2G7[20] and HMOX1,[21,22] have been reported to be associated with preeclampsia previously. Pathway analysis identified 7 significant canonical pathways.

In our patient cohort, a lower gestational age was found in the preeclamptic group (average, 32 weeks; range, 28–36) compared with the normal pregnancy group (39 weeks; range, 38–40). This is not unexpected due to the need for early delivery in patients with severe preeclampsia. Since gene expression in uteroplacental tissues

TABLE 2
Differentially expressed transcripts[a]

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1782259	NM_173078.2	SLITRK4	SLIT and NTRK-like family, member 4	X	−1.0363	4.6×10^{-8}	.0012
ILMN_1680465	NM_178815.3	ARL5B	ADP-ribosylation factor-like 5B	10	0.9122	4.5×10^{-7}	.0039
ILMN_1743367	NM_012193.2	FZD4	Frizzled homolog 4 (Drosophila)	11	−0.9122	4.1×10^{-7}	.0054
ILMN_1726210	NM_178172.2	LOC338328	High density lipoprotein–binding protein	8	−0.8672	3.7×10^{-6}	.0088
ILMN_1709222	NM_005692.3	ABCF2	ATP-binding cassette, subfamily F (GCN20), member 2, nuclear gene encoding mitochondrial protein, transcript variant 2	7	−0.8634	3.5×10^{-6}	.0093
ILMN_1772612	NM_012098.2	ANGPTL2	Angiopoietin-like 2	9	−0.8884	4.4×10^{-6}	.0097
ILMN_1659792	NM_014213.2	HOXD9	Homeobox D9	2	−0.8541	3.5×10^{-6}	.0102
ILMN_1813295	NM_018640.3	LMO3	LIM domain only 3 (rhombotin-like 2), transcript variant 1	12	−0.8992	3.3×10^{-6}	.0110
ILMN_1669023	NM_020482.3	FHL5	Four and a half LIM domains 5	6	−0.8489	3.2×10^{-6}	.0123
ILMN_1658677	NM_178502.2	DTX3	Deltex 3 homolog (Drosophila)	12	−0.9038	2.9×10^{-6}	.0130
ILMN_1812461	NM_003881.2	WISP2	WNT1 inducible signaling pathway protein 2	20	−0.8717	6.6×10^{-6}	.0134
ILMN_1776157	NM_080415.1	SEPT4	Septin 4, transcript variant 2	17	−0.8882	2.6×10^{-6}	.0140
ILMN_1794370	NM_001031702.2	SEMA5B	Sema domain, 7 thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B, transcript variant 1	3	−0.8695	8.0×10^{-6}	.0141
ILMN_1719069	NM_213596.1	FOXN4	Forkhead box N4	12	−0.8803	7.8×10^{-6}	.0147
ILMN_1733667	NM_021931.2	DHX35	DEAH (Asp-Glu-Ala-His) box polypeptide 35	20	−0.8537	9.0×10^{-6}	.0149
ILMN_1734276	NM_199169.1	TMEPAI	Transmembrane, prostate androgen-induced RNA, transcript variant 2	20	−0.8360	1.6×10^{-5}	.0153
ILMN_1701195	NM_005084.2	PLA2G7	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	6	0.8305	1.6×10^{-5}	.0155
ILMN_1687821	NM_033201.1	C16orf45	Chromosome 16 open reading frame 45	16	−0.8218	1.4×10^{-5}	.0156
ILMN_1736911	NM_003275.2	TMOD1	Tropomodulin 1	9	−0.8178	1.5×10^{-5}	.0157
ILMN_1744487	NM_015645.2	C1QTNF5	C1q and tumor necrosis factor-related protein 5	11	−0.8113	1.7×10^{-5}	.0157
ILMN_1767556	NM_007021.2	C10orf10	Chromosome 10 open reading frame 10	10	−0.7966	1.3×10^{-5}	.0158
ILMN_1668249	NM_022773.2	TMEM112	Transmembrane protein 112	16	−0.8079	1.6×10^{-5}	.0158
ILMN_1788462	NM_001033059.1	AMD1	Adenosylmethionine decarboxylase 1, transcript variant 2	6	0.8027	1.4×10^{-5}	.0160
ILMN_1665945	NM_022735.3	ACBD3	acyl-Coenzyme A binding domain containing 3	1	0.8297	1.3×10^{-5}	.0164

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TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1657803	NM_001014975.1	CFH	Complement factor H, transcript variant 2	1	−0.8780	2.5×10^{-6}	.0164
ILMN_1880012	NM_003966.2	SEMA5A	Sema domain, 7 thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	5	0.8208	2.7×10^{-5}	.0168
ILMN_1763036	NM_001286.2	CLCN6	Chloride channel 6, transcript variant ClC-6a	1	−0.8027	2.4×10^{-5}	.0170
ILMN_1710962	NM_014573.2	TMEM97	Transmembrane protein 97	17	0.8236	2.6×10^{-5}	.0171
ILMN_1801927	NM_001004311.2	FIGLA	Folliculogenesis-specific basic helix-loop-helix	2	−0.8616	1.1×10^{-5}	.0171
ILMN_1673773	NM_198516.1	GALNTL4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 4	11	−0.7998	2.3×10^{-5}	.0172
ILMN_1711516	NM_001690.2	ATP6V1A	ATPase, H+ transporting, lysosomal 70 kDa, V1 subunit A	3	0.8101	1.2×10^{-5}	.0172
ILMN_1715555	NM_001352.2	DBP	D site of albumin promoter (albumin D-box) binding protein	19	−0.7916	1.3×10^{-5}	.0172
ILMN_1779632	NM_001001723.1	TMEM1	Transmembrane protein 1, transcript variant 2	21	0.8054	2.6×10^{-5}	.0172
ILMN_1685703	NM_003500.2	ACOX2	acyl-Coenzyme A oxidase 2, branched chain	3	−0.8253	2.2×10^{-5}	.0173
ILMN_1711157	NM_004557.3	NOTCH4	Notch homolog 4 (Drosophila)	6	−0.7709	2.5×10^{-5}	.0174
ILMN_1740160	NM_182811.1	PLCG1	Phospholipase C, gamma 1, transcript variant 2	20	−0.8077	2.1×10^{-5}	.0176
ILMN_1834017	N25708	Hs.573236	yx79f04s1 Soares melanocyte 2NbHM cDNA clone IMAGE: 267967 3 sequence		0.8058	2.3×10^{-5}	.0176
ILMN_1798076	NM_006898.4	HOXD3	Homeobox D3	2	−0.8238	2.3×10^{-5}	.0176
ILMN_1705985	NM_020473.2	PIGA	Phosphatidylinositol glycan anchor biosynthesis, class A (paroxysmal nocturnal hemoglobinuria), transcript variant 3	X	0.7983	2.6×10^{-5}	.0177
ILMN_1772302	NM_006441.1	MTHFS	5,10-Methenyltetrahydrofolate synthetase (5-formyltetrahydrofolate cyclo-ligase)	15	0.7802	2.9×10^{-5}	.0178
ILMN_1781791	NM_000950.1	PRRG1	Proline-rich Gla (G-carboxyglutamic acid) 1	X	0.7681	3.2×10^{-5}	.0179
ILMN_1748812	NM_152913.1	TMEM130	Transmembrane protein 130	7	−0.7814	3.0×10^{-5}	.0179
ILMN_1680774	XM_001132373.1	LOC730994	Similar to NACHT, leucine-rich repeat and PYD (pyrin domain) containing 1, transcript variant 1	17	−0.8034	2.0×10^{-5}	.0179
ILMN_1755120	NM_006699.3	MAN1A2	Mannosidase, alpha, class 1A, member 2	1	0.8519	1.3×10^{-5}	.0180

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1788166	NM_003318.3	TTK	TTK protein kinase	6	0.8169	2.1×10^{-5}	.0181
ILMN_1685608	NM_002523.1	NPTX2	Neuronal pentraxin II	7	−0.7865	3.1×10^{-5}	.0181
ILMN_1678842	NM_003247.2	THBS2	Thrombospondin 2	6	−0.8054	3.2×10^{-5}	.0182
ILMN_1813430	NM_182985.3	TRIM69	Tripartite motif-containing 69, transcript variant a	15	−0.8120	3.5×10^{-5}	.0192
ILMN_1675936	NM_016438.2	HIGD1B	HIG1 domain family, member 1B	17	−0.8060	3.9×10^{-5}	.0202
ILMN_1877909	BX105647	Hs.125533	BX105647 Soares_NFL_T_GBC_S1 cDNA clone IMAGp998F143713 sequence		−0.7992	3.7×10^{-5}	.0202
ILMN_1803279	NM_016040.3	TMED5	Transmembrane emp24 protein transport domain containing 5	1	0.7904	3.8×10^{-5}	.0202
ILMN_1700202	NM_022918.2	TMEM135	Transmembrane protein 135	11	0.7615	4.1×10^{-5}	.0206
ILMN_1727589	NM_004605.2	SULT2B1	Sulfotransferase family, cytosolic, 2B, member 1, transcript variant 1	19	0.7826	4.2×10^{-5}	.0209
ILMN_1811873	NM_002889.2	RARRES2	Retinoic acid receptor responder (tazarotene induced) 2	7	−0.7690	4.4×10^{-5}	.0214
ILMN_1703955	NM_148177.1	FBXO32	F-box protein 32, transcript variant 2	8	−0.8049	4.8×10^{-5}	.0225
ILMN_1731358	NM_018181.4	ZNF532	Zinc finger protein 532	18	−0.7974	4.7×10^{-5}	.0226
ILMN_1682937	NM_001038633.2	RSPO1	R-spondin homolog (Xenopus laevis)	1	−0.7973	5.0×10^{-5}	.0230
ILMN_1695947	NM_174934.2	SCN4B	Sodium channel, voltage-gated, type IV, beta	11	−0.7948	5.8×10^{-5}	.0234
ILMN_1707342	NM_015541.2	LRIG1	Leucine-rich repeats and immunoglobulin-like domains 1	3	−0.7679	5.8×10^{-5}	.0235
ILMN_1781626	NM_001734.2	C1S	Complement component 1, s subcomponent, transcript variant 1	12	−0.7833	5.7×10^{-5}	.0236
ILMN_1676215	NM_001364.2	DLG2	Discs, large homolog 2, chapsyn-110 (Drosophila)	11	−0.7928	5.6×10^{-5}	.0238
ILMN_1880210	BC038188	Hs.179213	Homo sapiens, clone IMAGE: 3451765		0.7666	5.7×10^{-5}	.0239
ILMN_1767225	NM_006092.1	NOD1	Nucleotide-binding oligomerization domain containing 1	7	−0.7808	5.3×10^{-5}	.0239
ILMN_1793410	NM_021021.2	SNTB1	Syntrophin, beta 1 (dystrophin-associated protein A1, 59 kDa, basic component 1)	8	0.7636	5.5×10^{-5}	.0239
ILMN_1752837	NM_018184.2	ARL8B	ADP-ribosylation factor-like 8B	3	0.7644	5.3×10^{-5}	.0241
ILMN_1791949	NM_032507.2	PGBD1	PiggyBac transposable element-derived 1	6	−0.7478	5.5×10^{-5}	.0243
ILMN_1859863	BM458075	Hs.555181	AGENCOURT_6411402 NIH_MGC_71 cDNA clone IMAGE:5530423 5 sequence		0.7667	6.4×10^{-5}	.0248

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1782788	NM_003651.3	CSDA	Cold shock domain protein A	12	−0.7756	6.3×10^{-5}	.0251
ILMN_1727740	NM_006372.3	SYNCRIP	Synaptotagmin binding, cytoplasmic RNA interacting protein	6	0.6949	6.7×10^{-5}	.0253
ILMN_1677396	NM_019080.1	NDFIP2	Nedd4 family interacting protein 2	13	0.7591	6.6×10^{-5}	.0253
ILMN_1744191	NM_003042.2	SLC6A1	Solute carrier family 6 (neurotransmitter transporter, GABA), member 1	3	−0.7914	6.9×10^{-5}	.0253
ILMN_1656129	NM_020342.1	SLC39A10	Solute carrier family 39 (zinc transporter), member 10	2	0.7306	6.8×10^{-5}	.0253
ILMN_1809639	NM_178505.5	TMEM26	Transmembrane protein 26	10	0.7732	7.9×10^{-5}	.0287
ILMN_1786326	NM_024076.1	KCTD15	Potassium channel tetramerization domain containing 15	19	−0.7853	8.2×10^{-5}	.0291
ILMN_1651343	NM_001004439.1	ITGA11	Integrin, alpha 11	15	−0.7812	8.2×10^{-5}	.0292
ILMN_1739887	NM_031491.2	RBP5	Retinol-binding protein 5, cellular	12	−0.7607	8.7×10^{-5}	.0304
ILMN_1716247	NM_203371.1	FIBIN	Fin bud initiation factor	11	−0.7760	8.9×10^{-5}	.0307
ILMN_1752668	NM_015345.2	DAAM2	Disheveled-associated activator of morphogenesis 2	6	−0.7617	1.0×10^{-4}	.0309
ILMN_1789243	NM_018668.3	VPS33B	Vacuolar protein sorting 33 homolog B (yeast)	15	−0.7368	1.0×10^{-4}	.0312
ILMN_1763852	NM_001093.3	ACACB	acetyl-Coenzyme A carboxylase beta	12	−0.7651	9.6×10^{-5}	.0314
ILMN_1731561	NM_022370.2	ROBO3	Roundabout, axon guidance receptor, homolog 3 (Drosophila)	11	−0.7335	1.0×10^{-4}	.0314
ILMN_1672635	NM_182947.2	GEFT	RhoA/RAC/CDC42 exchange factor, transcript variant 1	12	−0.7711	9.3×10^{-5}	.0315
ILMN_1691181	NM_030755.4	TXNDC1	Thioredoxin domain containing 1	14	0.7498	1.1×10^{-4}	.0315
ILMN_1742034	NM_000261.1	MYOC	Myocilin, trabecular meshwork-inducible glucocorticoid response	1	−0.7416	1.0×10^{-4}	.0315
ILMN_1761968	NM_033256.1	PPP1R14A	Protein phosphatase 1, regulatory (inhibitor) subunit 14A	19	−0.7785	9.5×10^{-5}	.0315
ILMN_1703142	NM_001005416.1	MARCH2	Membrane-associated ring finger (C3HC4) 2, transcript variant 3	19	−0.7337	1.0×10^{-4}	.0316
ILMN_1752225	NR_002330.1	ST7OT1	ST7 overlapping transcript 1 (antisense noncoding RNA)	7	−0.7606	9.8×10^{-5}	.0318
ILMN_1667692	NM_000961.3	PTGIS	Prostaglandin I2 (prostacyclin) synthase	20	−0.7787	9.5×10^{-5}	.0318
ILMN_1691457	NM_004900.3	APOBEC3B	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	22	0.7343	1.0×10^{-4}	.0319

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1728979	NM_207310.1	CCDC74B	Coiled-coil domain containing 74B	2	−0.7428	1.2×10^{-4}	.0320
ILMN_1688346	NM_176814.3	ZNF800	Zinc finger protein 800	7	0.7259	1.2×10^{-4}	.0323
ILMN_1682428	NM_144584.1	C1orf59	Chromosome 1 open reading frame 59	1	0.7635	1.2×10^{-4}	.0323
ILMN_1755173	NM_020904.1	PLEKHA4	Pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 4	19	−0.7470	1.1×10^{-4}	.0324
ILMN_1782954	NM_005339.3	UBE2K	Ubiquitin-conjugating enzyme E2-25K	4	0.7190	1.2×10^{-4}	.0324
ILMN_1735996	NM_016931.2	NOX4	NADPH oxidase 4	11	−0.7504	1.3×10^{-4}	.0325
ILMN_1680110	NM_006829.2	C10orf116	Chromosome 10 open reading frame 116	10	−0.7497	1.2×10^{-4}	.0325
ILMN_1755832	NM_000435.2	NOTCH3	Notch homolog 3 (Drosophila)	19	−0.7589	1.1×10^{-4}	.0325
ILMN_1800463	NM_017859.2	UCKL1	Uridine-cytidine kinase 1-like 1	20	−0.7338	1.2×10^{-4}	.0326
ILMN_1674337	NM_004470.2	FKBP2	FK506 binding protein 2, 13 kDa, transcript variant 1	11	0.7401	1.2×10^{-4}	.0327
ILMN_1807171	NM_000929.2	PLA2G5	Phospholipase A2, group V	1	−0.7349	1.3×10^{-4}	.0327
ILMN_1724671	NM_207577.1	MAP6	Microtubule-associated protein 6, transcript variant 2	11	−0.7623	1.2×10^{-4}	.0328
ILMN_1655117	NM_025132.3	WDR19	WD repeat domain 19	4	−0.7425	1.3×10^{-4}	.0328
ILMN_1706511	NM_003216.2	TEF	Thyrotrophic embryonic factor	22	−0.7288	1.1×10^{-4}	.0328
ILMN_1677018	NM_002141.4	HOXA4	Homeobox A4	7	−0.7424	1.3×10^{-4}	.0333
ILMN_1785646	NM_153321.1	PMP22	Peripheral myelin protein 22, transcript variant 2	17	−0.7487	1.3×10^{-4}	.0334
ILMN_1709661	NM_145276.1	ZNF563	Zinc finger protein 563	19	−0.7481	1.4×10^{-4}	.0334
ILMN_1736863	NM_018295.2	TMEM140	Transmembrane protein 140	7	−0.7336	1.3×10^{-4}	.0337
ILMN_1807379	NM_023034.1	WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1, transcript variant long	8	0.7237	1.4×10^{-4}	.0338
ILMN_1740842	NM_005407.1	SALL2	Sal-like 2 (Drosophila)	14	−0.7458	1.4×10^{-4}	.0340
ILMN_1734229	NM_032802.3	SPPL2A	Signal peptide peptidase-like 2A	15	0.7168	1.4×10^{-4}	.0343
ILMN_1696003	NM_006496.1	GNAI3	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	1	0.7101	1.5×10^{-4}	.0343
ILMN_1793770	NM_058246.3	DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6, transcript variant 1	7	−0.7448	1.4×10^{-4}	.0343
ILMN_1797861	NM_002184.2	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor), transcript variant 1	5	0.7406	1.6×10^{-4}	.0353
ILMN_1720865	NM_145798.2	OSBPL7	Oxysterol binding protein-like 7, transcript variant 1	17	−0.7298	1.6×10^{-4}	.0355
ILMN_1713978	NM_006923.2	SDF2	Stromal cell-derived factor 2	17	0.7243	1.6×10^{-4}	.0356

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1682231	NM_001003682.2	TTMB	TTMB protein	1	−0.7581	1.6×10^{-4}	.0356
ILMN_1684554	NM_001856.3	COL16A1	Collagen, type XVI, alpha 1	1	−0.7316	1.5×10^{-4}	.0356
ILMN_1778595	NM_003063.2	SLN	Sarcolipin	11	−0.7375	1.6×10^{-4}	.0356
ILMN_1811790	NM_004118.3	FKHL18	Forkhead-like 18 (Drosophila)	20	−0.7197	1.6×10^{-4}	.0357
ILMN_1712461	NM_004352.1	CBLN1	Cerebellin 1 precursor	16	−0.7413	1.5×10^{-4}	.0358
ILMN_1815874	NM_018946.2	NANS	N-acetylneuraminic acid synthase (sialic acid synthase)	9	0.7205	1.7×10^{-4}	.0359
ILMN_1720819	XM_934796.2	LOC653566	Similar to signal peptidase complex subunit 2 (microsomal signal peptidase 25-kDa subunit) (SPase 25-kDa subunit), transcript variant 3	1	0.6515	1.7×10^{-4}	.0359
ILMN_1669898	NM_201446.1	EGFL7	EGF-like-domain, multiple 7, transcript variant 2	9	−0.6935	1.5×10^{-4}	.0359
ILMN_1740441	NM_000398.4	CYB5R3	Cytochrome b5 reductase 3, transcript variant M	22	−0.7263	1.7×10^{-4}	.0360
ILMN_1700274	NM_031442.2	TMEM47	Transmembrane protein 47	X	−0.7303	1.6×10^{-4}	.0360
ILMN_1720889	NM_001017369.1	SC4MOL	Sterol-C4-methyl oxidase-like, transcript variant 2	4	0.6822	1.7×10^{-4}	.0367
ILMN_1793543	NM_144697.2	C1orf51	Chromosome 1 open reading frame 51	1	−0.7115	1.8×10^{-4}	.0376
ILMN_1734288	NM_152511.3	DUSP18	Dual specificity phosphatase 18	22	−0.7243	1.9×10^{-4}	.0383
ILMN_1678998	NM_014665.1	LRRC14	Leucine-rich repeat containing 14	8	−0.7119	1.9×10^{-4}	.0383
ILMN_1791508	NM_024302.3	MMP28	Matrix metallopeptidase 28, transcript variant 1	17	−0.7246	1.9×10^{-4}	.0385
ILMN_1688295	NM_016423.1	ZNF219	Zinc finger protein 219	14	−0.7437	1.9×10^{-4}	.0388
ILMN_1770293	NM_001730.3	KLF5	Kruppel-like factor 5 (intestinal)	13	0.7122	1.9×10^{-4}	.0388
ILMN_1886424	BG621061	Hs.559870	602616941F1 NIH_MGC_79 cDNA clone IMAGE:4730410 5 sequence		−0.7236	1.9×10^{-4}	.0388
ILMN_1697006	XM_930748.2	LOC642361	Hypothetical protein LOC642361	10	−0.6926	2.2×10^{-4}	.0400
ILMN_1673543	NM_018290.2	PGM2	Phosphoglucomutase 2	4	0.6845	2.0×10^{-4}	.0401
ILMN_1742230	NM_182648.1	BAZ1A	Bromodomain adjacent to zinc finger domain, 1A, transcript variant 2	14	0.7376	2.1×10^{-4}	.0401
ILMN_1659843	NM_006260.2	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	13	0.7094	2.1×10^{-4}	.0401
ILMN_1696585	NM_017671.4	C20orf42	Chromosome 20 open reading frame 42	20	0.7335	2.1×10^{-4}	.0402
ILMN_1763641	NM_025040.2	ZNF614	Zinc finger protein 614	19	0.7013	2.1×10^{-4}	.0402
ILMN_1726678	NM_014147.1	HSPC047	HSPC047 protein	7	−0.7153	2.0×10^{-4}	.0402
ILMN_1779034	NM_018161.4	NADSYN1	NAD synthetase 1	11	−0.6854	2.1×10^{-4}	.0402
ILMN_1705253	NM_130393.2	PTPRD	Protein tyrosine phosphatase, receptor type, D, transcript variant 4	9	0.7342	2.1×10^{-4}	.0403

