Failing the stress test

Spiral artery pathology in pregnancy and the interplay with maternal cardiovascular health

Laura Brouwers

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Spiral artery pathology in pregnancy and the interplay with maternal cardiovascular health

De stress test falen

Spiraal arterie pathologie tijdens de zwangerschap en de wisselwerking met het maternale hart- en vaatstelsel (met een samenvatting in het Nederlands)

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General introduction

PREGNANCY AS A STRESS TEST

Pregnancy poses a comprehensive physiological challenge to all organ systems. In particular the cardiovascular, endocrine and respiratory systems require significant adaptations to guarantee a successful outcome for mother and child. Pregnancy complications, such as preeclampsia and fetal growth restriction (FGR), are therefore thought to reveal a predisposition for future disease. From this point of view pregnancy may act as a 'stress test' identifying women at risk for disease later in life. A well-known example is the increased risk for cardiovascular disease (CVD) in women who experience preeclampsia in pregnancy. Although vascular compromise subsides shortly after pregnancy, large cohort studies have consistently shown that an increased risk of CVD becomes apparent when these women age.[3,4] Not only the mother, but also her children, experience these pregnancy-related and lifetime risks. Besides the high risk of neonatal morbidity and mortality directly after (preterm) birth, an increased cardiovascular and metabolic risk has been shown in the adult offspring. This may be highlighting the influence the intrauterine environment has on life-long health of the baby.[5-7]

In this thesis we aim to elucidate factors leading to the actual 'failing of the stress test of pregnancy' by studying maternal spiral arteries and the surrounding uterine environment in normal pregnancy and in pregnancy complications **(part I)**. Furthermore we aim to elucidate some of the missing information regarding the development of CVD after having 'failed the stress test' previously (i.e. women with a history of preeclampsia). In other words, we investigate factors that may help to identify who is at increased risk, when cardiovascular damage develops and if timely screening and lifestyle interventions may have a positive health and economic impact **(part II)**.

PREGNANCY DISORDERS

Preeclampsia and FGR complicate around 3-5% of pregnancies and both conditions re-occur in approximately 15% of subsequent pregnancies.[8,9] Worldwide, ten million women develop preeclampsia each year and approximately 76,000 mothers and 500,00 babies die as a direct consequence of these two disorders.[10]

Preeclampsia is generally defined as new onset of hypertension after 20 weeks of gestation concurring with either significant proteinuria or other organ dysfunction.[11] Additionally, the mother is at risk for several severe complications such as pulmonary edema, eclamptic seizures and stroke.[10] When the unborn baby does not appear to achieve its genetically determined growth potential, it is determined as growth restricted. This is usually detected by prenatal ultrasound when either estimated fetal weight or abdominal circumference are below the tenth percentile or a reduction in the standardized growth curves of ≥20 percentile is apparent.[12-14] FGR poses significant intrauterine risks for the fetus as it is associated with

sudden fetal demise and postnatal morbidity related to chronic fetal hypoxia.[15]

Preeclampsia and FGR are often concurrent and have an overlapping, complex and multifactorial pathophysiology, for which no real treatment exists. Currently, careful clinical monitoring for imminent maternal complications and signs of fetal distress is aimed at correctly timing delivery of the baby when indicated. An unbalanced interplay between (pre-existent) maternal constitution with pregnancy-specific adaptations and possible fetal or placental factors is thought to lead to a heterogeneous presentation of the key symptoms mentioned above (Figure 1). Despite extensive research this interplay and ultimately the exact pathophysiology of preeclampsia and FGR is not completely understood.

Impaired vascular adaptation in the tissue underlying the placenta (the placental bed), known as spiral artery remodeling, is thought to be the cornerstone of the pathophysiology in a large proportion of cases. Impaired spiral artery remodeling is thought to lead to a chain of events ultimately resulting in a wide range symptoms occurring in interchangeable order. The release of inflammatory and antiangiogenic factors into the maternal circulation results in endothelial dysfunction and subsequent hypertension, proteinuria and end-organ dysfunction. Although estimated incidence varies between 30-80% depending on time of onset and severity, preeclampsia is often combined with restriction of the baby's growth potential.[16]