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1837017	CB269825	Hs.543359	1008732 Human Fat Cell 5-Stretch Plus cDNA Library cDNA 5' sequence		−0.7281	2.1×10^{-4}	.0405
ILMN_1829490	BX106357	Hs.445732	BX106357 Soares_NFL_T_GBC_S1 cDNA clone IMAGp998B055155 sequence		0.6957	2.2×10^{-4}	.0409
ILMN_1714691	NM_002148.3	HOXD10	Homeobox D10	2	−0.7265	2.3×10^{-4}	.0413
ILMN_1803213	NM_015419.2	MXRA5	Matrix-remodeling-associated 5	X	−0.7061	2.3×10^{-4}	.0416
ILMN_1732158	NM_001460.2	FMO2	Flavin containing monooxygenase 2 (nonfunctional)	1	−0.6950	2.4×10^{-4}	.0424
ILMN_1681938	NM_022568.2	ALDH8A1	Sldehyde dehydrogenase 8 family, member A1, transcript variant 1	6	0.6875	2.4×10^{-4}	.0424
ILMN_1753243	NM_016306.4	DNAJB11	DnaJ (Hsp40) homolog, subfamily B, member 11	3	0.7183	2.5×10^{-4}	.0431
ILMN_1793846	NM_014670.2	BZW1	Basic leucine zipper and W2 domains 1	2	0.7033	2.7×10^{-4}	.0431
ILMN_1852159	BF753039	Hs.557431	RC3-BN0425-011200-022-c08 BN0425 cDNA sequence		−0.7234	2.4×10^{-4}	.0432
ILMN_1805992	NM_018330.4	KIAA1598	KIAA1598	10	0.7077	2.4×10^{-4}	.0433
ILMN_1740512	XM_936687.1	MGC39900	Hypothetical protein MGC39900	X	−0.7227	2.6×10^{-4}	.0433
ILMN_1708916	NM_032512.2	PDZD4	PDZ domain containing 4	X	−0.7075	2.7×10^{-4}	.0434
ILMN_1773563	NM_015927.3	TGFB1I1	Transforming growth factor beta 1-induced transcript 1, transcript variant 2	16	−0.7335	2.6×10^{-4}	.0435
ILMN_1674184	NM_153022.2	C12orf59	Chromosome 12 open reading frame 59	12	0.7122	2.6×10^{-4}	.0436
ILMN_1657483	NM_032985.4	SEC23B	Sec23 homolog B (S cerevisiae), transcript variant 2	20	0.6717	2.7×10^{-4}	.0436
ILMN_1772540	NM_015251.2	ASCIZ	ATM/ATR-Substrate Chk2-Interacting Zn2+-finger protein	16	0.6872	2.6×10^{-4}	.0438
ILMN_1756862	NM_145641.1	APOL3	Apolipoprotein L, 3, transcript variant beta/a	22	−0.7021	2.8×10^{-4}	.0438
ILMN_1685413	NM_024079.4	ALG8	Asparagine-linked glycosylation 8 homolog (S cerevisiae, alpha-1,3-glucosyltransferase), transcript variant 1	11	0.6986	2.8×10^{-4}	.0439
ILMN_1686645	NM_021645.4	UTP14C	UTP14, U3 small nucleolar ribonucleoprotein, homolog C (yeast)	13	0.6746	2.8×10^{-4}	.0440
ILMN_1813746	NM_003389.2	CORO2A	Coronin, actin-binding protein, 2A, transcript variant 1	9	0.7135	2.6×10^{-4}	.0440
ILMN_1765557	NM_015441.1	OLFML2B	Olfactomedin-like 2B	1	−0.6714	2.7×10^{-4}	.0441
ILMN_1740586	NM_000300.2	PLA2G2A	Phospholipase A2, group IIA (platelets, synovial fluid)	1	−0.7049	2.6×10^{-4}	.0443

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1758750	NR_003501.1	EARS2	Glutamyl-tRNA synthetase 2, mitochondrial (putative), transcript variant 2, transcribed RNA	16	0.7199	2.6×10^{-4}	.0444
ILMN_1703178	NM_003469.3	SCG2	Secretogranin II (chromogranin C)	2	−0.7239	2.6×10^{-4}	.0444
ILMN_1710522	NM_175635.1	RUNX1T1	Runt-related transcription factor 1; translocated to, 1 (cyclin D-related), transcript variant 3	8	−0.6886	3.1×10^{-4}	.0444
ILMN_1730048	NM_024067.2	C7orf26	Chromosome 7 open reading frame 26	7	−0.6943	2.9×10^{-4}	.0444
ILMN_1722855	NM_003377.3	VEGFB	Vascular endothelial growth factor B	11	−0.7039	3.0×10^{-4}	.0445
ILMN_1752915	NM_004124.2	GMFB	Glia maturation factor, beta	14	0.6872	2.6×10^{-4}	.0445
ILMN_1702124	NM_153371.3	LNX2	Ligand of numb-protein X 2	13	0.7044	3.0×10^{-4}	.0445
ILMN_1695299	NM_014476.1	PDLIM3	PDZ and LIM domain 3	4	−0.7140	3.1×10^{-4}	.0445
ILMN_1666364	NM_144576.3	COQ10A	Coenzyme Q10 homolog A (S cerevisiae), transcript variant 1	12	−0.6949	2.6×10^{-4}	.0445
ILMN_1756942	NM_001017371.3	SP3	Sp3 transcription factor, transcript variant 2	2	0.6849	3.1×10^{-4}	.0445
ILMN_1750386	NM_006172.2	NPPA	Natriuretic peptide precursor A	1	−0.6947	3.1×10^{-4}	.0445
ILMN_1685433	NM_020351.2	COL8A1	Collagen, type VIII, alpha 1, transcript variant 2	3	−0.6900	2.9×10^{-4}	.0445
ILMN_1665095	NM_015537.3	NELF	Nasal embryonic LHRH factor	9	−0.7203	2.9×10^{-4}	.0445
ILMN_1695316	NM_022154.5	SLC39A8	Solute carrier family 39 (zinc transporter), member 8	4	0.6843	2.9×10^{-4}	.0446
ILMN_1749338	NM_173505.2	ANKRD29	Ankyrin repeat domain 29	18	−0.6916	3.0×10^{-4}	.0446
ILMN_1692340	NM_207404.2	ZNF662	Zinc finger protein 662	3	−0.7117	2.9×10^{-4}	.0447
ILMN_1730612	NM_001048223.1	DBNDD2	Dysbindin (dystrobrevin binding protein 1) domain containing 2, transcript variant 3	20	−0.7208	3.1×10^{-4}	.0447
ILMN_1778523	NM_001206.2	KLF9	Kruppel-like factor 9	9	−0.6988	3.0×10^{-4}	.0447
ILMN_1813175	NM_014921.3	LPHN1	Latrophilin 1, transcript variant 2	19	−0.6905	3.0×10^{-4}	.0447
ILMN_1800103	XM_001128785.1	LOC731196	Similar to proprotein convertase subtilisin/kexin type 7 precursor (proprotein convertase PC7) (subtilisin/kexin-like protease PC7) (prohormone convertase PC7) (PC8) (hPC8) (lymphoma proprotein convertase)	11	0.6977	2.9×10^{-4}	.0447
ILMN_1801583	NM_017680.3	ASPN	Asporin	9	−0.7233	2.9×10^{-4}	.0447
ILMN_1740024	NM_005467.2	NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2	11	−0.6949	3.0×10^{-4}	.0449
ILMN_1683133	NM_014079.2	KLF15	Kruppel-like factor 15	3	−0.6842	3.2×10^{-4}	.0451
ILMN_1801441	NM_144629.1	RFTN2	Raftlin family member 2	2	−0.6948	3.2×10^{-4}	.0452

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1719097	NM_013326.3	C18orf8	Chromosome 18 open reading frame 8	18	−0.7098	3.2×10^{-4}	.0452
ILMN_1723689	NM_003624.1	RANBP3	RAN binding protein 3, transcript variant RANBP3-a	19	−0.6899	3.2×10^{-4}	.0452
ILMN_1790052	NM_004659.1	MMP23A	Matrix metallopeptidase 23A	1	−0.7011	3.3×10^{-4}	.0456
ILMN_1679262	NM_001387.2	DPYSL3	Dihydropyrimidinase-like 3	5	−0.7208	3.3×10^{-4}	.0458
ILMN_1683487	NM_003444.1	ZNF154	Zinc finger protein 154 (pHZ-92)	19	−0.6905	3.3×10^{-4}	.0460
ILMN_1710284	NM_005524.2	HES1	Hairy and enhancer of split 1, (Drosophila)	3	−0.7019	3.4×10^{-4}	.0462
ILMN_1728710	NM_001031665.1	ZNF816A	Zinc finger protein 816A	19	0.6975	3.5×10^{-4}	.0462
ILMN_1685156	NM_020983.2	ADCY6	Adenylate cyclase 6, transcript variant 2	12	−0.6890	3.5×10^{-4}	.0464
ILMN_1721087	NM_012435.1	SHC2	SHC (Src homology 2 domain containing) transforming protein 2	19	−0.6788	3.5×10^{-4}	.0465
ILMN_1700811	NM_019116.2	UBFD1	Ubiquitin family domain containing 1	16	0.6888	3.5×10^{-4}	.0466
ILMN_1661066	XM_927710.1	LOC644596	Hypothetical protein LOC644596	X	−0.6663	3.5×10^{-4}	.0466
ILMN_1733769	NM_001033047.1	NPNT	Nephronectin	4	−0.7029	3.5×10^{-4}	.0466
ILMN_1784948	NM_144569.4	SPOCD1	SPOC domain containing 1	1	−0.7223	3.6×10^{-4}	.0467
ILMN_1727574	NM_178835.3	LOC152485	Hypothetical protein LOC152485		−0.6904	3.5×10^{-4}	.0467
ILMN_1724984	NM_004836.4	EIF2AK3	Eukaryotic translation initiation factor 2-alpha kinase 3	2	0.6981	3.7×10^{-4}	.0467
ILMN_1660305	NM_177966.4	2'-PDE	2'-Phosphodiesterase	3	0.7031	3.5×10^{-4}	.0468
ILMN_1782057	NM_020452.2	ATP8B2	ATPase, class I, type 8B, member 2, transcript variant 1	1	−0.7041	3.6×10^{-4}	.0468
ILMN_1751072	NM_021203.2	SRPRB	Signal recognition particle receptor, B subunit	3	0.6672	3.7×10^{-4}	.0468
ILMN_1740609	NM_032964.2	CCL15	Chemokine (C-C motif) ligand 15, transcript variant 1	17	−0.6697	3.7×10^{-4}	.0468
ILMN_1669982	NM_001080433.1	CCDC85A	Coiled-coil domain containing 85A	2	−0.6858	3.6×10^{-4}	.0468
ILMN_1807515	NM_015235.2	CSTF2T	Cleavage stimulation factor, 3' pre-RNA, subunit 2, 64 kDa, tau variant	10	0.6892	3.5×10^{-4}	.0469
ILMN_1657361	NM_175709.2	CBX7	Chromobox homolog 7	22	−0.6904	3.8×10^{-4}	.0469
ILMN_1801043	NM_198252.2	GSN	Gelsolin (amyloidosis, Finnish type), transcript variant 2	9	−0.7028	3.7×10^{-4}	.0469
ILMN_1738116	NM_181724.1	TMEM119	Transmembrane protein 119	12	−0.6425	3.7×10^{-4}	.0470
ILMN_1760890	NM_206926.1	SEPN1	Selenoprotein N, 1, transcript variant 2	1	−0.6762	3.8×10^{-4}	.0473
ILMN_1728785	NM_015234.4	GPR116	G protein-coupled receptor 116, transcript variant 1	6	−0.6903	3.8×10^{-4}	.0475

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TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1744647	NM_018448.2	CAND1	Cullin-associated and neddylation-dissociated 1	12	0.6883	3.9×10^{-4}	.0479
ILMN_1757440	XM_001130258.1	FAM69B	Family with sequence similarity 69, member B	9	−0.6706	3.9×10^{-4}	.0480
ILMN_1783805	NM_013364.4	PNMA3	Paraneoplastic antigen MA3	X	−0.7005	3.9×10^{-4}	.0482
ILMN_1809098	NM_019599.2	TAS2R1	Taste receptor, type 2, member 1	5	0.7013	4.1×10^{-4}	.0490
ILMN_1719759	NM_002160.2	TNC	Tenascin C (hexabrachion)	9	−0.7107	4.1×10^{-4}	.0491
ILMN_1811313	NM_003062.1	SLIT3	Slit homolog 3 (Drosophila)	5	−0.6810	4.0×10^{-4}	.0491
ILMN_1700432	NM_002221.2	ITPKB	Inositol 1,4,5-trisphosphate 3-kinase B	1	−0.6983	4.1×10^{-4}	.0495
ILMN_1809488	NM_014752.1	SPCS2	Signal peptidase complex subunit 2 homolog (S cerevisiae)	11	0.6204	4.2×10^{-4}	.0498
ILMN_1795338	NM_013313.3	YPEL1	Yippee-like 1 (Drosophila)	22	−0.6528	4.3×10^{-4}	.0505
ILMN_1736242	NM_015432.2	PLEKHG4	Pleckstrin homology domain containing, family G (with RhoGef domain) member 4	16	−0.6879	4.3×10^{-4}	.0506
ILMN_1696568	NM_014382.2	ATP2C1	ATPase, Ca++ transporting, type 2C, member 1, transcript variant 1	3	0.6572	4.3×10^{-4}	.0507
ILMN_1766925	NM_001257.3	CDH13	Cadherin 13, H-cadherin (heart)	16	−0.7020	4.3×10^{-4}	.0509
ILMN_1698252	NM_152633.2	FANCB	Fanconi anemia, complementation group B, transcript variant 2	X	0.6928	4.5×10^{-4}	.0526
ILMN_1781149	NM_006774.4	INMT	Indolethylamine N-methyltransferase	7	−0.6688	4.6×10^{-4}	.0530
ILMN_1665437	NM_000773.3	CYP2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1	10	−0.6839	4.6×10^{-4}	.0531
ILMN_1773395	NM_002905.2	RDH5	Retinol dehydrogenase 5 (11-cis/9-cis)	12	−0.6860	4.6×10^{-4}	.0533
ILMN_1665483	NM_014878.4	KIAA0020	KIAA0020	9	0.6892	4.6×10^{-4}	.0534
ILMN_1666545	NM_001097635.1	GCNT1	Glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase), transcript variant 4	9	0.6953	4.6×10^{-4}	.0535
ILMN_1743864	NM_001453.2	FOXC1	Forkhead box C1	6	−0.6694	4.8×10^{-4}	.0542
ILMN_1709486	NM_006307.3	SRPX	Sushi-repeat-containing protein, X-linked	X	−0.6834	4.8×10^{-4}	.0543
ILMN_1676088	NM_198080.2	MSRB3	Methionine sulfoxide reductase B3, transcript variant 1	12	−0.6889	4.8×10^{-4}	.0543
ILMN_1771238	NM_000390.2	CHM	Choroideremia (Rab escort protein 1), transcript variant 1	X	0.6744	4.9×10^{-4}	.0544
ILMN_1656807	NM_000988.3	RPL27	Ribosomal protein L27	17	−0.6987	4.9×10^{-4}	.0545
ILMN_1711826	NM_020344.1	SLC24A2	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 2	9	−0.6804	5.0×10^{-4}	.0545

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1660730	NM_032803.4	SLC7A3	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 3, transcript variant 1	X	−0.6816	4.9×10^{-4}	.0546
ILMN_1849218	BX451947	Hs.559564	BX451947 FETAL BRAIN cDNA clone CS0DF008YL16 5-PRIME sequence		−0.6910	4.9×10^{-4}	.0547
ILMN_1726752	NM_175071.1	APTX	Aprataxin, transcript variant 5	9	0.6732	4.9×10^{-4}	.0548
ILMN_1739640	NM_003737.2	DCHS1	Dachsous 1 (Drosophila)	11	−0.6808	5.0×10^{-4}	.0550
ILMN_1686968	NM_152493.2	FLJ25476	FLJ25476 protein	1	−0.6856	5.1×10^{-4}	.0557
ILMN_1718044	NM_018127.5	ELAC2	elaC Homolog 2 (E coli)	17	−0.6695	5.3×10^{-4}	.0562
ILMN_1799836	NM_006735.3	HOXA2	Homeobox A2	7	−0.7036	5.3×10^{-4}	.0563
ILMN_1658847	XM_939432.1	MGC61598	Similar to ankyrin-repeat protein Nrarp	9	−0.6363	5.3×10^{-4}	.0565
ILMN_1764619	NM_207443.1	FLJ45244	FLJ45244 protein	14	−0.6691	5.3×10^{-4}	.0567
ILMN_1739521	NM_014932.2	NLGN1	Neuroligin 1	3	0.6893	5.4×10^{-4}	.0568
ILMN_1710675	NM_005080.2	XBP1	X-box binding protein 1, transcript variant 1	22	0.6814	5.3×10^{-4}	.0568
ILMN_1772810	XM_946142.2	SHANK3	SH3 and multiple ankyrin repeat domains 3, transcript variant 4	22	−0.6733	5.4×10^{-4}	.0570
ILMN_1693481	NM_021949.2	ATP2B3	ATPase, Ca++ transporting, plasma membrane 3, transcript variant 1	X	0.6669	5.3×10^{-4}	.0570
ILMN_1671106	NM_002060.2	GJA4	Gap junction protein, alpha 4, 37 kDa	1	−0.6706	5.3×10^{-4}	.0572
ILMN_1773757	NM_138718.1	SLC26A8	Solute carrier family 26, member 8, transcript variant 2	6	0.6936	5.5×10^{-4}	.0573
ILMN_1680652	NM_003944.2	SELENBP1	Selenium binding protein 1	1	−0.6566	5.6×10^{-4}	.0585
ILMN_1813528	NM_133459.1	CCBE1	Collagen and calcium binding EGF domains 1	18	−0.6806	5.7×10^{-4}	.0587
ILMN_1715175	NM_000245.2	MET	Met protooncogene (hepatocyte growth factor receptor)	7	0.6834	5.7×10^{-4}	.0587
ILMN_1688160	NM_182552.3	WDR27	WD repeat domain 27	6	−0.6906	5.7×10^{-4}	.0587
ILMN_1805842	NM_001449.3	FHL1	Four and a half LIM domains 1	X	−0.6833	5.6×10^{-4}	.0587
ILMN_1806301	NM_002077.2	GOLGA1	Golgi autoantigen, golgin subfamily a, 1	9	−0.6603	5.8×10^{-4}	.0595
ILMN_1734653	NM_032532.2	FNDC1	Fibronectin type III domain containing 1	6	−0.6810	5.9×10^{-4}	.0596
ILMN_1706935	NM_022742.3	CCDC136	Coiled-coil domain containing 136	7	−0.6766	5.9×10^{-4}	.0597
ILMN_1727091	NM_138326.2	ACMSD	Aminocarboxymuconate semialdehyde decarboxylase	2	0.6688	5.9×10^{-4}	.0597
ILMN_1740385	NM_014956.4	CEP164	Centrosomal protein 164 kDa	11	−0.6244	5.9×10^{-4}	.0598
ILMN_1746517	NM_003937.2	KYNU	Kynureninase (L-kynurenine hydrolase), transcript variant 1	2	0.6445	6.0×10^{-4}	.0598

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1801246	NM_003641.3	IFITM1	Interferon-induced transmembrane protein 1 (9-27)	11	−0.6716	6.0×10^{-4}	.0599
ILMN_1756784	NM_014286.2	FREQ	Frequenin homolog (Drosophila)	9	−0.6824	6.1×10^{-4}	.0599
ILMN_1652389	NM_001031733.2	CALML4	Calmodulin-like 4, transcript variant 2	15	−0.6783	6.1×10^{-4}	.0600
ILMN_1794038	NM_030797.2	FAM49A	Family with sequence similarity 49, member A	2	0.6333	6.1×10^{-4}	.0601
ILMN_1758731	NM_000775.2	CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2	1	0.6767	6.1×10^{-4}	.0602
ILMN_1707380	NM_002725.3	PRELP	Proline/arginine-rich end leucine-rich repeat protein, transcript variant 1	1	−0.6844	6.1×10^{-4}	.0603
ILMN_1801226	NM_020812.1	DOCK6	Dedicator of cytokinesis 6	19	−0.6576	6.1×10^{-4}	.0605
ILMN_1766386	XR_017805.1	LOC401433	Hypothetical gene supported by AK127717, misc RNA	7	−0.6178	6.2×10^{-4}	.0607
ILMN_1763657	NM_025212.1	CXXC4	CXXC finger 4	4	−0.6560	6.3×10^{-4}	.0608
ILMN_1777221	NM_058182.2	C21orf51	Chromosome 21 open reading frame 51	21	−0.6266	6.3×10^{-4}	.0612
ILMN_1712199	NM_024738.1	C12orf49	Chromosome 12 open reading frame 49	12	0.6531	6.4×10^{-4}	.0619
ILMN_1741801	NM_003503.2	CDC7	Cell division cycle 7 homolog (S cerevisiae)	1	0.6725	6.6×10^{-4}	.0631
ILMN_1891067	AK127526	Hs.553187	cDNA FLJ45619 fis, clone BRTHA3027318		0.6413	6.6×10^{-4}	.0632
ILMN_1663843	NM_004161.3	RAB1A	RAB1A, member RAS oncogene family	2	0.6529	6.7×10^{-4}	.0632
ILMN_1792571	NM_173728.2	ARHGEF15	Rho guanine nucleotide exchange factor (GEF) 15	17	−0.6508	6.7×10^{-4}	.0632
ILMN_1790315	NM_001039706.1	FLJ21062	Hypothetical protein FLJ21062	7	−0.6657	6.6×10^{-4}	.0634
ILMN_1733756	NM_080645.2	COL12A1	Collagen, type XII, alpha 1, transcript variant short	6	−0.6799	6.8×10^{-4}	.0638
ILMN_1812701	NM_001099783.1	C4orf33	Chromosome 4 open reading frame 33, transcript variant 2	4	0.6666	6.8×10^{-4}	.0640
ILMN_1782257	NM_022734.2	METT11D1	Methyltransferase 11 domain containing 1, transcript variant 2	14	−0.6653	6.9×10^{-4}	.0643
ILMN_1691112	NM_176787.4	PIGN	Phosphatidylinositol glycan anchor biosynthesis, class N, transcript variant 1	18	0.6741	6.9×10^{-4}	.0646
ILMN_1756086	NM_023015.3	INTS3	Integrator complex subunit 3	1	−0.6306	6.9×10^{-4}	.0648
ILMN_1710303	NM_031421.2	TTC25	Tetratricopeptide repeat domain 25	17	−0.6482	7.0×10^{-4}	.0651
ILMN_1785765	NM_004800.1	TM9SF2	Transmembrane 9 superfamily member 2	13	0.6617	7.1×10^{-4}	.0656
ILMN_1684321	NM_030579.2	CYB5B	Cytochrome b5 type B (outer mitochondrial membrane)	16	0.6858	7.1×10^{-4}	.0658

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1722244	NM_001018011.1	ZBTB16	Zinc finger and BTB domain containing 16, transcript variant 2	11	−0.6734	7.2×10^{-4}	.0660
ILMN_1787906	NM_014629.2	ARHGEF10	Rho guanine nucleotide exchange factor (GEF) 10	8	−0.6634	7.3×10^{-4}	.0668
ILMN_1736974	NM_006943.2	SOX12	SRY (sex determining region Y)-box 12	20	−0.6435	7.4×10^{-4}	.0668
ILMN_1808590	NM_000856.3	GUCY1A3	Guanylate cyclase 1, soluble, alpha 3	4	−0.6252	7.3×10^{-4}	.0669
ILMN_1751559	NM_024600.2	C16orf30	Chromosome 16 open reading frame 30	16	−0.6442	7.4×10^{-4}	.0671
ILMN_1774427	NM_020898.1	CALCOCO1	Calcium binding and coiled-coil domain 1	12	−0.6804	7.7×10^{-4}	.0682
ILMN_1657502	NM_001098515.1	MRGPRF	MAS-related GPR, member F, transcript variant 1	11	−0.6781	7.6×10^{-4}	.0682
ILMN_1652128	NM_018368.2	LMBRD1	LMBR1 domain containing 1	6	0.6306	7.8×10^{-4}	.0683
ILMN_1808417	NM_015102.2	NPHP4	Nephronophthisis 4	1	−0.6615	7.7×10^{-4}	.0684
ILMN_1657194	NM_018430.2	TSNAXIP1	Translin-associated factor X interacting protein 1	16	−0.6513	7.7×10^{-4}	.0684
ILMN_1680948	NM_012134.2	LMOD1	Leiomodin 1 (smooth muscle)	1	−0.6757	7.7×10^{-4}	.0684
ILMN_1703471	NM_007348.2	ATF6	Activating transcription factor 6	1	0.6569	7.6×10^{-4}	.0684
ILMN_1728742	NM_032385.3	C5orf4	Chromosome 5 open reading frame 4, transcript variant 2	5	−0.6564	7.8×10^{-4}	.0686
ILMN_1702861	NM_172244.2	SGCD	Sarcoglycan, delta (35 kDa dystrophin-associated glycoprotein), transcript variant 2	5	−0.6460	7.6×10^{-4}	.0686
ILMN_1868150	BX537697	Hs.98581	mRNA; cDNA DKFZp686D0853 (from clone DKFZp686D0853)		−0.6645	7.9×10^{-4}	.0686
ILMN_1694325	NM_002501.2	NFIX	Nuclear factor I/X (CCAAT-binding transcription factor)	19	−0.6525	7.8×10^{-4}	.0687
ILMN_1748432	XM_375646.3	ZNF525	Zinc finger protein 525	19	0.6710	8.0×10^{-4}	.0691
ILMN_1743357	NM_003399.5	XPNPEP2	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	X	−0.6388	8.0×10^{-4}	.0694
ILMN_1782125	NM_024422.2	DSC2	Desmocollin 2, transcript variant Dsc2a	18	0.6142	8.0×10^{-4}	.0695
ILMN_1687967	NM_001007156.1	NTRK3	Neurotrophic tyrosine kinase, receptor, type 3, transcript variant 3	15	−0.6630	8.1×10^{-4}	.0699
ILMN_1685286	NM_017607.2	PPP1R12C	Protein phosphatase 1, regulatory (inhibitor) subunit 12C	19	−0.6754	8.3×10^{-4}	.0710
ILMN_1756937	NM_005668.3	ST8SIA4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4, transcript variant 1	5	0.6470	8.3×10^{-4}	.0711

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1794534	NM_021827.3	CCDC81	Coiled-coil domain containing 81	11	−0.6588	8.4×10^{-4}	.0712
ILMN_1793615	NM_001014811.1	ME3	Malic enzyme 3, NADP(+)-dependent, mitochondrial, nuclear gene encoding mitochondrial protein, transcript variant 2	11	−0.6720	8.5×10^{-4}	.0716
ILMN_1885397	BM311228	Hs.503590	ig62e09y1 HR85 islet cDNA 5 sequence		−0.6606	8.5×10^{-4}	.0717
ILMN_1759375	NM_001083330.1	ZNF133	Zinc finger protein 133, transcript variant 2	20	−0.6532	8.5×10^{-4}	.0719
ILMN_1796851	XM_001131060.1	FOXL2	Forkhead box L2	3	−0.6417	8.6×10^{-4}	.0720
ILMN_1703105	NM_139178.2	ALKBH3	alkB, Alkylation repair homolog 3 (E coli)	11	−0.6275	8.5×10^{-4}	.0720
ILMN_1678710	NM_032439.1	PHYHIPL	Phytanoyl-CoA 2-hydroxylase interacting protein-like	10	0.6064	8.8×10^{-4}	.0733
ILMN_1758398	NM_000858.4	GUK1	Guanylate kinase 1	1	−0.6158	8.9×10^{-4}	.0735
ILMN_1796734	NM_003118.2	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	5	−0.6468	8.8×10^{-4}	.0737
ILMN_1653856	NM_032873.3	STS-1	Cbl-interacting protein Sts-1	11	0.6288	8.9×10^{-4}	.0739
ILMN_1795251	NM_004684.3	SPARCL1	SPARC-like 1 (mast9, hevin)	4	−0.6301	9.0×10^{-4}	.0746
ILMN_1717206	NM_175060.1	CLEC14A	C-type lectin domain family 14, member A	14	−0.6376	9.2×10^{-4}	.0754
ILMN_1739496	NM_006902.3	PRRX1	Paired related homeobox 1, transcript variant pmx-1a	1	−0.6477	9.2×10^{-4}	.0754
ILMN_1718552	NM_006419.1	CXCL13	Chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)	4	−0.6037	9.2×10^{-4}	.0755
ILMN_1797191	NM_014656.1	KIAA0040	KIAA0040	1	0.6227	9.2×10^{-4}	.0757
ILMN_1737705	NM_015054.1	KIAA0701	KIAA0701 protein, transcript variant 1	12	0.6459	9.5×10^{-4}	.0770
ILMN_1682781	NM_003598.1	TEAD2	TEA domain family member 2	19	−0.6440	9.5×10^{-4}	.0771
ILMN_1673352	NM_006435.2	IFITM2	Interferon-induced transmembrane protein 2 (1-8D)	11	−0.6409	9.6×10^{-4}	.0772
ILMN_1750158	NM_007292.4	ACOX1	acyl-Coenzyme A oxidase 1, palmitoyl, transcript variant 2	17	0.6554	9.7×10^{-4}	.0772
ILMN_1657156	NM_207306.2	KIAA0495	KIAA0495	1	−0.6716	9.7×10^{-4}	.0773
ILMN_1787576	NM_004070.3	CLCNKA	Chloride channel Ka, transcript variant 1	1	−0.6609	9.6×10^{-4}	.0773
ILMN_1665449	NM_019055.4	ROBO4	Roundabout homolog 4, magic roundabout (Drosophila)	11	−0.6256	9.7×10^{-4}	.0773
ILMN_1796018	NM_004554.3	NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	14	−0.6726	9.6×10^{-4}	.0774
ILMN_1765118	NM_003627.4	SLC43A1	Solute carrier family 43, member 1	11	−0.6174	9.9×10^{-4}	.0784

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1785424	NM_006720.3	ABLIM1	Actin-binding LIM protein 1, transcript variant 4	10	−0.6004	9.9×10^{-4}	.0784
ILMN_1701204	NM_005429.2	VEGFC	Vascular endothelial growth factor C	4	−0.6393	9.9×10^{-4}	.0784
ILMN_1769186	NM_001755.2	CBFB	Core-binding factor, beta subunit, transcript variant 2	16	0.6454	1.0×10^{-3}	.0785
ILMN_1651958	NM_000900.2	MGP	Matrix Gla protein	12	−0.6404	1.0×10^{-3}	.0786
ILMN_1770803	NM_004330.1	BNIP2	BCL2/adenovirus E1B 19 kDa interacting protein 2	15	0.6074	1.0×10^{-3}	.0786
ILMN_1720452	NM_001031855.1	LONRF3	LON peptidase N-terminal domain and ring finger 3, transcript variant 1	X	0.6477	1.0×10^{-3}	.0786
ILMN_1780349	NM_003292.2	TPR	Translocated promoter region (to activated MET oncogene)	1	−0.6328	1.0×10^{-3}	.0792
ILMN_1818018	DA321576	Hs.576997	DA321576 BRHIP3 cDNA clone BRHIP3014850 5 sequence		0.6451	1.0×10^{-3}	.0793
ILMN_1724424	NM_145239.1	PRRT2	Proline-rich transmembrane protein 2	16	−0.6496	1.0×10^{-3}	.0794
ILMN_1760849	NM_018092.3	NETO2	Neuropilin (NRP) and tolloid (TLL)-like 2	16	0.6201	1.0×10^{-3}	.0794
ILMN_1773742	NM_012328.1	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	7	0.6452	1.0×10^{-3}	.0794
ILMN_1792529	NM_004783.2	TAOK2	TAO kinase 2, transcript variant 1	16	−0.6358	1.0×10^{-3}	.0796
ILMN_1740772	NM_133172.2	APBB3	Amyloid beta (A4) precursor protein-binding, family B, member 3, transcript variant 3	5	−0.6393	1.1×10^{-3}	.0796
ILMN_1737604	NM_018291.2	FLJ10986	Hypothetical protein FLJ10986	1	0.6581	1.0×10^{-3}	.0796
ILMN_1742272	NM_000537.2	REN	Renin	1	−0.6262	1.1×10^{-3}	.0798
ILMN_1806403	NM_016563.2	RASL12	RAS-like, family 12	15	−0.6338	1.1×10^{-3}	.0798
ILMN_1715647	NM_020335.1	VANGL2	Vang-like 2 (van gogh, Drosophila)	1	−0.6544	1.1×10^{-3}	.0800
ILMN_1655913	NM_005013.2	NUCB2	Nucleobindin 2	11	0.6408	1.1×10^{-3}	.0801
ILMN_1736080	NM_012432.2	SETDB1	SET domain, bifurcated 1	1	−0.6417	1.1×10^{-3}	.0805
ILMN_1663033	NM_138385.2	TMEM129	Transmembrane protein 129	4	−0.6319	1.1×10^{-3}	.0809
ILMN_1697585	NM_022496.3	ACTR6	ARP6 actin-related protein 6 homolog (yeast)	12	0.6055	1.1×10^{-3}	.0817
ILMN_1711124	NM_144724.1	MARVELD2	MARVEL domain containing 2, transcript variant 2	5	0.6463	1.1×10^{-3}	.0820
ILMN_1711919	NM_017988.4	SCYL2	SCY1-like 2 (S cerevisiae)	12	0.5937	1.1×10^{-3}	.0821
ILMN_1669142	NM_057175.3	NARG1	NMDA receptor regulated 1	4	0.6007	1.1×10^{-3}	.0822
ILMN_1678862	NM_173540.2	FUT11	Fucosyltransferase 11 (alpha [1,3] fucosyltransferase)	10	0.6192	1.1×10^{-3}	.0822
ILMN_1768393	NM_006938.2	SNRPD1	Small nuclear ribonucleoprotein D1 polypeptide 16 kDa	18	0.5974	1.1×10^{-3}	.0822

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1782938	NM_018593.3	SLC16A10	Solute carrier family 16, member 10 (aromatic amino acid transporter)	6	0.5965	1.1×10^{-3}	.0822
ILMN_1899428	AW173494	Hs.483540	xj07f12x1 NCI_CGAP_Ut2 cDNA clone IMAGE:2656559 3 sequence		−0.6320	1.1×10^{-3}	.0825
ILMN_1748845	NM_002506.2	NGFB	Nerve growth factor, beta polypeptide	1	−0.6390	1.1×10^{-3}	.0832
ILMN_1767722	NM_203437.2	AFTPH	Aftiphilin, transcript variant 1	2	0.6445	1.2×10^{-3}	.0837
ILMN_1791545	NM_015515.3	KRT23	Keratin 23 (histone deacetylase inducible)	17	0.6391	1.2×10^{-3}	.0838
ILMN_1747183	NM_001099650.1	GLT8D3	Glycosyltransferase 8 domain containing 3, transcript variant 2	12	0.6353	1.2×10^{-3}	.0839
ILMN_1815666	NM_170665.2	ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2, transcript variant 1	12	0.6340	1.2×10^{-3}	.0840
ILMN_1761425	NM_182487.2	OLFML2A	Olfactomedin-like 2A	9	−0.6347	1.2×10^{-3}	.0843
ILMN_1794825	NM_000382.2	ALDH3A2	Aldehyde dehydrogenase 3 family, member A2, transcript variant 2	17	−0.6101	1.2×10^{-3}	.0845
ILMN_1767459	NM_018082.4	POLR3B	Polymerase (RNA) III (DNA directed) polypeptide B	12	0.6220	1.2×10^{-3}	.0851
ILMN_1717905	NM_015726.2	WDR42A	WD repeat domain 42A	1	−0.6202	1.2×10^{-3}	.0853
ILMN_1682404	NM_006515.1	SETMAR	SET domain and mariner transposase fusion gene	3	−0.6306	1.2×10^{-3}	.0861
ILMN_1725338	NM_194284.2	CLDN23	Claudin 23	8	0.6369	1.2×10^{-3}	.0863
ILMN_1765371	NM_018032.3	LUC7L	LUC7-like (S cerevisiae), transcript variant 1	16	−0.6428	1.2×10^{-3}	.0863
ILMN_1756118	NM_014634.2	PPM1F	Protein phosphatase 1F (PP2C domain containing)	22	−0.5914	1.2×10^{-3}	.0864
ILMN_1793621	NM_001002262.1	ZFYVE27	Zinc finger, FYVE domain containing 27, transcript variant 3	10	0.6345	1.2×10^{-3}	.0865
ILMN_1654945	NM_153759.2	DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha, transcript variant 2	2	0.6434	1.2×10^{-3}	.0866
ILMN_1754364	NM_001868.1	CPA1	Carboxypeptidase A1 (pancreatic)	7	0.6196	1.2×10^{-3}	.0866
ILMN_1663640	NM_000240.2	MAOA	Monoamine oxidase A, nuclear gene encoding mitochondrial protein	X	0.6353	1.3×10^{-3}	.0874
ILMN_1728581	NM_016210.2	C3orf18	Chromosome 3 open reading frame 18	3	−0.6159	1.3×10^{-3}	.0876
ILMN_1736834	NM_005414.2	SKIL	SKI-like oncogene	3	0.6206	1.3×10^{-3}	.0883
ILMN_1800731	NM_018328.3	MBD5	Methyl-CpG binding domain protein 5	2	−0.6235	1.3×10^{-3}	.0884

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1805098	NM_000924.2	PDE1B	Phosphodiesterase 1B, calmodulin-dependent	12	−0.6434	1.3×10^{-3}	.0884
ILMN_1769764	NM_001039935.1	ANKRD55	Ankyrin repeat domain 55, transcript variant 2	5	0.6090	1.3×10^{-3}	.0885
ILMN_1814015	NM_004063.2	CDH17	Cadherin 17, LI cadherin (liver-intestine)	8	−0.6315	1.3×10^{-3}	.0885
ILMN_1802669	NM_021132.1	PPP3CB	Protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	10	−0.6361	1.3×10^{-3}	.0888
ILMN_1800512	NM_002133.1	HMOX1	Heme oxygenase (decycling) 1	22	0.5922	1.3×10^{-3}	.0888
ILMN_1772731	NM_005326.4	HAGH	Hydroxyacylglutathione hydrolase, transcript variant 1	16	−0.6280	1.3×10^{-3}	.0890
ILMN_1756573	NM_020142.3	NDUFA4L2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	12	−0.6186	1.3×10^{-3}	.0891
ILMN_1686464	NM_180991.4	SLCO4C1	Solute carrier organic anion transporter family, member 4C1	5	0.6381	1.3×10^{-3}	.0903
ILMN_1769083	NM_000847.3	GSTA3	Glutathione S-transferase A3	6	0.6117	1.3×10^{-3}	.0904
ILMN_1687410	NM_022776.3	OSBPL11	Oxysterol binding protein-like 11	3	0.6035	1.4×10^{-3}	.0918
ILMN_1651611	NM_000527.2	LDLR	Low-density lipoprotein receptor (familial hypercholesterolemia)	19	0.6122	1.4×10^{-3}	.0937
ILMN_1665123	NM_178177.2	NMNAT3	Nicotinamide nucleotide adenylyltransferase 3	3	−0.6152	1.4×10^{-3}	.0938
ILMN_1651370	NM_001014443.2	USP21	Ubiquitin-specific peptidase 21, transcript variant 3	1	−0.6228	1.4×10^{-3}	.0939
ILMN_1774110	NM_004067.2	CHN2	Chimerin (chimaerin) 2, transcript variant 2	7	0.6275	1.4×10^{-3}	.0950
ILMN_1730662	NM_001008745.1	LOC401431	Hypothetical gene LOC401431	7	−0.6048	1.4×10^{-3}	.0950
ILMN_1753554	NM_022763.2	FNDC3B	Fibronectin type III domain containing 3B	3	0.6167	1.4×10^{-3}	.0950
ILMN_1734254	NM_014106.2	ZNF770	Zinc finger protein 770	15	0.5801	1.4×10^{-3}	.0951
ILMN_1801889	NM_015011.1	MYO16	Myosin XVI	13	−0.6401	1.4×10^{-3}	.0951
ILMN_1703074	NM_001304.3	CPD	Carboxypeptidase D	17	0.6178	1.4×10^{-3}	.0952
ILMN_1885728	XM_001130020.1	KIAA1147	KIAA1147	7	0.6203	1.5×10^{-3}	.0952
ILMN_1652594	NM_024855.3	ACTR5	ARP5 actin-related protein 5 homolog (yeast)	20	−0.6197	1.4×10^{-3}	.0953
ILMN_1672287	NM_018657.3	MYNN	Myoneurin	3	0.6237	1.5×10^{-3}	.0954
ILMN_1680113	NM_004758.1	BZRAP1	Benzodiazepine receptor (peripheral)-associated protein 1	17	−0.6340	1.5×10^{-3}	.0966
ILMN_1660282	NM_022135.2	POPDC2	Popeye domain containing 2	3	−0.6316	1.5×10^{-3}	.0967
ILMN_1683441	NM_015261.2	NCAPD3	Non-SMC condensin II complex, subunit D3	11	−0.6169	1.5×10^{-3}	.0968
ILMN_1761486	NM_024808.2	C13orf34	Chromosome 13 open reading frame 34	13	0.6187	1.5×10^{-3}	.0970

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1894569	BX093121	Hs.571048	BX093121 Soares_placenta_ 8to9weeks_2NbHP8to9W cDNA clone IMAGp998K13561; IMAGE:257796 sequence		0.6106	1.5×10^{-3}	.0970
ILMN_1751086	NM_015459.3	DKFZP564J0863	DKFZP564J0863 protein	11	0.5780	1.5×10^{-3}	.0970
ILMN_1656386	NM_014822.1	SEC24D	SEC24-related gene family, member D (S cerevisiae)	4	0.6073	1.5×10^{-3}	.0971
ILMN_1760271	NM_194314.2	ZBTB41	Zinc finger and BTB domain containing 41	1	0.6038	1.5×10^{-3}	.0972
ILMN_1702683	NM_004733.2	SLC33A1	Solute carrier family 33 (acetyl-CoA transporter), member 1	3	0.6196	1.5×10^{-3}	.0972
ILMN_1878019	AL512695	Hs.278285	mRNA; cDNA DKFZp547G133 (from clone DKFZp547G133)		0.6348	1.5×10^{-3}	.0973
ILMN_1806487	NM_001002034.2	FAM109B	Family with sequence similarity 109, member B	22	−0.5735	1.5×10^{-3}	.0973
ILMN_1779748	NM_004192.2	ASMTL	Acetylserotonin O-methyltransferase-like	X,Y	−0.6146	1.5×10^{-3}	.0974
ILMN_1770084	NM_006283.1	TACC1	Transforming, acidic coiled-coil containing protein 1	8	−0.6145	1.6×10^{-3}	.0974
ILMN_1707534	NM_017544.2	NKRF	NF-kappaB repressing factor	X	0.6044	1.6×10^{-3}	.0974
ILMN_1678086	NM_138770.1	CCDC74A	Coiled-coil domain containing 74A	2	−0.6070	1.6×10^{-3}	.0975
ILMN_1669064	NM_001080493.2	HSZFP36	ZFP-36 for a zinc finger protein	19	0.6087	1.5×10^{-3}	.0975
ILMN_1810093	NM_005725.3	TSPAN2	Tetraspanin 2	1	−0.5941	1.5×10^{-3}	.0976
ILMN_1673522	NM_017947.1	MOCOS	Molybdenum cofactor sulfurase	18	0.6122	1.6×10^{-3}	.0976
ILMN_1764309	NM_000667.2	ADH1A	Alcohol dehydrogenase 1A (class I), alpha polypeptide	4	−0.6229	1.6×10^{-3}	.0977
ILMN_1795325	NM_001615.3	ACTG2	Actin, gamma 2, smooth muscle, enteric	2	−0.6151	1.6×10^{-3}	.0977
ILMN_1773814	NM_205853.2	MUSTN1	Musculoskeletal, embryonic nuclear protein 1	3	−0.6206	1.5×10^{-3}	.0977
ILMN_1703576	NM_012334.2	MYO10	Myosin X	5	0.6086	1.6×10^{-3}	.0977
ILMN_1780937	NM_025128.3	MUS81	MUS81 endonuclease homolog (S cerevisiae)	11	−0.6341	1.6×10^{-3}	.0977
ILMN_1757162	XM_945736.2	LOC654085	Similar to Glycine cleavage system H protein, mitochondrial precursor, transcript variant 2	19	0.6181	1.5×10^{-3}	.0977
ILMN_1832155	AK094744	Hs.167721	cDNA FLJ37425 fis, clone BRAWH2001530		−0.6007	1.6×10^{-3}	.0977
ILMN_1782688	NM_024838.4	THNSL1	Threonine synthase-like 1 (S cerevisiae)	10	0.6374	1.6×10^{-3}	.0977
ILMN_1757298	NM_018167.3	BTBD7	BTB (POZ) domain containing 7, transcript variant 2	14	0.6056	1.6×10^{-3}	.0978
ILMN_1798975	NM_005228.3	EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral [v-erb-b] oncogene homolog, avian), transcript variant 1	7	0.6186	1.6×10^{-3}	.0978

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1775974	NM_019012.2	PLEKHA5	Pleckstrin homology domain containing, family A member 5	12	0.5995	1.6×10^{-3}	.0979
ILMN_1872404	AK055652	Hs.478682	cDNA FLJ31090 fis, clone IMR321000102		−0.6351	1.6×10^{-3}	.0979
ILMN_1808999	NM_153213.3	ARHGEF19	Rho guanine nucleotide exchange factor (GEF) 19	1	−0.6104	1.6×10^{-3}	.0979
ILMN_1733703	NM_018006.4	TRMU	tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase, nuclear gene encoding mitochondrial protein, transcript variant 1	22	−0.6098	1.6×10^{-3}	.0980
ILMN_1795574	XM_928045.1	LOC644968	Hypothetical protein LOC644968	4	0.5916	1.6×10^{-3}	.0980
ILMN_1700994	NM_001039571.1	KREMEN1	Kringle containing transmembrane protein 1, transcript variant 4	22	0.6255	1.6×10^{-3}	.0980
ILMN_1737146	NM_014294.4	TRAM1	Translocation-associated membrane protein 1	8	0.6212	1.6×10^{-3}	.0980
ILMN_1809889	NM_173510.1	CCDC117	Coiled-coil domain containing 117	22	0.6109	1.6×10^{-3}	.0981
ILMN_1735909	NM_001033678.2	TRPT1	tRNA phosphotransferase 1, transcript variant 1	11	−0.6155	1.6×10^{-3}	.0982
ILMN_1670472	NM_014613.2	UBXD8	UBX domain containing 8	5	0.6387	1.7×10^{-3}	.0986
ILMN_1700633	NM_022060.2	ABHD4	Abhydrolase domain containing 4	14	−0.5964	1.7×10^{-3}	.0988
ILMN_1914072	BQ718005	Hs.562762	AGENCOURT_8100698 Lupski_sympathetic_trunk cDNA clone IMAGE:6190431 5 sequence		0.6098	1.7×10^{-3}	.0989
ILMN_1651642	NM_152742.1	GPC2	Glypican 2	7	−0.6187	1.7×10^{-3}	.0990
ILMN_1671046	NM_001541.2	HSPB2	Heat shock 27-kDa protein 2	11	−0.6162	1.7×10^{-3}	.0990
ILMN_1662578	NM_020156.1	C1GALT1	Core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase, 1	7	0.5897	1.7×10^{-3}	.0990
ILMN_1693514	NM_001014795.1	ILK	Integrin-linked kinase, transcript variant 3	11	−0.6264	1.7×10^{-3}	.0992
ILMN_1800447	NM_001031835.1	PHKB	Phosphorylase kinase, beta, transcript variant 2	16	0.5895	1.7×10^{-3}	.0992
ILMN_1701933	NM_007308.1	SNCA	Synuclein, alpha (non-A4 component of amyloid precursor), transcript variant NACP112	4	−0.5903	1.7×10^{-3}	.0993
ILMN_1779547	NM_006665.2	HPSE	Heparanase	4	0.6297	1.7×10^{-3}	.0995
ILMN_1883624	DA589983	Hs.582952	DA589983 HLUNG2 cDNA clone HLUNG2011800 5 sequence		0.5802	1.7×10^{-3}	.0997
ILMN_1774717	NM_020182.3	TMEPAI	Transmembrane, prostate androgen-induced RNA, transcript variant 1	20	−0.5933	1.7×10^{-3}	.0998

Løset. A transcriptional profile of the decidua in preeclampsia. Am J Obstet Gynecol 2011. *(continued)*

TABLE 2
Differentially expressed transcripts[a] *(continued)*

Illumina ID	GenBank ID	Symbol	Definition	Ch	Beta value[b]	*P* value[c]	FDR *P* value[d]
ILMN_1789463	NM_021902.2	FXYD1	FXYD domain containing ion transport regulator 1 (phospholemman), transcript variant b	19	−0.6164	1.7×10^{-3}	.0999
ILMN_1651900	NM_002233.2	KCNA4	Potassium voltage-gated channel, shaker-related subfamily, member 4	11	0.6164	1.7×10^{-3}	.0999

FDR, false discovery rate; *mRNA*, messenger RNA.

[a] Gene expression in decidual tissue from preeclamptic and normal pregnancies has been compared; [b] Values are given in beta, measure of distance between group means, expressed in SD units–positive beta implies up-regulation and negative beta implies down-regulation in preeclamptic group compared with normal pregnant group; [c] $P < .05$, obtained with SOLAR; [d] FDR $P < .10$.

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may be influenced by gestational age,[23,24] it cannot be excluded that some of the differences observed between the preeclamptic and normal pregnancy groups are, in fact, gestational age related. Winn et al[23] compared global gene expression in basal plate (decidual) biopsies from normal pregnancies at mid-gestation (14-24 weeks) and at term (37-40 weeks) and found that 418 genes (of 39,000 transcripts examined) changed expression throughout gestation. This provides a useful dataset for comparison with the data obtained in this current study, albeit different profiling platforms were used. Winn et al[23] used the Affymetrix HG-U133 A&B chip for transcriptional profiling, whereas we used the Illumina HumanWG-6 v2 Expression BeadChip. By this, the number of possible comparisons was restricted to the 16,799 genes shared in both systems. Of the 455 transcripts found to be differentially expressed in this current study, 368 genes demonstrate no gestational age-influenced changes, according to the data of Winn et al.[23] It is therefore tempting to speculate that the differential expression of these 368 genes may be related to disease mechanisms at play in preeclampsia. Seventeen of our differentially expressed genes (TEMEM97, KIAA1598, SULT2B1, EGFR, FHL1, PLA2G7, SHANK3, NOTCH4, UBASH3B, ROBO4, NRARP, GPR116, IL6ST, LDLR, ANGPTL2, SRPRB, and KREMEN1) are reported to change expression with gestational age.[23] For 2 of these genes (SULT2B1 and EGFR), expression increases toward term.[23] Thus, isolated gestational age-related influences in the preeclampsia group would suggest a lower expression of SULT2B1 and EGFR, but both were up-regulated in our dataset. Similarly, the ANGPTL2 gene is down-regulated toward term,[23] but in contrast to what might be expected from gestational age–related changes, expression was lower in the preeclampsia group than in the normal pregnancy group. Based on this, we conclude that the differential expression of these 3 genes may also be ascribed to disease-related mechanisms. However, with regard to the remaining 14 genes in our dataset previously shown to exhibit gestational age-dependent changes in expression, conclusions are hampered by the fact that gestational age may have contributed to the differences observed between preeclamptic and normal pregnancies. To illustrate: expression of FHL1, SHANK3, NOTCH4, ROBO4, NRARP, and GPR116 increases toward term[23] and was down-regulated in the preeclampsia group, whereas TMEM97, KIAA1598, PLA2G7, UBASH3B, IL6ST, LDLR, SRPRB, and

TABLE 3
Results for selected genes from microarray and real-time quantitative polymerase chain reaction expression

		Microarray		RT-qPCR	
Gene symbol	Up/down	Beta value	*P* value[a]	Fold change	*P* value[b]
SLITRK4	↓	−1.04	4.59×10^{-8}	−1.98	< .0001 1.73 x 10-5
FZD4	↓	−0.91	4.05×10^{-7}	−1.35	.001 7.71 x 10-4
ANGPTL2	↓	−0.89	4.39×10^{-6}	−1.74	< .0001 4.79 x 10-5
PLA2G7	↑	0.83	1.58×10^{-5}	1.26	.068 6.79 x 10-2
MAN1A	↑	0.85	1.29×10^{-5}	1.30	.025 2.49 x 10-2
ARL5B	↑	0.91	4.46×10^{-7}	1.22	.017 1.66 x 10-2

RT-qPCR, real-time quantitative polymerase chain reaction.

[a] $P < .05$, obtained with SOLAR; [b] $P < .10$, obtained with *t* test statistics with software (SPSS, version 16; SPSS, Inc, Chicago, IL).

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TABLE 4
Canonical pathway analysis

Canonical pathway[a]	Genes	*P* value[b] Ingenuity Pathway Analysis	*P* value[c] Rotation Gene Set Enrichment Analysis
Tryptophan metabolism	ACMSD, ALDH3A2, ASMTL, CYP2E1, CYP2J2, INMT, KYNU, MAOA	5.51×10^{-4}	2.0×10^{-4}
Endoplasmic reticulum stress pathway	ATF6, DNAJC3, EIF2AK3, XBP1	5.81×10^{-4}	5.3×10^{-3}
Linoleic acid metabolism	CYP2E1, CYP2J2, PLA2G5, PLA2G2A, WISP2	3.91×10^{-3}	1.5×10^{-3}
Notch signaling	DTX3, HES1, NOTCH3, NOTCH4	6.72×10^{-3}	7.9×10^{-3}
Fatty acid metabolism	ACOX1, ACOX2, ADH1A, ALDH3A2, CYP2E1, CYP2J2	7.90×10^{-3}	10.0×10^{-5}
Arachidonic acid metabolism	CYP2E1, CYP2J2, PLA2G5, PLA2G2A, PTGIS, WISP2	8.66×10^{-3}	10.0×10^{-5}
NRF2-mediated oxidative stress response	ACTG2, DNAJB6, DNAJB9, DNAJB11, DNAJC3, EIF2AK3, GSTA3, HMOX1, UBE2K	9.99×10^{-3}	6.7×10^{-2}

[a] Ingenuity Pathway Analysis (www.ingenuity.com; Ingenuity Systems, Redwood City, CA) was used to bioinformatically identify canonical (ie, cell signaling and metabolic) pathways potentially involved in preeclampsia within our dataset; [b] *P* value obtained with Fisher's exact test; [c] *P* value obtained with use of *limma* package.

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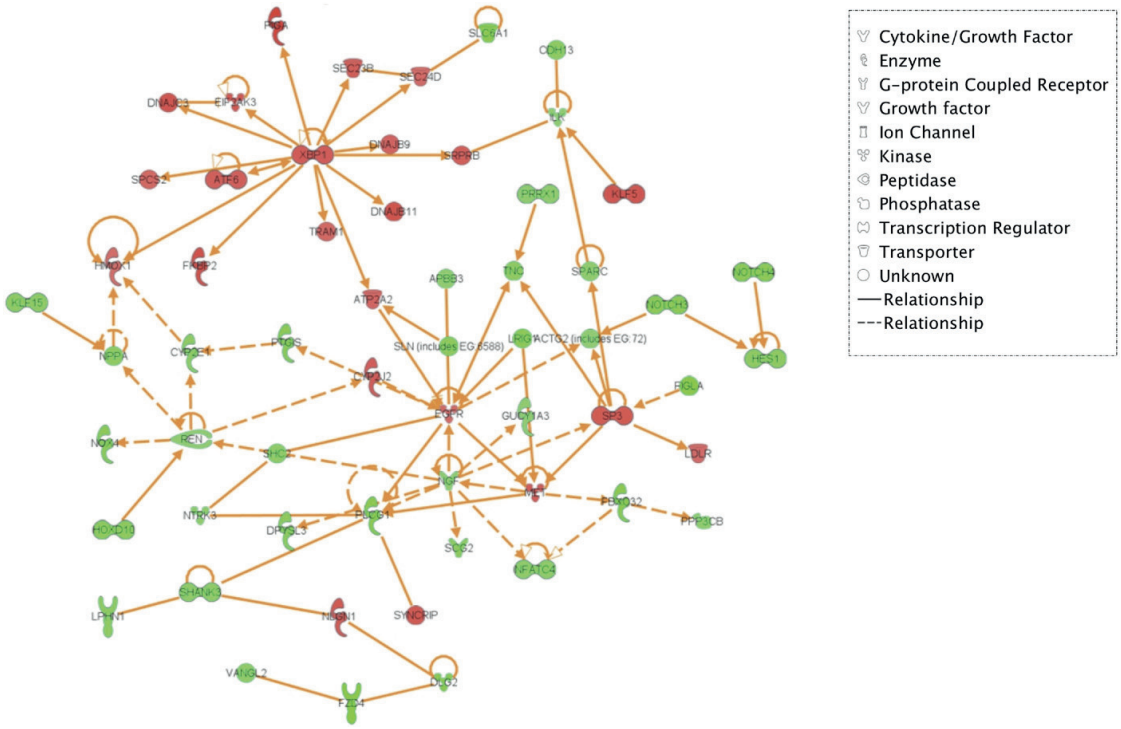
KREMEN1 expression decreases toward term[23] and was up-regulated in the preeclampsia group.

In genomewide transcriptional profiling, analysis of *groups* of genes is a strategy to increase power and reduce the dimensionality of the underlying statistical problem following multiple testing.[25] Further, it may be advantageous to put focus on canonical pathways and networks instead of single genes when the aim is to obtain insight in the pathophysiology of complex diseases, such as preeclampsia. The high interconnectivity of focus genes with other correlated genes within a biological network may imply functional and biological importance of these genes.[26,27] To be able to assess this in a comprehensive manner, we increased the FDR cutoff to 0.1 and consequently the number of genes included in the analysis. Using this approach, 7 significant canonical pathways were found to be represented by the differentially expressed genes identified in this current study (Table 4).

The most significant canonical pathway detected was tryptophan metabolism. The metabolism of tryptophan, through the kynurenine pathway, has previously been suggested to be involved in preeclampsia pathogenesis,[28,29] and, in accordance with this, the activity of the first enzyme of the kynurenine pathway, indoleamine 2,3 dioxygenase, has been reported to be reduced in placenta from preeclamptic pregnancies.[28] We found no disease-associated changes in indoleamine 2,3 dioxygenase expression, but the transcript encoding the enzyme kynureninase (KYNU) was up-regulated. KYNU metabolizes L-kynurenine, which suppresses T-cell proliferation and natural killer cells and influences immunotolerance to foreign antigens.[30] This implies that a consequence of KYNU up-regulation may be an increased inflammatory response (due to lack of L-kynurenine). An additional 7 genes were assigned to this canonical pathway (Table 4).

The second most significant canonical pathway identified was the ER stress pathway. Three genes (EIF2AK3, ATF6, and XBP1) included in the unfolded protein response, a coordinated adaptive response to ER stress, were up-regulated. ER stress has previously been suggested as one of the main sources for generation of placental oxidative stress.[31] Yung et al[32] have reported similar associations of the unfolded protein response signaling pathways to preeclampsia in placental tissue, but these findings are reported for the first time in decidual tissue. There is a close connection between oxidative stress and ER stress,[31,33] also indicated by the many direct relationships of the ER and oxidative stress–related genes in the generated network (Figure). The canonical pathway NRF2-mediated oxidative stress response was also among the significant pathways identified (Table 4). The nuclear factor NFR2 plays an essential role in the defense of oxidative stress by regulating the expression of antioxidant response elements.[34] In case of excessive oxidative stress, activation by reactive oxygen species, nitrogen oxide, and proinflammatory cytokines results in translocation of NRF2 to the nucleus. NRF2 binds to antioxidant response element sequences, leading to transcriptional activation of antioxidant genes (eg, glutathione and HMOX1). NRF2-mediated oxidative stress response included 9 genes, of which 3 genes have pre-

FIGURE
Network of preeclampsia-correlated genes



Ingenuity Pathway Analysis (www.ingenuity.com; Ingenuity Systems, Redwood City, CA) generated gene-gene product interaction network of preeclampsia-correlated genes. Genes or gene products are represented as nodes, and biological relationship between 2 nodes is represented as edge (*line*). All edges are supported by at least 1 published reference. *Solid edges* represent direct relationship, and *dashed edges* represent indirect relationship. Node color represents correlation of expression level with preeclampsia, and color intensity indicates degree of correlation (*red* is positive and *green* negative). Shape of each node represents functional class of gene product, as shown in *key*.

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viously been associated with preeclampsia (EIF2AK3,[32] GSTA3,[10] and HMOX1[21,22]). Several enzymes metabolize reactive oxygen species to exportable compounds, and in this study the transcripts encoding the antioxidant enzymes GSTA3, HMOX1, and UBE2K were up-regulated.

Three of the remaining significant canonical pathways generated by IPA represented metabolism of fatty acids: linoleic acid metabolism, fatty acid metabolism, and arachidonic acid metabolism. The genes included in these pathways were partly overlapping, as shown in Table 4. Decidual arterioles of preeclamptic women show atherosclerotic-like lesions,[35] suggesting an underlying atherogenic process of low-density lipoprotein lipid peroxidation.[36] Lipid peroxidation contributes to the development of preeclampsia,[37] and decidua basalis tissue from preeclamptic women has an increased content of lipid peroxides.[4] The first enzyme of the fatty acid β-oxidation pathway, acyl-coenzyme A oxidase (ACOX)1/palmitoyl-coA oxidase, donates electrons directly to molecular oxygen, thereby producing hydrogen peroxides. ACOX1 was found to be up-regulated, whereas ACOX2/branched chain ACOX, which is involved in the degradation of long branched fatty acids and bile acid intermediates in peroxisomes, was found to be down-regulated. Two genes involved in elimination of lipid peroxidation products were also down-regulated in the material: alcohol dehydrogenase 1a, which metabolizes a wide variety of substrates including lipid peroxidation products, and aldehyd-edehydrogenase 3 family member A2 isozymes, thought to play a major role in the detoxification of aldehydes generated by alcohol metabolism and lipid peroxidation. Increased generation or decreased elimination of lipid peroxidation products may be among the factors activating the maternal endothelium[38] and triggering systemic inflammation in preeclampsia.

Finally, the pathway analysis suggested a role of notch signaling, with inclusion of 4 down-regulated genes: DTX3, HES1, NOTCH 3, and NOTCH 4. Notch signaling is known to be involved in cell differentiation, proliferation, apopto-

sis,[39] and blood vessel formation,[40] processes neatly regulated in the placenta to maintain a normal pregnancy. Notch receptors are expressed on extravillous trophoblasts and are hypothesized to be involved in the differentiation and proliferation of both extravillous trophoblasts and endothelial cells.[41] Placental villi from preeclamptic pregnancies show down-regulation of notch pathway members.[42] Notch signaling in placenta has been suggested to play a role in the development of preeclampsia,[42,43] and the altered expression of DTX and HES1 in tissue from preeclamptic pregnancies compared with normal pregnancies is presented for the first time.

In summary, we have provided a comprehensive transcriptional profile of the decidua in preeclampsia. Our network analysis has demonstrated extensive connectivity between the differently expressed genes. Alteration of the expression level of 1 gene may influence the transcription of others included in the network. Due to this, it is difficult to pinpoint the genes having primary roles in perpetuating preeclampsia from our dataset. Some of our findings confirm and elaborate the current knowledge on the pathophysiology of preeclampsia, while others are novel. Further studies are warranted to replicate findings and confirm involvement of specific genes that have been identified. ■

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Paper II

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MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

STOX2 but not *STOX1* is differentially expressed in decidua from pre-eclamptic women: data from the Second Nord-Trøndelag Health Study

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ABSTRACT: Variation in the Storkhead box-1 (*STOX1*) gene has previously been associated with pre-eclampsia. In this study, we assess candidate single nucleotide polymorphisms (SNPs) in *STOX1* in an independent population cohort of pre-eclamptic ($n = 1.139$) and non-pre-eclamptic ($n = 2.269$) women (the HUNT2 study). We also compare gene expression levels of *STOX1* and its paralogue, Storkhead box-2 (*STOX2*) in decidual tissue from pregnancies complicated by pre-eclampsia and/or fetal growth restriction (FGR) ($n = 40$) to expression levels in decidual tissue from uncomplicated pregnancies ($n = 59$). We cannot confirm association of the candidate SNPs to pre-eclampsia ($P > 0.05$). For *STOX1*, no differential gene expression was observed in any of the case groups, whereas *STOX2* showed significantly lower expression in deciduas from pregnancies complicated by both pre-eclampsia and FGR as compared with controls ($P = 0.01$). We further report a strong correlation between transcriptional alterations reported previously in choriocarcinoma cells over expressing *STOX1A* and alterations observed in decidual tissue of pre-eclamptic women with FGR.

Key words: intrauterine growth restriction (IUGR) / decidua basalis / pre-eclampsia / HUNT2 / *STOX* genes

Introduction

Pre-eclampsia is a serious complication of pregnancy and a major cause of preterm intervention by Caesarean section, as delivery relieves symptoms. The condition presents with *de novo* elevated blood pressure and proteinuria after 20 weeks of pregnancy. Severe pre-eclampsia is not well defined, but assessment for severity includes both the mother (severe hypertension, end organ manifestations and preterm disease), and the fetus (intrauterine growth restriction, fetal movement assessment and oligohydramnios) (Gifford *et al.*, 2000). As defined in clinical practice, ~20% of pre-eclamptic cases are severe. Early identification, as well as more refined treatment options, is particularly important for these women, and this remains a major challenge in obstetric medicine. Thus, there is a rationale for concentrating research efforts on severe cases (Gifford *et al.*, 2000).

Pre-eclampsia is a complex disorder, including both placental and maternal components. Disease heritability is as high as 54% (Salonen *et al.*, 2000), but a distinct pattern of inheritance is not known. Whole genome linkage studies of families with an increased prevalence of pre-eclampsia have identified several genetic susceptibility loci for pre-eclampsia (Harrison *et al.*, 1997; Arngrimsson *et al.*, 1999; Moses *et al.*, 2000, 2006; Lachmeijer *et al.*, 2001; Laivuori *et al.*, 2003; Johnson *et al.*, 2007). Several studies have suggested the involvement of epigenetic mechanisms for the disease (Graves, 1998), and evidence for a maternal effect in one of the pre-

eclampsia susceptibility loci has been observed for a region of genes on chromosome 10q22 (Oudejans *et al*., 2004). The exons of 17 positional candidate genes in this region were sequenced in a Dutch cohort of families with two or more sibling pairs affected by pre-eclampsia (van Dijk *et al*., 2005). All families with increased prevalence of pre-eclampsia showed missense mutations within the Storkhead box-1 (*STOX1*, AK057891) gene on chromosome 10q22, identical between affected sisters (van Dijk *et al*., 2005).

The *STOX1* gene encodes a winged-helix domain-containing transcription factor, and is believed to play a role in the differentiation of trophoblast cells (van Dijk *et al*., 2005). The longest isoform of the STOX1 protein, *STOX1A* (NM_152709) exerts regulatory effects in several tissues (van Dijk *et al*., 2010a). Over expression of *STOX1A* in choriocarcinoma cells (JEG-3 cell line) was shown to be associated with transcriptional alterations similar to those observed in third-trimester pre-eclamptic placentas (Rigourd *et al*., 2008). The predominant variation co-segregating with pre-eclampsia disease status in the Dutch families (van Dijk *et al*., 2005), *STOX1A*-Y153H (rs1341667) is located in the DNA binding domain. Recently published data suggest that this variant may negatively regulate trophoblast invasion by up-regulation of the cell–cell adhesion protein a-T-catenin (CTNNA3) (van Dijk *et al*., 2010b).

Storkhead box 2 (*STOX2*, AB037813) has been identified as the only known human paralogue to *STOX1* (van Dijk *et al*., 2005; Kivinen *et al*., 2007), but to our knowledge little is known of its function. In humans, there is evidence that *STOX2* is a component of a molecular profile unique and globally characteristic of uncommitted stem cells (Thomas *et al*., 2008). The *STOX2* transcript is also included in a transcriptional profile observed with increased inflammatory response to air pollutants, differing between pregnant and non-pregnant mice (Fedulov *et al*., 2008). The gene is situated at chromosome 4q35, near a replicated region of known, suggestive linkage to pre-eclampsia on chromosome 4q31-q32 (Harrison *et al*., 1997; Moses *et al*., 2000; Laivuori *et al*., 2003).

The involvement of *STOX1* in pre-eclampsia observed in the Dutch families (Oudejans *et al*., 2004; van Dijk *et al*., 2005) has not been confirmed in independent studies (Berends *et al*., 2007; Kivinen *et al*., 2007; Iglesias-Platas *et al*., 2007). It has therefore been hypothesized that *STOX1* is relevant to a clinically severe subgroup of women with early onset of the disease, and fetal growth restriction (FGR) (Oudejans *et al*., 2007; Oudejans and van Dijk, 2008; van Dijk *et al*., 2007).

In the present study, we investigated candidate functional single nucleotide polymorphisms (SNPs) within *STOX1* in an independent large population-based pre-eclampsia cohort. The proposed disease-causing *STOX1A*-Y153H (rs1341667) variant was tested for association with defined clinical subgroups of pre-eclamptic women. We also compared decidual gene expression of *STOX1* and *STOX2* in uncomplicated pregnancies and pregnancies complicated by pre-eclampsia, FGR or both. Furthermore, we compared transcriptional alterations in Rigourd's cultured trophoblast cells with increased expression of *STOX1A* (Rigourd *et al*., 2008), with transcriptional alterations seen in our clinically defined subgroups.

Materials and Methods

Candidate gene expression study

Human subjects

Women with pregnancies complicated by pre-eclampsia, FGR, pre-eclampsia with FGR and women with uncomplicated pregnancies were recruited at St Olavs Hospital, Trondheim, Norway and Haukeland University Hospital, Bergen, Norway from 2002 to 2006. Pre-eclampsia was defined as hypertension (blood pressure $\geq$140/90 mmHg) plus proteinuria ($\geq$0.3 g/24 h or $\geq 1+$ according to a dipstick test) on at least two occasions, developing after 20 weeks of pregnancy (Gifford *et al*., 2000). FGR was defined by a stringent small for gestational age (SGA) definition (birthweight $\leq$2 SD below the expected weight for gestational age and sex, corresponding to the 2.5 percentile) (Marsal *et al*., 1996), confirmed by at least one of the following: reduced fundal height in serial measurements, serial ultrasound biometry identifying failure to grow along a consistent centile or abnormal umbilical artery wave form. Multiple pregnancies and pregnancies with chromosomal aberrations, fetal and placental structural abnormalities or suspected perinatal infections were excluded.

Decidual tissue collection

Decidua basalis tissue samples were collected by vacuum aspiration of the placental bed during Caesarean section as previously described (Staff *et al*., 1999; Harsem *et al*., 2004). Caesarean section in the control group was done for reasons considered irrelevant to the study hypotheses (breech presentation, cephalopelvic disproportion or maternal request). Samples were flushed with 500 ml sterile saline solution at room temperature to remove excess blood. The decidual tissue was immediately submerged in RNA-later (Ambion, Huntington, UK), incubated at 4°C and stored at −80°C for later RNA extraction. The quality of the decidual material was assessed by immune histochemistry, as described in Eide *et al*. (2006). Only specimens containing extravillous trophoblast (decidua basalis) were included in the study.

Total RNA processing

Decidua basalis tissue was disrupted in a 2:1 (w/v) trizol:sample tissue mix using a Polytron® PT 1300 D digital, handheld homogenizer with a PT-DA 1307/2EC generator (Kinematica Inc., Lucerne, Switzerland). Total RNA was isolated using a trizol extraction protocol with chloroform interphase separation; isopropanol precipitation and ethanol wash steps. Isolated total RNA was purified with an RNeasy Mini Kit using spin technology (Qiagen, Valencia, CA, USA). Spectrophotometric determination of purified total RNA yield (μg) was performed using the NanoDrop ND-1000 (Wilmington, DE, USA). Total RNA quality was measured using the total RNA Nano Series II kit on the Agilent BioAnalyzer 2100 using the 2100 Expert software (Agilent Technologies, Germany). Synthesis, amplification, purification and biotin labelled complementary RNA (cRNA) was produced from a total RNA template using the Illumina TotalPrep RNA Amplification Kit (Ambion, TX, USA), according to manufacturer's instructions. Purified cRNA yield was determined spectrophotometrically using the NanoDrop ND-1000.

A total of 1.5 μg purified cRNA per sample was hybridized onto Illumina's HumanWG-6 v2 Expression BeadChip® following Illumina's 6 × 2 BeadChip protocol (Illumina, San Diego, CA, USA). After hybridization, the 6 × 2 expression arrays were washed, blocked, stained with streptavidin-Cy3 (GE Healthcare, Buckinghamshire, UK) and dried prior to them being scanned on the Illumina BeadArrayer 500GX reader using Illumina BeadScan image data acquisition software (version 2.3.0.13). Illumina's BeadStudio Gene Expression software module

(version 3.2.7) was used to subtract background noise signals for each individual sample and generate an output file for statistical analysis. The control summary report generated by the Gene Expression software module was used to evaluate the performance of the built-in controls for each BeadChip. The control summary report summarizes signal intensity, hybridization signal, background signal and the background to noise level for all samples scanned in a particular batch.

Candidate gene association study

HUNT2 case/control subjects

The samples used for our candidate gene association study were retrospectively identified from the second Nord-Trøndelag Health Study in Norway (HUNT2). The HUNT2 study was conducted from 1995 to 1997. All residents above 19 years were invited and 75.5% of the invited women ($n = 35.280$) participated. This population is considered well suited for genetic studies as it is homogeneous (<3% non-Caucasians) and stable (net out migration of 0.3% each year). The HUNT2 survey included a questionnaire, a clinical examination and collection of biological samples as previously described (Holmen *et al*., 2003). Obstetrical data from these women were obtained by linking the HUNT2 data with the The Medical Birth Registry of Norway (MBRN). Physicians and midwives have been registering obstetrical data from all deliveries in Norway after 16 weeks of gestation in standard questionnaires since 1967. More than 1.8 million births are included. Each resident in Norway is registered with an 11-digit personal identification number, also used for all national registries such as the MBRN and HUNT. The women having experienced pre-eclampsia in one or more pregnancies, defined as new onset of hypertension (blood pressure ≥140/90 mmHg) and proteinuria (≥0.3 g/d or ≥1 + according to a dipstick test), developing after 20 weeks of pregnancy (Gifford *et al*., 2000) were identified using diagnosis codes ICD-8 (before 1998) and ICD-10 (after 1998). Pre-eclamptic women with multiple pregnancies were excluded. Two controls per case were identified at random among parous women in the HUNT2 cohort with no registered pre-eclamptic pregnancy in the MBRN. All personal identification numbers in the total data set were randomly replaced by a serial number in order to ensure patient anonymity.

Clinical characterization of the HUNT2 pre-eclampsia-cohort

Preterm delivery was defined as delivery before 37.0 weeks (Gifford *et al*., 2000), and FGR as delivery of a SGA infant (birthweight ≤2 SD below the expected weight for gestational age and sex, corresponding to the 2.5 percentile) (Marsal *et al*., 1996). As fasting blood glucose was not available for all the individuals in the study cohort, an International Diabetes Federation (IDF)-proxy definition [waist circumference ≥80 cm plus any two of the following: high-density lipoprotein (HDL) cholesterol <1.29 mmol/l, treatment for hypertension or blood pressure ≥130/85 mmHg, diabetes diagnosed after the age of 30] (Hildrum *et al*., 2007) was used for assessment of metabolic syndrome. This method has been evaluated in a cross-sectional analysis of 10.206 participants in HUNT2 and no differences in the prevalence of metabolic syndrome between fasting and non-fasting groups were shown using the IDF-proxy definition (Hildrum *et al*., 2007).

Genotyping

DNA from pre-eclamptic (case) and non-pre-eclamptic (control) pregnancies was extracted from peripheral blood leukocytes from blood clots or from EDTA blood stored in the HUNT biobank, using the PURGENE chemistry (Gentra Systems Inc, Minneapolis, MN, USA) for large volumes or the MagAttract DNA Blood M48 kit (Qiagen, Hilden, Germany) for low volumes (<400 μl) as previously described (Holmen *et al*., 2003; Moses *et al*., 2007).

The four *STOX1* missense SNPs described by van Dijk *et al*. (2005) plus two additional known *STOX1* missense SNPs were selected for genotyping in the HUNT2 case/control cohort. The additional *STOX1* SNP was selected from the NCBI SNP database (*Homo sapiens* NCBI Build 35; dbSNP build 124). The six *STOX1* SNPs were genotyped using Applied Biosystems' SNPlex™ Genotyping System and sample genotypes were interrogated using Applied Biosystems' GeneMapper (version 4.0) software as previously described (Moses *et al*., 2007; Roten *et al*., 2008).

Statistical analysis

Gene expression analysis

Decidual gene expression profiles were analysed using SOLAR (Almasy and Blangero, 1998) as previously described (Goring *et al*., 2007). All raw gene expression signals were initially log-transformed. To identify significantly expressed genes in decidua, the distribution of expression values for a given gene across all samples was compared with the distribution of the expression values of the control targets implemented in each expression array. For each gene, we performed a χ^2 tail test using a false discovery rate (Hochberg and Benjamini, 1990) of 0.01 to determine whether there was a significant excess of samples with expression values above the 95th percentile of the control null distribution (Goring *et al*., 2007). The pre-processing of gene expression signals produces a sub-set of significantly expressed (detected) genes from the total number of targets synthesized onto each expression array. Within each sample, the mean log expression signal for each detected gene was ranked and grouped into bin sets of 2.000 genes. Each bin set was then *z*-scored independently. We applied a direct normalization procedure using an inverse Gaussian transformation for each detected same gene across all samples. This conservative procedure results in normalized expression phenotypes that are comparable between individuals and across all genes (Goring *et al*., 2007). To evaluate the magnitude of differential gene expression between case (pre-eclampsia and/or FGR) and control women, we measured the displacement of each detected gene's mean expression value between the two groups. We performed a standard regression analysis on the case group to test whether the mean gene expression level is significantly different (up- or down-regulated) in the case group compared with the control group.

When evaluating the correlation between transcriptional alterations in Rigourd's data set (Rigourd *et al*., 2008) and our data set (E-TABM-682), we performed a Spearman rank test, as the beta-values and the fold change values generated from these two studies are not directly comparable.

The microarray data have been reported to ArrayExpress according to MIAME standards with accession number E-TABM-682.

SNP association analysis

SNP association analyses of all selected SNPs were performed for the total case/control cohort using Pearson's χ^2 statistic in the software package SPSS 16.0 for Windows. The Y153H variation was analyzed separately for subgroups of pre-eclamptic women and control women (FGR, recurrence) for both a dominant (CC + CT genotype frequency versus TT genotype frequency) and additive (C allele frequency versus T allele frequency) genetic model. Multivariate logistic regression was used to model pre-eclampsia as the (dichotomous) dependent variable against maternal age. Odds ratios (OR) with 95% confidence intervals were calculated. Concordance with Hardy–Weinberg proportions was tested using a χ^2 goodness-of-fit statistic. A threshold of $\alpha = 0.05$ was set for statistical significance of all computed analyses.

Clinical characterization

Descriptive statistics means and standard deviations were computed in the software package SPSS 16.0 for Windows. *P*-values were computed based on *t*-test statistics. Recurrent and non-recurrent pre-eclamptic cases were

Table I Clinical characteristics of the cohort used in the gene expression analysis.

	Pre-eclampsia ($n = 13$)	FGR[a] ($n = 9$)	Pre-eclampsia with FGR ($n = 24$)	Controls ($n = 58$)
Maternal age (years)	30 ± 5*	34 ± 4	31 ± 5	31 ± 5
Gestational age (weeks)	35 ± 3**	32 ± 5**	30 ± 4**	39 ± 1
Systolic blood pressure (mmHg)	153 ± 18**	128 ± 15	151 ± 16**	116 ± 11
Diastolic blood pressure (mmHg)	95 ± 9**	74 ± 8	97 ± 12*	70 ± 9
Birthweight (g)	2364 ± 510**	1225 ± 21**	1118 ± 470**	3619 ± 469

Data are presented as mean ± SD. All case groups were compared with controls and *P*-values were computed based on *t*-test statistics.
[a]≤2 SD of expected weight, confirmed by at least one of the following: reduced fundal height in serial measurements, serial ultrasound biometry identifying failure to grow along a consistent centile or abnormal umbilical artery wave form.
*$P < 0.05$; **$P < 0.001$.

analyzed separately. Each pre-eclamptic group (recurrent and non-recurrent) was compared with the non-pre-eclamptic group. Multivariate logistic regression was used to model pre-eclampsia as the (dichotomous) dependent variable against maternal age. A threshold of $\alpha = 0.05$ was set for statistical significance of all computed analyses.

Ethical approval

Informed written consent was obtained from all participants in this study. The study was approved by the Regional Committees for Medical Research Ethics, the National Data Inspectorate and The Directorate of Health and Social Welfare, Norway. Ethical approval for genotyping the Norwegian case/control cohort (HUNT2), decidual RNA processing and statistical analysis of decidual RNA was obtained from The University of Texas Health Science Center at San Antonio, Institutional Review Board.

Results

Decidual tissue candidate gene expression analysis

Decidua basalis tissue was vacuum aspirated from the placental bed of 14 women with pre-eclampsia, 9 non-pre-eclamptic women with FGR neonates, 29 women with both pre-clampsia and FGR (pre-eclampsia + FGR) and 59 normal pregnancies. Seven samples of low RNA quality were excluded (assessed by the RIN value and visual evaluation of digital electrophoretic gel pictures). We therefore processed a total of 104 samples. Clinical characteristics of the study groups are presented in Table I.

Both the *STOX1* (NM_152709.3) and *STOX2* (NM_020225.1) targets were represented on each expression array as a single known transcript, respectively and were annotated from NCBI's Human Genome Build 36.2, RefSeq content release 17. The expression of both the *STOX1* and *STOX2* targets were significantly detected in decidual tissue using a conservative false detection rate of 1%. To evaluate the magnitude of differential gene expression, we measured the displacement of the mean *STOX1* or *STOX2* expression signals in the case group from the control group. We performed this measurement on the three different sample groups and found no significant difference in *STOX1* gene expression levels ($P > 0.05$) (Table II). The expression of *STOX2* however, was significantly lower in the group of pre-eclamptic women delivering FGR neonates compared with the control group ($P = 0.01$) (Table II).

Table II Decidua basalis differential *STOX1* and *STOX2* expression analysis.

Sample group comparison	Gene			
	STOX1		*STOX2*	
	β*	*P*-value	β*	*P*-value
Pre-eclampsia versus control	0.39	0.19	0.07	0.81
FGR versus control	0.49	0.18	−0.17	0.61
Pre-eclampsia with FGR versus control	0.04	0.86	**−0.59**	**0.01**

*The β (beta) value is the measure of displacement between the case and control group mean expression signals and is expressed in standard deviation units. A positive β implies a higher mean transcript expression signal in the case group compared with the control group and is analogous to a gene/transcript being up-regulated. A negative β implies a lower mean transcript expression signal in the case group compared with the control group and is analogous to a gene/transcript being down-regulated. Values significant at the 1% level are outlined in bold.

Table III Correlation between transcriptional alterations seen in JEG-3 cells over expressing *STOX1A* and alterations observed in decidua basalis tissue from pre-eclamptic and/or FGR pregnancies.

	Correlation coefficient[2] (Spearman *r*)	*P*-value
Pre-eclampsia	0.16	0.004*
Pre-eclampsia with FGR[1]	0.23	0.00008*
FGR	0.09	0.17

[1] ≤2 SD of expected weight, confirmed by at least one of the following: reduced fundal height in serial measurements, serial ultrasound biometry identifying failure to grow along a consistent centile or abnormal umbilical artery wave form.
[2]correlation between the degrees of up/down-regulation of 259 pre-eclampsia related genes from the study of Rigourd *et al.* (comparing JEG-3 cells over expressing STOX1 with mock-transfected JEG-3 cells) with the present study (comparing decidua basalis samples from patients with pre-eclampsia and/or FGR with control decidua basalis samples).
*$P < 0.01$.

In the study by Rigourd *et al.* (2008), 259 of the 500 most up/down-regulated genes in pre-eclamptic placentas had detectable transcript levels in the microarray experiment comparing JEG-3 cells over

expressing *STOX1A* with mock-transfected JEG-3 cells. Of these 259 genes, 242 were found to have transcripts expressed above the background in the transcriptomic data from our material (E-TABM-682). We observed a strong correlation (Spearman $r = 0.23$, $P = 0.00008$) between the beta-values (ratio of up/down-regulation compared with controls) for these genes in our subgroup of women experiencing pre-eclampsia with FGR and the ratio of up/down-regulation in the study by Rigourd *et al.* (2008). The beta-values for the women with pre-eclampsia only, showed a weaker correlation (Spearman $r = 0.16$, $P = 0.004$), and there was no correlation to the beta-values for the non-pre-eclamptic women delivering FGR neonates (Spearman $r = 0.09$, $P = 0.17$) (Table III).

The HUNT2 case/control cohort

DNA samples were available for 1139 women registered with pre-eclamptic pregnancies (cases) and 2269 non-pre-eclamptic women (controls) (Moses *et al.*, 2007). Of the available cases, 1003 women were registered with one and 136 women with more than one pre-eclamptic pregnancy. Mean follow-up time from diagnosis in the MBRN to inclusion in the present study was 25 ± 10 years. As expected, gestational age and birthweight differed between the neonates in pre-eclamptic and non-pre-eclamptic pregnancies; the pre-eclamptic women had a higher risk of delivering preterm, and of delivering a FGR neonate (Table IV, $P < 0.001$). The metabolic syndrome, evaluated by data from the HUNT2 study, was also higher in the case groups as compared with controls (Table IV, $P < 0.001$). After adjusting for maternal age, the differences in clinical phenotype between case and control groups remained significant at $\alpha < 0.001$ level (Table IV).

We also observed clinical differences between the recurrent and non-recurrent pre-eclamptic groups (Table IV). The women in the recurrent group delivered earlier ($P = 0.018$) and the neonates had a lower birthweight (adjusted for gestational age, $P = 0.055$). The prevalence of preterm birth was higher in the recurrent group (22%) compared with the non-recurrent group (14%) ($P < 0.01$), but the seemingly different prevalence of FGR (20 versus 15%) was not statistically significant ($P = 0.2$). The *P*-values are adjusted for maternal age. Metabolic syndrome at inclusion in the HUNT2 study was more prevalent in the recurrent group compared with the non-recurrent when adjusting for age at inclusion ($P = 0.019$).

Statistical power analysis for the HUNT2 case/control cohort

A priori power calculations *ad modum* Lalouel and Rohrwasser (2002) for the *STOX1A* Y153H (rs1341667) variant demonstrated 90% power to detect an effect size (OR) difference of 1.3 for the total case/control (HUNT2) cohort and 1.9 for the recurrent pre-eclampsia subgroup ($n = 136$).

STOX1 *genotyping and association analysis*

The R18P *STOX1* SNP failed the SNPlex assay design due to high homology and another two *STOX1* SNPs were non-polymorphic. There were no significant differences in allele frequencies between the case and control groups for the genotyped SNPs (Table V). There was a high average sample genotype success rate of 87% (range 84–88%) and all SNPs conformed to Hardy–Weinberg proportions ($P > 0.05$).

Assuming a dominant genetic model for the Y153H variant, it is proposed that this variant becomes mutagenic through mechanisms of imprinting either of *STOX1* itself, or of downstream proteins (van Dijk *et al.*, 2005). Under this analysis model, the pre-eclampsia + FGR subgroup did not show any association with the *STOX1A*-Y153H variant when compared with the control group (Table VI). However, the recurrent pre-eclampsia subgroup showed a tendency towards higher incidence of the C-genotype ($P = 0.09$) (Table VI).

Discussion

In this study, we demonstrate reduced decidual gene expression of *STOX2* in pre-eclamptic women delivering FGR neonates. To our

Table IV Clinical characteristics of the HUNT2 case/control cohort.

	Pre-eclampsia (recurrent[1], *n* = 136)	Pre-eclampsia (non-recurrent, *n* = 1.003)	Control (*n* = 2.269)
Maternal age at index pregnancy (years)	25 ± 5	27 ± 6*	25 ± 5
Gestational age (days)	271 ± 20*	275 ± 22*	282 ± 18
Birthweight (g)	3.040 ± 846*	3.238 ± 837*	3.483 ± 592
FGR[2]	26 (20)*	147 (15)*	87 (4)
Preterm birth[3]	29 (22)*	132 (14)*	114 (5)
Maternal age at inclusion in HUNT2	37 ± 9*	40 ± 11	40 ± 11
Metabolic syndrome[4]	30 (22)*	163 (16)*	212 (9)

Data presented as mean ± SD or number (percentage). *P*-values are computed based on *t*-test statistics, each pre-eclamptic group is compared with the non-pre-eclamptic group. IDF, the International Diabetes Federation; HDL, high-density lipoprotein; CI, confidence interval.
[1]More than one pre-eclamptic pregnancy.
[2]≤2 SD of expected weight.
[3]Delivery before week 37.
[4]IDF-proxy definition; waist circumference ≥80 cm plus any two of the following: (HDL cholesterol <1.29 mmol/l, treatment for hypertension or blood pressure ≥130/85 mmHg, diabetes diagnosed after age of 30 or fasting plasma glucose ≥5.6 mmol/l) [43].
*$P < 0.001$.

Table V Genotyped SNPs within *STOX1*.

SNP	Genotype (NN), Allele (N)	Pre-eclampsia (proportion of total)	Control (proportion of total)	*P*-value
R18P		Failed assay design		
rs1341667 (Y153H)	CC	412 (0.418)	840 (0.416)	0.9
	CT	450 (0.456)	931 (0.462)	
	TT	124 (0.126)	245 (0.122)	
	C	1274 (0.646)	2611 (0.648)	
	T	698 (0.354)	1421 (0.352)	
rs41278530 (L582F)		Non-polymorphic		
rs10509305 (E608D)	AA	583 (0.591)	1206 (0.597)	0.5
	AC	346 (0.351)	716 (0.355)	
	CC	57 (0.058)	97 (0.048)	
	A	1512 (0.767)	3128 (0.775)	
	C	460 (0.233)	910 (0.225)	
rs41278532 (N825I)	AA	953 (0.968)	1827 (0.972)	0.4
	AT	28 (0.028)	50 (0.027)	
	TT	3 (0.004)	2 (0.001)	
	A	1934 (0.983)	3704 (0.986)	
	T	34 (0.017)	54 (0.014)	
rs7904300 (A865T)		Non-polymorphic		

P-values were computed using Pearson's χ^2 statistic.

Table VI The *STOX1A*-Y153H variation in severe subgroups of pre-eclamptic women.

Y153H variation	Pre-eclampsia + FGR[a]	Recurrent[b] pre-eclampsia	Control
Number of individuals genotyped	151	115	2010
Dominant model frequency CC + TC	0.881	0.930	0.878
OR* (CI)	1.0 (0.6–1.7)	1.9 (0.9–3.9)	
P-value	0.92	0.09	

[a]FGR, fetal growth restriction measured by birthweight below 2 SD for gestational age.
[b]More than one pre-eclamptic pregnancy.
*OR for each of the subgroups of pre-eclamptic women compared with controls with 95% confidence intervals (CIS) using χ^2 statistics.

knowledge, this is a novel finding. We also elaborate the results of Rigourd *et al.* showing correlation between transcriptional alterations of pre-eclamptic placentas (relative to controls) and JEG-3 cells over expressing *STOX1A* (relative to mock-transfected JEG-3 cells) (Rigourd *et al.*, 2008). Transcriptional alterations in our decidua basalis tissue (E-TABM-682) show the strongest correlation to Rigourd's data set in pre-eclamptic pregnancies complicated by FGR, but not in non-pre-eclamptic pregnancies with FGR. Furthermore, in a population- and registry-based cohort, we find that women experiencing pre-eclampsia more than once are at a higher risk of complications and co-morbidity (preterm birth, lower birthweight and development of metabolic syndrome) compared with those experiencing pre-eclampsia once. There is also a tendency towards higher frequency of the C genotypes for the previously reported *STOX1A*-Y153H variation (van Dijk *et al.*, 2005) in this group of women.

Reduced placental perfusion due to impairment of trophoblast invasion and failed spiral artery remodelling are proposed to be pathogenic features of both fetal growth restriction and pre-eclampsia (Brosens *et al.*, 1972, 1977). We believe the present study of decidua basalis tissue, where these disease processes are taking place, to be an important supplement to previous reports of *STOX1* gene expression in placental tissues (van Dijk *et al.*, 2005; Iglesias-Platas *et al.*, 2007; Kivinen *et al.*, 2007) and cultured trophoblast cells (van Dijk *et al.*, 2005, 2010; Rigourd *et al.*, 2009). We show novel evidence that the *STOX2* gene is down-regulated in term decidua basalis from pre-eclamptic women delivering FGR neonates. *STOX2* resides on chromosome 4q35, near a replicated region of suggestive linkage to pre-eclampsia on chromosome 4q31-q32 (Harrison *et al.*, 1997; Moses *et al.*, 2000; Laivuori *et al.*, 2003). To our knowledge, the role of *STOX2* in the maternal–fetal interface is not known. Gene duplication is an important evolutionary mechanism, and is a continuous matter of research in evolutionary systems biology (Hughes, 2005; Hittinger and Carroll, 2007). Studies of paralogous genes show that duplication does not create genes with novel functions, but rather daughter genes performing specialized sub functions of the ancestral gene (Jensen, 1976; Conant and Wolfe, 2008). *STOX2* has a high sequence similarity to *STOX1* (van Dijk *et al.*, 2005; Kivinen *et al.*, 2007), and it is reasonable to hypothesize that it is involved in some of the same biological processes. Therefore, the role of *STOX2* in

normal placental development as well as in the pathogenesis of pre-eclampsia with FGR warrants further investigation. A comprehensive assessment of genetic regulatory variation as well as a molecular functional evaluation is required to confirm the biological importance of our observation.

Rigourd *et al.* recently showed a strong correlation between transcriptional alterations in trophoblast cell-lines over expressing *STOX1A* and transcriptional alterations shown in term pre-eclamptic placentas (Rigourd *et al.*, 2008). This supports the observation made by others, that the possible disease-causing effects of *STOX1* dysregulation are mediated through aberrant transcriptional regulation of trophoblasts (van Dijk *et al.*, 2005, 2010). The dysregulation of *STOX1* can potentially have deleterious effects as it affects the transcription level of many other genes shown to be important in the development of pre-eclampsia (such as Endoglidin, human Chorionic Gonadotrophin, and Glial Cell Missing Homolog (Rigourd *et al.*, 2009). Furthermore, a direct effect of *STOX1* on *CTNNA3* expression was recently confirmed, affecting trophoblast differentiation and growth (van Dijk *et al.*, 2010b). When comparing the transcriptional alterations of our defined subgroups of women with the transcriptional alterations in Rigourd's trophoblast cell line over expressing *STOX1A*, we confirm their results from placental tissue in decidua basalis. Furthermore, the observed correlation is strongest in the group of women with both pre-eclampsia and FGR. Our findings suggest that the *STOX* genes are associated with a severe fetal phenotype (FGR), interestingly though, only in pre-eclamptic patients, and not in non-pre-eclamptic women delivering FGR neonates. This supports the opinion that FGR in pre-eclampsia and FGR alone represent different pathogenic conditions, as discussed by others (Ness and Sibai, 2006; Huppertz, 2008; Srinivas *et al.*, 2009).

The analysis of gene expression in whole tissue harvested from patients with manifest disease benefits from describing the *in vivo* situation. However, interesting differences will potentially be masked due to the heterogeneity of the material. Approximately 40% of cells in decidua basalis are maternal leukocytes, 20% are extravillous trophoblast and 30% are decidual stromal cells (Benirschke *et al.*, 2006). This is a possible confounding factor; however, our results were interpreted in relation to, and are consistent with, earlier observations (Rigourd *et al.*, 2009; van Dijk *et al.*, 2010b). Our findings are limited to describing gene expression in decidua basalis samples collected at birth; we do not report expression throughout the pregnancy. The strength of this study, however, is a thorough monitoring by an obstetrician; multiple blood pressure and proteinuria measurements, prenatal ultrasound measures and birthweight confirmation, providing a sound diagnostic basis (Eide *et al.*, 2006).

There were no significant differences in allele frequencies between the case and control groups in the population-based cohort for the genotyped candidate SNPs within *STOX1*. This is in agreement with smaller population samples included in other studies (Berends *et al.*, 2007; Kivinen *et al.*, 2007).

Severe pre-eclampsia is diagnosed by assessment of both maternal and fetal phenotypes (Gifford *et al.*, 2000). It has been shown that mothers developing early onset pre-eclampsia have a higher prevalence of fetal growth restriction, as well as increased maternal morbidity and cardiovascular risk later in life (Ness and Roberts, 1996; Mostello *et al.*, 2002; Brown *et al.*, 2007). The MBRN did not include information about onset of disease until 1998, and we are therefore not able to include this parameter in our analyses. It has been shown, however, that women developing severe pre-eclampsia in the second trimester are at high risk of recurrent pre-eclampsia as well as later life chronic hypertension and increased maternal morbidity and mortality (Sibai *et al.*, 1986, 1991; Odegard *et al.*, 2000). Therefore, research on multiparous pre-eclamptic women is encouraged as a tool of better understanding of disposition to, and development of strategies for treatment and prevention of pre-eclampsia (Gifford *et al.*, 2000). Findings in our cohort are consistent with earlier studies, as the recurrent group shows the highest risk of preterm labour, of low fetal birthweight and the highest later life cardiovascular risk, assessed as metabolic syndrome. We do find a tendency towards higher incidence of the C genotypes of the Y153H mutation in the recurrent group under a dominant genetic model. This finding is however, not significant at the 5% level. Assessment of the Y153H mutation in pre-eclamptic women delivering FGR neonates did not show association. Thus, we cannot confirm the hypothesis that this variant is linked to severe pre-eclampsia, or pre-eclampsia with FGR in the Norwegian population.

In the population-based part of the study, we are using registry data, and some misclassification can be expected. This will limit the power to detect a true association. The prevalence and recurrence rates of pre-eclampsia in MBRN are, however comparable to those reported in other Nordic countries (Trogstad *et al.*, 2001). Being born SGA does not necessarily imply FGR, but can also reflect e.g. different ethnicity, constitutional smallness and chromosomal aberrations. However, the growth curves used in this study are based on ultrasonically estimated fetal weights, appropriate for the population (which is homogeneous) and considers the sex of the fetus (Marsal *et al.*, 1996). Furthermore, a stringent SGA definition of ± 2 SD is used, which is more likely to identify the true FGR cases within the SGA group (ACOG, 2002; Sheridan, 2005).This leads us to believe that SGA is a good approximation to FGR in our study. Also, the possible maternal imprinting effect on *STOX1* is a matter of debate (Berends *et al.*, 2007; Iglesias-Platas *et al.*, 2007; van Dijk *et al.*, 2007). We are not able to evaluate this in a case–control cohort, and epigenetic effects will lessen our power to detect a true association. However, the strength of this study is a large sample size, and the extensive clinical information available.

In conclusion, we present novel observations suggesting involvement of *STOX2* in the pathogenesis of pre-eclampsia with FGR. We confirm Rigourd's findings in decidual tissue, and link the transcriptional alterations observed with *STOX1A* over expression to alterations seen in deciduas of pre-eclamptic women delivering FGR neonates. Conceivably, these findings might reflect a role for the *STOX* genes in the pathophysiology of pre-eclampsia with FGR.

Authors' roles

M.H.F. wrote the paper, contributed substantially to acquisition of gene expression and epidemiology data and to data analysis and interpretation of results. M.P.J., J.B., E.K.M. and R.A. contributed substantially to conception and design, analysis and interpretation of data, revising and final approval of manuscript. M.L., S.B.M., A.K.J. and T.D.D. contributed substantially to acquisition and analysis of expression data, revising and final approval of manuscript. L.T.R. acquisition and interpretation of genotype data, revising and final approval

of manuscript. I.P.E. contributed substantially to acquisition of data, revising and final approval of manuscript. S.F. contributed substantially to acquisition of epidemiology data, analysis and interpretation, revising and final approval of manuscript. L.B. and R.K.S. recruitment of patients, collection and cryopreservation of decidual tissues, establishment of clinical database, revising and final approval of manuscript.

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Paper III

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Increased endoplasmic reticulum stress in decidual tissue from pregnancies complicated by fetal growth restriction with and without pre-eclampsia

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ABSTRACT

Objectives: Endoplasmic reticulum (ER) stress has been implicated in both pre-eclampsia (PE) and fetal growth restriction (FGR), and is characterised by activation of three signalling branches: 1) PERK-pEIF2α, 2) ATF6 and 3) splicing of XBP1(U) into XBP1(S). To evaluate the contribution of ER stress in the pathogenesis of PE relative to FGR, we compared levels of ER stress markers in decidual tissue from pregnancies complicated by PE and/or FGR.
Study design: Whole-genome transcriptional profiling was performed on decidual tissue from women with PE ($n = 13$), FGR ($n = 9$), PE+FGR ($n = 24$) and controls ($n = 58$), and used for pathway and targeted transcriptional analyses of ER stress markers. The expression and cellular localisation of ER stress markers was assesses by Western blot and immunofluorescence analyses.
Results: Increased ER stress was observed in FGR and PE+FGR, including both the PERK-pEIF2α and ATF6 signalling branches, whereas ER stress was less evident in isolated PE. However, these cases demonstrated elevated levels of XBP1(U) protein. ATF6 and XBP1 immunoreactivity was detected in most (>80%) extravillous trophoblasts, decidual cells and macrophages. No difference in the proportion of immunopositive cells or staining pattern was observed between study groups.
Conclusions: Increased PERK-pEIF2α and ATF6 signalling have been associated with decreased cellular proliferation and may contribute to the impaired placental growth characterising pregnancies with FGR and PE+FGR. XBP1(U) has been proposed as a negative regulator of ER stress, and increased levels in PE may reflect a protective mechanism against the detrimental effects of ER stress.

1. Introduction

Impaired spiral artery remodelling is a prominent feature of the decidua basalis in pregnancies complicated by pre-eclampsia (PE) and/or fetal growth restriction (FGR) [1]. The incompletely remodelled spiral arteries retain their musculo-elastic structure, which renders the vessels more responsive to vasoactive stimuli. Subsequent fluctuation in uteroplacental blood supply is proposed to cause ischaemia-reperfusion insults and oxidative stress [2]. Several studies have shown that decidual tissue is an important source of oxidative stress [3,4], and increased levels of oxidative stress have been reported in pregnancies complicated by PE and FGR [3,5,6]. Furthermore, ischaemia-reperfusion insults can induce endoplasmic reticulum (ER) stress in trophoblast-like cell lines [7].

The ER is an eukaryotic organelle involved in protein folding and maturation, lipid synthesis and calcium homeostasis [8], as well as sensing, coordinating and mediating stress responses [9,10]. Conditions that interfere with ER functions are collectively called ER stress. Stimuli such as accumulation of unfolded proteins, nutrient deprivation and oxidative stress can induce ER stress and activate the unfolded protein response (UPR) [8,9]. The UPR is a cellular self-defence mechanism, aiming to alleviate ER stress and re-establish homeostasis [8]. Three ER transmembrane sensors,

Abbrevations: ER, endoplasmic reticulum; UPR, unfolded protein response; PERK, PKR-like ER kinase; IRE1, inositol-requiring enzyme 1; ATF6, activating transcription factor 6; EIF2α, eukaryotic translation initiation factor 2α; XBP1, X-box binding protein 1.

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Table 1
Primary and secondary antibodies used for Western blot and immunohistochemical analyses.

	Species	Manufacturer	Catalogue no.	Dilution Western blot	Dilution immunofluorescence
Primary antibodies					
XBP1	Rabbit anti-human	Santa Cruz Biotechnology, CA	sc-7160	1:500	1:5
ATF6	Mouse anti-human	Imgenex, San Diego, CA	IMG-273	1:200	1:300
pEIF2α	Rabbit anti-human	Cell Signalling Technology, Danvers, MA	#9721	1:1500	
EIF2α	Rabbit anti-human	Cell Signalling Technology, Danvers, MA	#9722	1:3000	
β-actin	Mouse anti-human	Abcam, Cambridge, UK	ab6276-100	1:30,000	
CK7	Mouse anti-human	Dako, Glostrup, Denmark	M7018		1:750
CK7	Rabbit anti-human	Abcam, Cambridge, UK	ab52870		1:50
Vimentin	Mouse anti-human	Dako, Glostrup, Denmark	M0725		1:200
Prolactin	Rabbit anti-human	Dako, Glostrup, Denmark	A0569		1:60
CD68	Mouse anti-human	Dako, Glostrup, Denmark	M0814		1:1000
CD68	Rabbit anti-human	Santa Cruz Biotechnology, CA	sc-9139		1:5
Secondary antibodies					
IRDye 680	Goat anti-rabbit	Li-Cor Biosciences, Cambridge, UK	#926-32221	1:5000	
IRDye 800CW	Donkey anti-mouse	Li-Cor Biosciences, Cambridge, UK	#926-32212	1:5000	
FITC[a]	Goat anti-mouse	Dako, Glostrup, Denmark	F0479		1:10
TRITC[b]	Swine anti-rabbit	Dako, Glostrup, Denmark	R0156		1:10

[a] Fluorescein iso-thiocyanate.
[b] Tetramethyl rhodamine iso-thiocyanate.

PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6), regulate the UPR through their respective signalling cascades [9]. Activated PERK phosphorylates the eukaryotic translation initiation factor 2α (EIF2α), which leads to attenuation of mRNA translation and a reduced ER workload [8]. IRE1 splices the constitutively expressed (unspliced) mRNA of the X-box binding protein 1, *XBP1(U)*, into a spliced isoform, *XBP1(S)* [11]. Both *XBP1(U)* and *XBP1(S)* mRNA are translated into transcription factors, but XBP1(S) has higher transcriptional activator activity [11]. The transcription factor ATF6 is activated by proteolytic cleavage in the Golgi compartment [12]. With partly overlapping functions, XBP1 and ATF6 initiate transcription of genes that aim to increase protein folding capacity and degradation of misfolded proteins in ER [8]. These multiple signalling pathways allow for diversity in responses to ER stress, from minor homeostatic adjustments to oxidative stress [13] and activation of inflammatory pathways [10]. Additionally, if the cell fails to combat ER stress, the UPR can trigger apoptosis to eliminate damaged cells [9], but the manner in which the UPR switches from a protective to an apoptotic role is complex and not fully understood [14].

Increased levels of ER stress have been detected in FGR and PE+FGR placentas, where ER stress was associated with decreased cellular proliferation and apoptosis, and proposed as an important cause for the reduced placental growth characterising these phenotypes [15,16]. We recently performed whole-genome transcriptional profiling of decidual tissue from pre-eclamptic and normal pregnancies, identifying upregulation of several transcripts involved in ER stress in PE [17]. Thus, current data indicate that ER stress is involved in the pathogenesis of both PE and FGR, but whether the degree of ER stress differs between these pregnancy complications is unknown. Emerging observations indicate that PE and/or FGR may represent more or less severe stages on a continuous spectrum of responses to impaired placentation, where ischaemia-reperfusion insults and oxidative stress following impaired spiral artery remodelling appear to be common pathophysiological events [16,18]. However, as the clinical outcomes differ, disparate phenomena must take place at some point during the pathogenesis. Given the multiple responses ER stress may elicit, differential activation of UPR signalling branches might explain some of the differences in clinical outcome. The present study aimed to compare the degree of ER stress in pregnancies complicated by PE and/or FGR by analysing transcriptional- and protein expression of key mediators in each branch of the ER stress response.

2. Materials and methods

2.1. Study subjects

Women with pregnancies complicated by PE and/or FGR (cases) and women with uncomplicated pregnancies (controls) were recruited at Trondheim University Hospital (Norway) and Haukeland University Hospital (Bergen, Norway) from 2002 to 2006. PE was defined as persistent hypertension (blood pressure of ≥140 mmHg systolic or 90 mmHg diastolic), plus proteinuria (≥0.3 g in a 24 h urine collection or ≥ 1+ according to a dipstick test), developing after 20 weeks of gestation [19]. PE was sub-classified as severe in accordance with criteria recommended by Sibai et al. [20]. FGR was defined as birthweight <2.5 percentile adjusted for gestational age and sex according to a Scandinavian normogram [21], in addition to at least one of the following criteria: 1) reduced fundal height in serial measurements; 2) serial ultrasound biometry identifying failure to grow along a consistent percentile; or 3) abnormal umbilical artery waveform. Severe FGR was defined as birthweigh <1.7 percentile [22]. Cases diagnosed with PE or FGR before gestational week 34 were classified as early onset. Exclusively healthy women with no prior pregnancy complications were included as controls. Pregnancies with chromosomal aberrations, fetal and placental structural abnormalities or suspected perinatal infections were excluded from both study groups. Cases had caesarean section (CS) performed due to medical indications, whereas controls were undergoing CS for reasons considered irrelevant to the aim of this study (i.e. breech presentation, previous CS or maternal request). Only singleton pregnancies delivered by CS without labour activity were included. The study was approved by the Norwegian Regional Committee for Medical Research Ethics (REK no. 054-02) and informed consent was obtained from all participants.

2.2. Decidual tissue

Decidua basalis tissue was collected by vacuum suction immediately after separation of the placenta from the placental bed during CS [3,23]. The vacuum suction procedure was performed in less than 1 min. Collected tissue was flushed with saline to remove excessive blood. Decidual tissue was placed in RNAlater (for microarray analyses), 10% neutral-buffered formalin and paraffin embedded (for immunohistochemical analyses) and snap frozen in liquid nitrogen (for Western blot analyses) within 15 min of tissue collection.

2.3. Illumina microarray analysis

Total RNA extracted from decidual tissue was used for synthesis of biotin labelled anti-sense RNA (aRNA) which was hybridised to Illumina HumanWG-6 v2 Expression BeadChips (Illumina Inc., San Diego, CA) as previously described [17]. Microarray expression data were preprocessed and analysed using Sequential Oligogenic Linkage Analysis Routines (SOLAR) [24] as previously described [25]. Microarray were performed in accordance with the Minimum Information About a Microarray Experiment (MIAME) guidelines [26]. Experimental data have been submitted to ArrayExpress (www.ebi.ac.uk/arrayexpress/) under accession no. E-TABM-682. The microarray dataset was validated by quantitative real-time polymerase chain reaction (qRT-PCR) analyses for six of the most differentially expressed transcripts as previously described [17].

Table 2
Clinical characteristics of the study subjects included in the Microarray analysis ($n = 104$).

	PE ($n = 13$)	FGR ($n = 9$)	PE+FGR ($n = 24$)	Controls ($n = 58$)
Maternal age (y)	30.1 ± 5.4	32.7 ± 6.4	29.7 ± 5.5	32.4 ± 5.1
Systolic BP (mmHg)	153.1 ± 17.5**	128.3 ± 14.7*	151.0 ± 14.9**	116.2 ± 10.4
Diastolic BP (mmHg)	94.7 ± 8.6**	74.4 ± 8.3	96.2 ± 12.1**	69.9 ± 8.8
BMI (kg/m^2)[a]	29.2 ± 6.3	28.5 ± 7.5	26.4 ± 6.1	26.0 ± 4.5
Primipara	8/13	4/9	16/24	29/55[b]
GA (weeks)	34.8 ± 2.7**	31.9 ± 5.0**	30.3 ± 3.5**	38.7 ± 0.8
Birthweight (g)	2363.9 ± 509.7**	1224.7 ± 672.4**	1118.2 ± 470.1**	3608.3 ± 491.9

Data are presented as means ± SD, *$p < 0.05$ and **$p < 0.001$ comparing pre-eclampsia (PE), fetal growth restriction (FGR) and PE+FGR to controls; BP, blood pressure.
[a] BMI, Body Mass Index measured at first antenatal care visit.
[b] No info available for three of the controls; GA, gestational age.

2.4. Pathway and targeted transcriptional analyses of ER stress markers

We recently performed whole-genome transcriptional profiling of decidual tissue from pre-eclamptic and normal pregnancies, identifying upregulation of several transcripts involved in ER stress in PE. In the previous study, we solely focused on *PE associated* transcriptional changes, i.e. using only a subset of the total microarray dataset used in this work [17]. To further explore the role of ER stress in the pathogenesis of *both* PE and FGR, we performed pathway analyses on a group of 20 ER stress related transcripts (as annotated by Ingenuity Pathway Analysis) (Supplementary Table 1) in cases and controls. Pathway analyses were performed using Rotation Gene Set Tests (ROAST) and Rotation Gene Set Enrichment Analysis (ROMER), implemented in the *limma* package [27] available via the Bioconductor Project (www.bioconductor.org). ROAST was used to test whether any of the transcripts in the pathway were differentially expressed [28] and ROMER was used to test whether the subset of transcripts in the pathway was more differentially expressed than any other subset of transcripts in the total dataset [29]. As a second step in our approach, three central ER stress marker from each branch of the UPR (ATF6, XBP1 and PERK), known to be upregulated by ER stress [30,31], were selected for a targeted transcriptional comparison to test if there was any differential UPR activation between cases with PE, FGR, PE+FGR and controls.

2.5. Western blotting

Decidual tissue was homogenised in lysis buffer (Active Motif, Rixensart, Belgium) using a rotor-stator homogeniser (Ultra-Turrax T25, Janke & Kunkel IKA Labortechnik, Staufen, Germany). Total protein extracts were prepared using a Nuclear extract kit (#40010, Active Motif), following manufacturer's instructions. Homogenised decidual tissue was lysed on ice for 30 min and cell debris pelleted at 14,000 g at 4 °C for 20 min. The supernatant was recovered and equal amounts (100 μg) of protein were separated on precast 10% denaturing NuPAGE gels (Invitrogen Life Technologies, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were blocked in Odyssey blocking buffer (Li-Cor Biosciences, Cambridge, UK) for 1 h at room temperature (RT) before incubating with primary antibodies against pEIF2α, EIF2α, XBP1 and ATF6. The antibody against XBP1 detected both spliced and unspliced variants of XBP1 [32,33], and the antibody against ATF6 detected both cleaved and uncleaved ATF6 [33,34]. Blots were reprobed with β-actin as a loading control. Primary antibodies were diluted in Odyssey blocking buffer (Li-Cor Biosciences) and hybridised to the membranes overnight at 4 °C. Membranes were washed 3 × 10 min in tris-buffered saline (TBS) and incubated with fluorescently labelled secondary antibodies diluted in Odyssey blocking buffer (Li-Cor Biosciences) for 1 h at RT. Specifications for the primary and secondary antibodies are listed in Table 1. Membranes were scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Specific bands for pEIF2α (37 kDa), EIF2α (37 kDa), XBP1(U) (31 kDa), XBP1(S) (54 kDA), ATF6 (50 kDA) and β-actin (42 kDa) were detected in all samples. Band intensities were determined from two or three scans, normalised relative to loading control and quantified by densitometric analysis using Odyssey imaging software v3.0 (Li-Cor Biosciences).

2.6. Immunohisotchemical analyses

Cellular localisation and expression of ATF6 and XBP1 in decidual tissue was assesses using the same antibodies against XBP1 and ATF6 as used in Western blot experiments, in combination with antibodies against cytokeratin 7 (CK7) to detect extravillous trophoblasts (EVTs), vimentin and prolactin to detect decidual cells (DeCs), and CD68 to detect macrophages (Mφs). Double immunofluorescence staining was performed manually after deparaffination in xylen, rehydration and heat-induced antigen retrieval in TBS. Protein Block (X0909, Dako, Glostrup, Denmark) was added for 10 min to inhibit non-specific binding, and slides were incubated in a primary antibody mixture overnight at 4 °C. The slides were incubated with appropriate species-specific secondary antibodies for 30 min in a dark chamber. Slides were examined using a fluorescent microscope (Nikon Eclipse 90i with CytoVision 3.7 software, Applied Imaging, New Milton Hampshire, UK) at magnification ×600. Decidual glands, tonsil tissue and pituitary glands were used as positive controls for CK7, vimentin/CD68 and prolactin, respectively. For negative controls, primary antibodies were substituted with isotype-matched rabbit- (#011-000-120, Jackson ImmunoResearch, PA) and mouse (#400102, BioLegend, CA) immunoglobulins. Specifications for the primary and secondary antibodies are listed in Table 1. The proportion of XBP1 and ATF6 immunopositive EVTs, DeCs and Mφs was counted in five randomly selected fields on each slide, and calculated as the number of positive cells among the total number of CK7, vimentin/prolactin or CD68 positive cells, respectively.

2.7. Statistical analyses

Nonparametric data were analysed using Mann–Whitney *U* test, whereas parametric data were analysed using the Students *t*-test. Fisher's exact test and χ^2 test were used for categorical data, and Spearman's rank correlation was used for correlation analyses. The significance threshold was set to 0.05. All analyses were performed using SPSS v15 (SPSS, Chicago, IL).

3. Results

3.1. Clinical characteristics of the study subjects

Clinical characteristics of the study subjects included in the microarray analysis are presented in Table 2. A total of 104 samples were included (PE, $n = 13$; FGR, $n = 9$; PE+FGR, $n = 24$ and controls, $n = 58$). As expected, elevated blood pressure was observed in pregnancies with PE and PE+FGR, and lower gestational age and birthweight were observed in all case groups (Table 2).

A total of 30 samples (PE, $n = 7$; FGR, $n = 7$; PE+FGR, $n = 8$ and controls, $n = 8$) were included in Western blot analysis (Table 3).

Table 3
Clinical characteristics of the study subjects included in the Western blot analysis ($n = 30$).

	PE ($n = 7$)	FGR ($n = 7$)	PE+FGR ($n = 8$)	Controls ($n = 8$)
Birthweight (g)	2137.1 ± 259.6**	1363.1 ± 753.8**	1105.3 ± 329.1**	3576.3 ± 507.1
Placenta weight (g)	447.8 ± 30.3*	269.2 ± 115.0*	261.3 ± 55.9**	657.9 ± 163.9
Placenta Weight Ratio[a]	1.0 ± 0.3	0.5 ± 0.2*	0.7 ± 0.1*	0.9 ± 0.1
Severe PE or FGR	5/7	6/7	8/8	
Early onset < 34 weeks	7/7	5/7	8/8	
Mean uterine artery score[b]	2.4**	2.2*	3.5**	0

Data are presented as means ± SD; *$p < 0.05$, **$p < 0.001$ comparing pre-eclampsia (PE), fetal growth restriction (FGR) and PE+FGR to controls.
[a] Placenta Weight Ratio was calculated as observed/expected placenta weight according to gestational age and sex according to a Norwegian normogram [42].
[b] Uterine artery score is based on pulsatility index and notching in Aa.uterinae [43]; Blood pressure, maternal age, gestational age, Body Mass Index and parity did not differ statistically from that of women included in the microarray analysis (Table 2).

Twenty-four of these were also included in the microarray study population, whereas the remaining six were selected from our total study population, but not included in microarray analyses due to low RNA quality. As ER stress probably arises secondary to ischaemia-reperfusion insults, we selected cases with clinical characteristics suggesting vascular malperfusion, which is closely associated with abnormal uterine artery Doppler findings and more common in early onset, severe PE [35] and severe FGR [36]. Thus, only cases with severe clinical characteristics were included, i.e. cases with uterine artery score ≥ 1 and/or early onset PE or FGR (Table 3). The mean uterine artery score was higher in all case groups compared to controls, whereas the placental weight ratio was lower in cases with FGR and PE+FGR as compared to controls (Table 3). Sixteen decidual samples selected from the Western blot study population were included in immunohistochemical analyses (PE, $n = 4$; FGR, $n = 4$; PE+FGR, $n = 4$ and controls, $n = 4$). The clinical characteristics of these samples did not differ from those included in Western blot analyses.

3.2. Pathway and targeted transcriptional analyses of ER stress markers

Pathway analyses (ROAST and ROMER) showed that the ER stress pathway was upregulated in cases with FGR and PE+FGR, whereas in PE, a trend towards upregulation was observed (Table 4). The targeted comparison of transcript data for *XBP1*, *ATF6* and *PERK* demonstrated that cases with FGR had increased expression of *ATF6*, cases with PE+FGR had increased expression of *XBP1*, *ATF6* and *PERK*, whereas *XBP1* was increased in PE (Table 4). The Illumina probe interrogating *XBP1* (ILMN_1710675) detected both the spliced and unspliced *XBP1* variants. No differences between the case groups were observed for any of these three transcripts *(data not shown)*.

3.3. Western blot analyses

Cases with FGR and PE+FGR showed increased phosphorylation of EIF2α ($p < 0.05$), increased ratio of pEIF2α/EIF2α ($p < 0.05$) and increased levels of ATF6 ($p < 0.01$) (Fig. 1A–C). Although cases with PE had high mean levels of pEIF2α/EIF2α and ATF6, large variations within this group rendered the results non-significant in comparison to controls ($p = 0.09$ and 0.43, respectively), as indicated by the larger standard deviation bars for the PE group in Fig. 1B–C. No differences in protein levels of the spliced form of XBP1, XBP1(S), were observed between any of the case groups compared to controls. However, increased levels of the unspliced form of XBP1, XBP1(U), and a decreased ratio of XBP1(S)/XBP1(U) were observed in isolated PE ($p < 0.05$ and 0.01, respectively) (Fig. 1A and C). The levels of XBP1(U) was higher in PE compared to that of FGR, and the ratio of XBP1(U)/XBP1(S) was lower in PE compared to FGR (all p's < 0.05). No differences in protein levels of pEIF2α/EIF2α, ATF6 or XBP1(S) were observed between case groups.

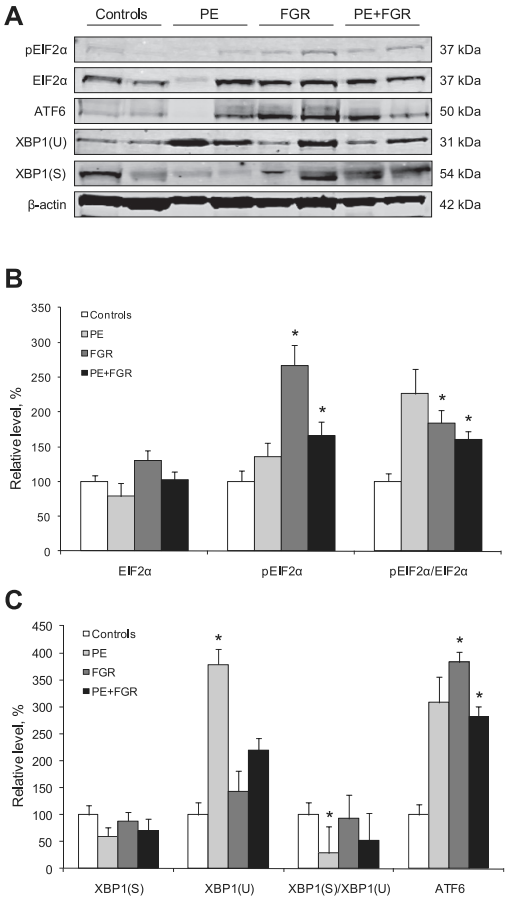

Fig. 1. Western blot analyses of endoplasmic reticulum (ER) stress markers in decidual tissue from pregnancies complicated by pre-eclampsia (PE, $n = 7$), fetal growth restriction (FGR, $n = 7$), PE+FGR ($n = 8$) and controls ($n = 8$). (A) A representative Western blot of phosphorylated and total EIF2α, ATF6, spliced (S) and unspliced (U) forms of XBP1 and loading control (β-actin). (B and C) Densitometry of bands expressed relative to controls (100%), showing increased levels of pEIF2α and ATF6 in pregnancies complicated by FGR and PE+FGR. Increased levels of XBP1(U) and a decreased XBP1(S)/XBP1(U) ratio were observed in pregnancies complicated by PE. Phosphorylation status of EIF2α is presented as the ratio between phosphorylated and total level of EIF2α. No significant differences in EIF2α were observed between any of the study groups. Data are presented as means ± SE, $^{*}p < 0.05$ as compared to controls.

Table 4
Pathway and targeted transcriptional analyses of ER stress related transcripts.

	PE vs. controls	FGR vs. controls	PE+FGR vs. controls
Pathway analysis[a]			
ROAST	$p = 0.06$ (NS)	↑, $p < 0.05$	↑, $p < 0.001$
ROMER	$p = 0.09$ (NS)	↑, $p < 0.05$	↑, $p < 0.05$
Microarray analysis[b]			
XBP1	↑, $p < 0.01$	$p = 0.07$ (NS)	↑, $p < 0.05$
ATF6	$p = 0.22$ (NS)	↑, $p < 0.05$	↑, $p < 0.001$
PERK	$p = 0.09$ (NS)	$p = 0.55$ (NS)	↑, $p < 0.05$

[a] Rotation Gene Set Tests (ROAST) and Rotation Gene Set Enrichment Analysis (ROMER) pathway analyses of 20 transcripts involved in the endoplasmic reticulum (ER) stress response (Supplementary Table 1).
[b] A targeted comparison of transcription data for ER stress markers from each branch of ER stress response; NS, not significant; ↑, upregulation.

3.4. Immunohistochemical analyses

Both cytoplasmic and nuclear immunoreactivity for XBP1 and ATF6 was observed in most (>80%) EVTs, DeCs and Mφs (Fig. 2 A,A′-R,R′). No differences in the proportion of XBP1 or ATF6 immunopositive cells or staining pattern were observed between the study groups. In general, XBP1 and ATF6 immunoreactivity was present in most cells in the decidual tissue, in contrast to tonsil tissue and pituitary glands, in which only a few cells were positive (*not shown*). Decidual glands displayed intense nuclear immunoreactivity for XBP1 in both cases and controls (Fig. 2S). No differences in staining

intensity of XBP1 and ATF6 were observed between the study groups in any of the evaluated cell types.

3.5. Correlation analyses

Correlation analyses demonstrated a negative correlation between the pEIF2α/EIF2α ratio and the placental weight ratio ($r_s = -0.561$, $p < 0.05$). A similar tendency was observed for ATF6 ($r_s = -0.352$, $p = 0.072$). None of the ER stress markers were correlated with gestational age.

4. Discussion

In this work, we have shown that decidual ER stress is increased in pregnancies complicated by FGR and PE+FGR. Initially, this was demonstrated by pathway analyses, showing upregulation of the ER stress pathway in FGR and PE+FGR. In addition, targeted transcriptional and protein analyses showed upregulation of the PERK-pEIF2α and ATF6 signalling branches of the UPR. In PE, none of these branches were significantly altered However, a marked increase in XBP1(U) was observed. These results indicate divergent activation of the UPR in FGR and PE+FGR compared to isolated PE.

Consistent with our finding of increased pEIF2α in deciduas from FGR and PE+FGR, increased placental levels of pEIF2α have previously been reported in these cases [15]. In trophoblast-like cell lines, increased levels of pEIF2α were associated with reduced proliferation through suppression of protein synthesis and decreased survival [15]. The net effect of reduced proliferation and cell survival was proposed as a cause for reduced placental growth in pregnancies with FGR and PE+FGR [15,16], which are characterised by decreased placental villous tissue volume and surface area [37]. Of relevance, we observed that the pEIF2α/EIF2α ratio was negatively correlated with placental weight ratio, with a similar tendency for ATF6, suggesting an association between ATF6 and PERK-pEIF2α signalling and reduced placental weight.

Cases with isolated PE only showed a trend towards upregulation of the ER stress response. However, we observed increased levels of *XBP1* mRNA and XBP1(U) protein in isolated PE. It was recently shown that XBP1(U), the constitutively expressed form of XBP1, accumulates in the recovery phase of ER stress [38] and is

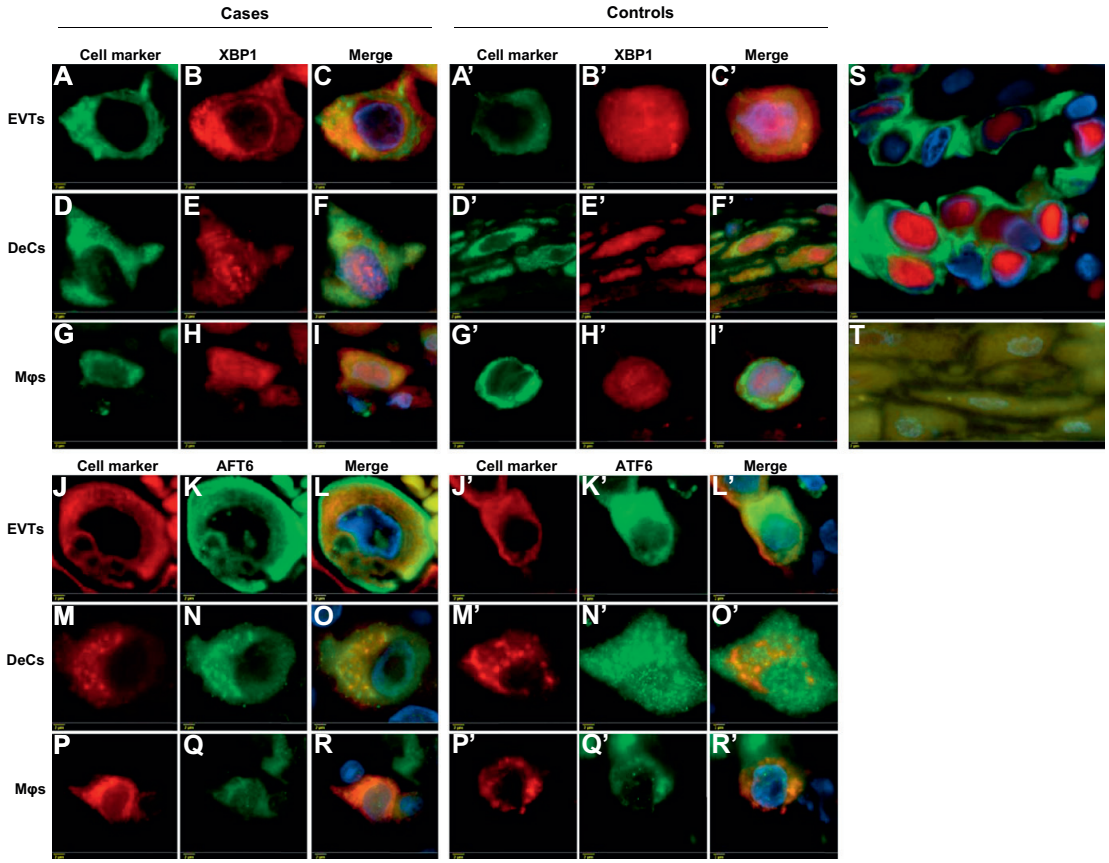

Fig. 2. Immunofluorescensce analyses of XBP1 and ATF6 in extravillous trophoblasts (EVTs), decidual cells (DeCs) and macrophages (Mφs), identified using antibodies against cytokeratin 7, vimentin/prolactin and CD68, respectively. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (blue signal). Cytoplasmic and nuclear immunoreactivity of XBP1 (red signal) was observed in EVTs, DeCs and Mφs in both cases with PE and/or FGR (A–I) and controls (A′–I′). A similar staining pattern was observed for ATF6 (green signal) for the same cell types in cases with PE and/or FGR (J–R) and controls (J′-R′). In decidual glands, strong nuclear immunoreactivity for XBP1 (red signal) was observed (S). No positive staining was found in negative controls where primary antibodies were substituted with isotype-matched immunoglobulins of the appropriate species (T). Magnification ×600 in D′-F′, S and T, otherwise ×1000. Scale bar 2 μm.

able to inhibit XBP1(S) and ATF6, implying that XBP1(U) functions as a negative regulator during ER stress [39,40]. This correlates well with our finding of the highest level of XBP1(U) and the lowest level of XBP1(S) in PE, and that ATF6 was not significantly increased in these cases. Based on this, it is tempting to speculate that IRE1-XBP1 signalling via upregulation of XBP1(U) may protect against or decrease the effects of ER stress in PE, which could be reflected by the normal placental weigh ratio observed in this group. Correspondingly, reduced placental weight in FGR and PE+FGR, but not in isolated PE, was recently reported in a Norwegian cohort of 317,688 pregnancies [41].

Both nuclear and cytoplasmic immunoreactivity for XBP1 and ATF6 was observed in EVTs, DeCs and Mφs, but no differences in the proportion of immunoreactive cells or staining pattern was observed between the study groups. No differences in cytoplasmic staining intensity for XBP1, representing XBP1(U), or nuclear staining intensity of ATF6, representing cleaved ATF6, were detected in any of these cell types between the study groups. In general, nuclear staining intensity was difficult to assess due to photobleaching. Thus, no determination of the cellular source of increased levels of XBP1(U) or cleaved ATF6 in decidual tissue, as detected by Western blot, could be made by immunofluorescence analysis. However, we cannot exclude that other cell types in the decidua, that were not evaluated in the present work may have contributed to the increased ATF6 and XBP1(U) levels.

In summary, we found that decidua basalis is a source of ER stress, and that ER stress is increased in pregnancies complicated by FGR and PE+FGR. In PE, we found increased levels of XBP1(U), which may be a protective mechanism against the detrimental effects of ER stress. This could explain some of the observed clinical differences in between PE and FGR. However, future studies are warranted to test this hypothesis and elucidate the implications of our findings.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.placenta.2011.08.005.

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Paper IV

Preeclampsia and cardiovascular disease share genetic risk factors on chromosome 2q22

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Short title:
Genetic risk preeclampsia and CVD

ABSTRACT

Objective: Four putative single nucleotide polymorphism (SNP) risk variants at the preeclampsia susceptibility locus on chromosome 2q22; rs2322659 (*LCT*), rs35821928 (*LRP1B*), rs115015150 (*RND3*) and rs17783344 (*GCA*), were recently shown to associate with known cardiovascular risk factors in a Mexican American cohort. This study aimed to further evaluate the pleiotropic effects of these preeclampsia risk variants in an independent Australian population-based cohort.
Methods: The four SNPs were genotyped in the Western Australian Pregnancy Cohort (Raine) Study that included DNA, clinical and biochemical data from 1,246 mothers and 1,404 of their now adolescent offspring. Genotype association analyses were undertaken using the SOLAR software.
Results: Nominal associations ($P < 0.05$) with cardiovascular risk factors were detected for all four SNPs. The *LCT* SNP was associated with decreased maternal height ($P = 0.005$) and decreased blood glucose levels in adolescents ($P = 0.022$). The *LRP1B* SNP was associated with increased maternal height ($P = 0.026$) and decreased maternal weight ($P = 0.044$). The *RND3* SNP was associated with decreased triglycerides in adolescents ($P = 0.001$). The *GCA* SNP was associated with lower risk in adolescents to be born of a preeclamptic pregnancy ($P = 0.003$) and having a mother with prior preeclamptic pregnancy ($P = 0.033$).
Conclusions: Our collective findings support the hypothesis that genetic mechanisms for preeclampsia and CVD are, at least in part, shared, but need to be interpreted with some caution as a Bonferroni correction for multiple testing adjusted the statistical significance threshold (adjusted $P < 0.001$).

Key words
2q22; cardiovascular disease (CVD); genetic association; preeclampsia; Raine Study

Introduction

Women with a history of preeclampsia and offspring exposed to preeclampsia in utero are at increased risk of cardiovascular disease (CVD) later in life [1-3]. A large review and meta-analysis found that women with a history of preeclampsia have approximately four-fold increased risk of chronic hypertension, and two-fold increased risk of coronary artery disease and stroke 10-15 years after pregnancy [1]. The offspring of women with preeclampsia have higher mean systolic and diastolic blood pressure in childhood and early adult life in both genders, including those with normal birth weight [4-6]. Furthermore, they have almost a two-fold greater risk of stroke in adulthood [3]. Preeclampsia is now widely viewed as an early screening criterion for CVD in women. Pregnancy is a unique opportunity to identify both women and offspring at increased risk of premature CVD [7], and clinical risk assessments and preventive programmes are under development [8].

Preeclampsia and CVD share several constitutional risk factors (e.g. hypertension and obesity) [9], pathological features (e.g. endothelial dysfunction and inflammation) [10, 11], and tend to occur in the same families [12]. These common antecedents have drawn attention to the likelihood of shared genetic susceptibility [13, 14]. Supporting this notion are several cardiovascular risk factors present years before a preeclamptic pregnancy, including increased blood pressure, higher levels of serum cholesterol, higher levels of low density lipoprotein (LDL)-cholesterol and higher levels of triglycerides [15]. Moreover, the positive association between preeclampsia and CVD is more dependent on these shared pre-pregnancy risk factors than the influence of the hypertensive disorder in the pregnancy itself [16]. This has encouraged the search for genetic determinants common to both disorders. However, to date only a few shared genetic risk factors have been identified [17-20].

Recently, our genetic dissection of the 2q22 preeclampsia susceptibility locus identified four independent single nucleotide polymorphism (SNP) risk variants, residing within four genes, to associate with preeclampsia in an Australian family cohort [19]: lactase (*LCT*, rs2322659), low density lipoprotein receptor-related protein 1B (*LRP1B*, rs35821928), rho family GTPase 3 (*RND3*, rs115015150) and grancalcin (*GCA*, rs17783344). Furthermore, these same four SNPs were associated with cardiovascular risk factors in an independent cohort of Mexican American families, suggesting pleiotropic effects for these SNPs [19]. The aim of this study was to determine whether these four SNPs exhibited pleiotropic characteristics with preeclampsia

susceptibility and cardiovascular risk factors in an independent Australian population-based cohort consisting of mothers and their adolescents. Identifying common genetic factors influencing preeclampsia and CVD may provide insight into pathophysiological mechanisms relevant to both disorders.

Materials and methods

Study population

The Western Australian Pregnancy Cohort (Raine) Study is a pregnancy cohort where women were recruited prior to 18 weeks' gestation from the public antenatal clinic at King Edward Memorial Hospital or surrounding private clinics in Perth, Western Australia. The study has been described in detail elsewhere [21]. Pregnant women (n = 2,900) were enrolled between August 1989 and April 1992, and they gave birth to 2,868 live babies. From the original cohort of women, their children have been followed up over the last two decades with detailed assessments performed every 2-3 years. In the current study, data from the pregnant women, the neonates, and the 8-, 14- and 17-year cohort follow-ups were assessed, as shown in Fig. 1. Only subjects that had two Caucasian parents, were biologically unrelated, and who had no congenital deformities, were included in the current study.

Informed written consent was obtained at recruitment and at each follow-up from the mother or legal guardian as well as from the adolescent during the 14- and 17-year cohort follow-ups. Ethical approval was obtained for all protocols from the Human Ethics Committees of King Edward Memorial Hospital, Princess Margaret Hospital Ethics Committee, Perth, Western Australia and The University of Western Australia.

Antenatal information

At recruitment the mothers completed self-administrated questionnaires concerning their pregnancies and demographic information. The presence of preeclampsia and history of preeclampsia were collected from the mother at antenatal visits at the delivery units and later assessed from the medical records. The medical records were reviewed by obstetricians and research midwives to confirm a standardised diagnosis of preeclampsia as a pregnancy-induced increase in systolic blood pressure ≥140 mmHg and/or a diastolic blood pressure ≥90 mmHg in

women who were normotensive before the 24th week of pregnancy, combined with significant new onset proteinuria (≥ 0.3 g/l in a 24-hour specimen) [22].

Blood pressure, anthropometry and blood samples

Detailed information on measures of blood pressure, anthropometry and biochemistry, is given in detail elsewhere [23, 24]. Briefly, blood pressure was measured with an automatic device (Dinamap Vital Signs Monitor 8100, Dinamap XL Vital Signs Monitor or Dinamap ProCare 100; GE Healthcare) after 5 minutes rest and using the appropriate cuff size. Six readings were recorded, and the average value was calculated after excluding the first reading. Height and weight were measured with light clothing and without shoes. Height was measured with Holtain Infantometer and Stadiometer (to the nearest 0.1 cm), and weight was measured on Wedderburn Scales (to the nearest 100 g). Fasting venous blood samples were drawn for DNA and biochemical analyses. Serum insulin, glucose, total cholesterol, high density lipoprotein (HDL)-cholesterol, LDL-cholesterol and triglycerides were measured in the PathWest Laboratory at Royal Perth Hospital as described previously [23, 24].

Cardiovascular risk factors

Cardiovascular risk factors assessed included resting systolic and diastolic blood pressure, height, weight, waist-hip ratio, abdominal skinfold, and fasting insulin, glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides. Maternal data was obtained from an examination and blood sample taken when their children attended the 8-year cohort follow-up and included 1,685 mothers. Adolescent data was obtained during the 14- and 17-year cohort follow-ups and included 1,293 [23] and 1,053 [24] participants, respectively.

DNA extraction and SNP genotyping

DNA was extracted from blood samples taken from mothers and adolescents at the 14- or 17-year cohort follow-ups as described elsewhere [25]. Briefly, DNA was extracted from 4 mL ethylenediaminetetraacetic acid (EDTA) anticoagulated blood using Qiagen PureGene chemistry (Qiagen, Hilden, Germany). Four independent SNPs in four genes, rs2322659 (*LCT*), rs35821928 (*LRP1B*), rs115015150 (*RND3*) and rs17783344 (*GCA*), were genotyped for mothers and adolescents. For the mothers *de novo* genotyping of the four SNPs was performed. For the

adolescents *de novo* genotyping was performed for rs35821928 and rs115015150. The rs2322659 and rs17783344 SNPs had already been genotyped in a previously performed genome wide association study (GWAS) [26]. *De novo* genotyping was commercially performed by KBioscience (KBioscience, Hertfordshire, UK), with the use of their proprietary fluorescence-based competitive allele-specific PCR genotyping assay, KASP™. Genotyping and quality control of GWAS data have been described in detail elsewhere [26]. Briefly, the Raine adolescent samples were genotyped on the Illumina Human 660W-Quad SNP Chip (Illumina Inc., San Diego, CA, USA) at the Centre for Applied Genomics (Toronto, Ontario, Canada). Individual samples were checked (and excluded accordingly) for gender inconsistencies, levels of heterozygosity and inter-sample relatedness.

Statistical analysis

The software package R (www.r-project.org) was used to compute descriptive statistics, means and 95% confidence intervals (CI). Phenotypes of interest included cardiovascular risk factor measurements and maternal pregnancy characteristics.

SNP association analysis

Measured genotype association analyses were undertaken for all phenotypes applying variance-component methods as implemented in SOLAR [27]. Because variance-component methods are sensitive to kurtosis, all quantitative phenotypes were transformed using SOLAR's inverse normalization procedure. Genetic association was tested for each SNP under an additive genetic model allowing mean phenotype value to vary by minor allele. This model was compared with the null model of no difference in mean phenotype value by SNP genotype using a likelihood ratio test. Twice the difference in log-likelihoods of these models was distributed as a χ^2 random variable with 1 degree of freedom. Concordance with Hardy-Weinberg proportions was tested using χ^2 goodness-of-fit statistic. A threshold of $\alpha = 0.05$ was set for statistical significance of all computed analyses. Adjustment for multiple hypothesis testing was performed using Bonferroni corrections (α/(total number of SNPs x total number of phenotypic traits)).

Cardiovascular risk factors including height, weight, blood pressure, and cholesterol, have consistently been demonstrated to correlate between relatives. This could reflect genetic- and/or shared life style effects [28]. Therefore we performed genetic association analysis to examine the

association between total maternal genotype and total adolescent phenotype, and vice versa. We did not look specifically at mother-offspring pairs. In addition, we performed separate association analyses for girls and boys for all cardiovascular risk factors aiming to detect differences in genetic risk profiles between the adolescent's genders.

Results

Clinical characterisation

At the 14-year follow-up 629 (48.6%) girls and 664 (51.3%) boys participated, whereas at the 17-year follow-up 509 (48.3%) girls and 544 (51.7%) boys participated. Of the enrolled women (mothers of the adolescents) with accessible DNA for genotyping, 40 (3.2%) were diagnosed with preeclampsia in the index pregnancy, and 31 (2.5%) had previously experienced a preeclamptic pregnancy. The mean age for the index pregnancy was 28.2 years. Clinical and biochemical characteristics of mothers and adolescents are presented in Table 1.

SNP genotyping and association analysis

De novo DNA data was available for 1,246 of the mothers. *De novo* DNA data was available for 1,461 of the adolescents and GWAS DNA data was available for 1,494 adolescents. After exclusion of children with congenital deformities, siblings and non-Caucasians, DNA from 1,246 mothers and 1,404 adolescents were included in the final analysis. We observed a high genotyping success rate for all four SNPs (>97%). Allele frequencies for mothers and adolescents are presented in Table 2, and are consistent with frequencies observed by Johnson et al. [19]. Except for the *LCT* (rs2322659) SNP for mothers, all SNPs confirmed to Hardy-Weinberg proportions ($P > 0.05$).

Measured genotype association results were undertaken for all phenotypes and adjusted for sex and maternal age (raw $P < 0.05$). The results are presented in Table 3 and 4 for mothers and adolescents respectively. Carrying the A allele of *LCT* rs2322659 was associated with decreased levels of the adolescent's blood glucose in both mothers and adolescents ($P = 0.003$ and $P = 0.022$, respectively) and decreased maternal height ($P = 0.005$) in mothers. Carrying the T allele of *LRP1B* rs35821928 was associated with increased maternal height in both mothers and adolescents ($P = 0.026$ and $P = 0.013$, respectively) and decreased maternal weight ($P = 0.044$) in

mothers. An association between the A allele of *RND3* rs115015150 and decreased adolescent's waist-hip ratio ($P = 0.030$) was observed in mothers, whereas this SNP was associated with decreased level of adolescent's triglycerides ($P = 0.001$) in adolescents. In mothers, carrying the C allele of *GCA* rs17783344 was associated with increased adolescent's height ($P = 0.045$), whereas in adolescents carrying the C allele was associated with lower risk to be born of a preeclamptic pregnancy ($P = 0.003$) and lower risk to have a mother who previously had experienced a preeclamptic pregnancy ($P = 0.033$). The two latter associations were related to male gender ($P = 0.009$ and $P = 0.0372$, respectively). However, after accounting for the four SNPs tested across the 14 phenotypes, none of the association results satisfy our Bonferroni-adjusted statistical significance threshold (adjusted $P < 0.001$).

Discussion

The basis for this study was the recently reported shared genetic mechanisms putatively influencing preeclampsia and cardiovascular risk factors [19]. We have now assessed these independent putative pleiotropic variants representing four genes; rs2322659 (*LCT*), rs35821928 (*LRP1B*), rs115015150 (*RND3*) and rs17783344 (*GCA*), in an independent Australian population-based pregnancy cohort. We observed shared genetic associations between specific SNPs and known cardiovascular risk factors, for mothers and their offspring. However, we were unable to replicate many of the genetic associations previously reported by Johnson et al. [19]. To our knowledge, this is the first published study that has assessed possible shared genetic risk factors for preeclampsia and CVD in both mothers and their offspring.

Johnson et al. found the A allele of the *LCT* SNP protective for preeclampsia, and nominally associated with oxidative stress indicators, inflammatory- and diabetic biomarkers [19]. Supportive of protective pleiotropic effects on preeclampsia and cardiovascular risk factors, we found the A allele of the *LCT* SNP to be nominally associated with decreased glucose levels in the adolescents. To date, there is limited evidence of the association between exposure to preeclampsia in utero and the offspring's fasting glucose metabolism later in life [5, 6]. The *LCT* SNP was out of Hardy-Weinberg equilibrium for mothers, the same observation made by Johnson et al. in their Australian preeclampsia case-control cohort [19]. This could possibly be explained by locus-specific population stratification, and has been thoroughly discussed elsewhere [19, 29].

LRP1B, a member of the LDL receptor gene superfamily, has recently been shown to be involved in cell migration and invasion *in vitro* [30], central elements in the development of preeclampsia. Further, SNPs in the *LRP1B* gene were associated with body mass index (BMI) in a large GWAS [31], and insulin resistance in a follow-up study [32], suggesting that this gene may be involved in body weight regulation. Johnson et al. found the T allele of the *LRP1B* SNP protective for preeclampsia [19]. We observed the T allele of the *LRP1B* SNP to be associated with decreased weight, and increased height. The possibility of *LRP1B* harbouring genetic variants influencing preeclampsia and CVD is possible, as obesity and short stature are risk factors for both preeclampsia and coronary heart disease [9, 33, 34].

RND3 (RhoE) plays a role in human cytotrophoblast fusion, suggesting an important role in the regulation of trophoblast fusion in pregnancy [35]. *RND3* inhibits the biological activity of a downstream effector protein, Rho-associated protein kinase (ROCK) [36]. ROCK proteins have important roles in abnormal vascular tone, endothelial dysfunction, inflammation, oxidative stress and vascular re-modelling, all of which are influential factors in preeclampsia and CVD pathogenesis. Johnson et al. found the A allele of the *RND3* SNP associated with higher preeclampsia risk and nominally associated with increased adiponectin levels [19], a protein which is inversely correlated with body fat percentage in adults. In accordance with the latter, we found reduced levels of triglycerides and reduced waist-hip ratio for the A allele of the *RND3* SNP. Hence, these data add to the possibility of *RND3* harbouring genetic variants that may have a role in obesity-related pathology.

Grancalcin (*GCA*), a calcium binding protein, is specifically expressed in neutrophils and monocytes/macrophages, and displays calcium-dependent translocation to the granules and plasma membrane upon activation of these innate immune responders [37]. Neutrophil activation leads to the release of toxic factors (e.g. myeloperoxidase) promoting an inflammatory response, oxidative stress and vascular dysfunction [38]. While grancalcin deficiency does not adversely affect neutrophil function, it does however, impact their adhesive properties to fibronectin [39]. Plasma cellular fibronectin, a marker for endothelial and vascular injury, has been reported in several studies to be elevated in preeclampsia [40, 41]. Furthermore, neutrophil adhesion to fibronectin promotes cytokines such as IL-8 to exert their chemotactic effects, which may explain the pronounced abundance of neutrophils in the maternal systemic vasculature of both preeclamptic [38] and obese [42] women. We observed that the C allele of the *GCA* SNP was

associated with lower risk to be born of a preeclamptic pregnancy and lower risk to have a mother who previously had experienced preeclampsia. These results showed association to male gender, and were not associated with preeclampsia in the mothers. This could possibly indicate a paternally inherited role for this SNP. However, Johnson et al. [19] assessed the maternal genotype, and we cannot exclude our association results to preeclampsia as false positives.

There was only a partial replication between the results of Johnson et al. [19] and our study. This could be explained by differences between the studies including constitution of study populations (e.g. ethnicity, sex and age), sampling procedures and the undertaken biochemical measurements. A limitation to our study is that the number of women with preeclampsia is limited, which reduces the power to detect significant associations and making subgroups analysis assessing severe preeclampsia (e.g. early versus late onset) impossible. Severe preeclampsia may be associated with an even greater risk of CVD later in life [1]. However, this relationship was not confirmed in a recently published large review and meta-analysis [2]. Another limitation of our study is that we did not access paternal data due to insufficient information on paternal cardiovascular risk factors. On the other hand, there is no clear evidence of association between preeclampsia and paternal cardiovascular risk factors [43, 44], suggesting that influence of paternal genes for increasing preeclampsia risk differs to the influence of genes increasing cardiovascular risk [44]. Strengths of our study include a large homogeneous study population, assessment of both maternal and adolescent data, a relatively high attendance at cohort follow-ups, inclusion of fasting blood samples, standardized endpoint measurements and an accurate diagnosis of preeclampsia [21].

In conclusion, our study has demonstrated in an independent population that all four genetic variants tested (rs2322659 (*LCT*), rs35821928 (*LRP1B*), rs115015150 (*RND3*) and rs17783344 (*GCA*)) were nominally associated with known cardiovascular risk factors including height, weight, waist-hip ratio, blood glucose and triglycerides. The *GCA* SNP was associated with lower risk to be born of a preeclamptic pregnancy and lower risk to have a mother who previously had experienced a preeclamptic pregnancy, increasing the putative role for this gene locus in preeclampsia susceptibility. Our findings support the hypothesis that underlying genetic mechanisms for preeclampsia and CVD are, at least in part, shared. These results warrant further investigation to determine the potential roles of these variants in preeclampsia and CVD. The complex etiology of these disorders are striking, and targeted analyses and more comprehensive

investigation strategies made possible by new technologies will be important in further revealing the genetic susceptibility to preeclampsia and CVD.

Contributors

M.L. contributed to conception and design, data analysis, interpretation of results and was responsible for manuscript preparation. E.K.M. conceived the idea for the project, contributed substantially to conception and design, revision and final approval of the manuscript. M.P.J. and P.E.M. contributed substantially to data analysis, interpretation of results, revision and final approval of the manuscript. W.A., R.C.H, T.A.M., L.J.B. and C.P. were involved in the planning of the project, contributed substantially to acquisition of data, revision and final approval of the manuscript. L.T.R. A.C.I., R.A., C.E.E., J.B. and S.P.B. contributed to interpretation of data, revision and final approval of the manuscript.

Conflict of interest statement

The authors declare that they do not have any conflict of interest.

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Table 1
Clinical and biochemical characteristics of mothers at their children's 8-year follow-up, and adolescents at the 14- and 17-year follow-ups.

Trait description	Mothers[ab]	Adolescents 14 yr[a]	Adolescents 17 yr[a]
Systolic blood pressure (mmHg)	118.8 (118.0, 119.6)	111.5 (110.9, 112.1)	118.1 (114.3, 121.9)
Diastolic blood pressure (mmHg)	69.5 (68.9, 70.0)	58.7 (58.4, 59.1)	63.7 (59.7, 67.8)
Height (cm)	163.9 (163.5, 164.3)	165.1 (164.6, 165.5)	174.3 (172.1, 176.4)
Weight (kg)	70.6 (69.6, 71.5)	58.7 (57.9, 59.5)	71.5 (68.3, 74.6)
Waist-hip ratio	-	0.83 (0.83, 0.84)	0.81 (0.80, 0.81)
Abdominal skinfold (cm)	-	-	26.8 (25.3, 28.3)
Insulin (mU/liter)	3.56 (3.45, 3.67)	12.58 (11.85-13.31)	9.49 (8.83, 10.15)
Glucose (mmol/liter)	4.81 (4.72, 4.90)	4.81 (4.78-4.84)	4.77 (4.73, 4.80)
Total cholesterol (mmol/liter)	5.07 (4.99, 5.16)	4.17 (4.13, 4.22)	4.12 (4.07, 4.17)
HDL-cholesterol (mmol/liter)	1.51 (1.48, 1.55)	1.39 (1.37, 1.41)	1.30 (1.28, 1.32)
LDL-cholesterol (mmol/liter)	3.10 (3.02, 3.18)	2.32 (2.28, 2.35)	2.34 (2.30, 2.38)
Triglycerides (mmol/liter)	1.02 (0.96, 1.08)	1.02 (0.98, 1.05)	1.06 (1.02, 1.09)

[a]Data are expressed as mean (95% CI) or *n* (%).
[b]Clinical and biochemical characteristics were obtained when their children attended the 8-year follow-up.

Table 2
Distribution of alleles for mothers (*n* = 1,246) and adolescents (*n* = 1,404) in the Raine Study.

Gene	SNP	Mothers		Adolescents	
		Major allele *n* (proportion of total)	Minor allele *n* (proportion of total)	Major allele *n* (proportion of total)	Minor allele *n* (proportion of total)
LCT	rs2322659	G 1684 (0.76)	A 522 (0.24)	G 2138 (0.77)	A 656 (0.23)
LRP1B	rs35821928	C 2072 (0.94)	T 142 (0.06)	C 2272 (0.94)	T 156 (0.06)
RND3	rs115015150	G 2171 (0.98)	A 37 (0.02)	G 2392 (0.98)	A 42 (0.02)
GCA	rs17783344	A 1914 (0.86)	C 300 (0.14)	A 2398 (0.85)	C 410 (0.15)

Table 3
SNPs nominally associated ($P < 0.05$) with cardiovascular risk factors for the mothers.

Gene	SNP	Trait description	*n*	*P* value[a]	Direction of association[b]
LCT	rs2322659	Blood glucose*	875	0.003	↓
		Height	900	0.005	↓
LRP1B	rs35821928	Height	902	0.026	↑
		Weight	863	0.044	↓
RND3	rs115015150	Waist-hip ratio*	739	0.030	↓
GCA	rs17783344	Height*	830	0.045	↑

*Associated with the adolescent phenotype.
[a]Observed measured genotype *P* value.
[b]Direction of association, for the minor allele.

Table 4
SNPs nominally associated ($P < 0.05$) with cardiovascular risk factors for the adolescents.

Gene	SNP	Trait description	*n*	*P* value[a]	Direction of association[b]
LCT	rs2322659	Blood glucose	969	0.022	↓
LRP1B	rs35821928	Height*	960	0.013	↑
RND3	rs115015150	Triglycerides	935	0.001	↓
GCA	rs17783344	Born of a preeclamptic pregnancy	1404	0.003	↓
		Mother with prior preeclampsia	1403	0.033	↓

*Associated with the maternal phenotype.
[a]Observed measured genotype *P* value.
[b]Direction of association, for the minor allele.

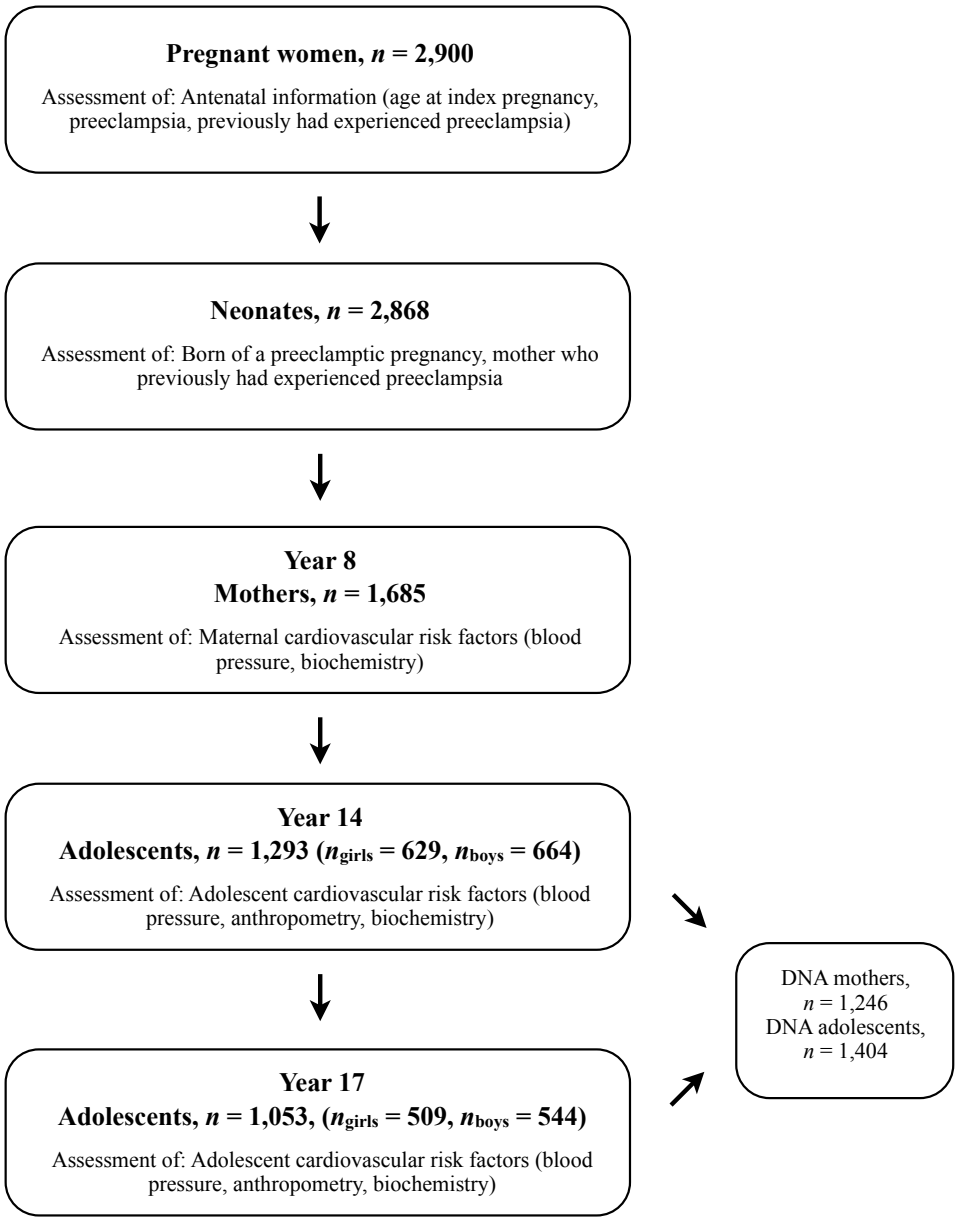

Fig. 1. Diagram showing numbers of mothers and offspring at the cohort follow-ups which were included in the analysis for the current study.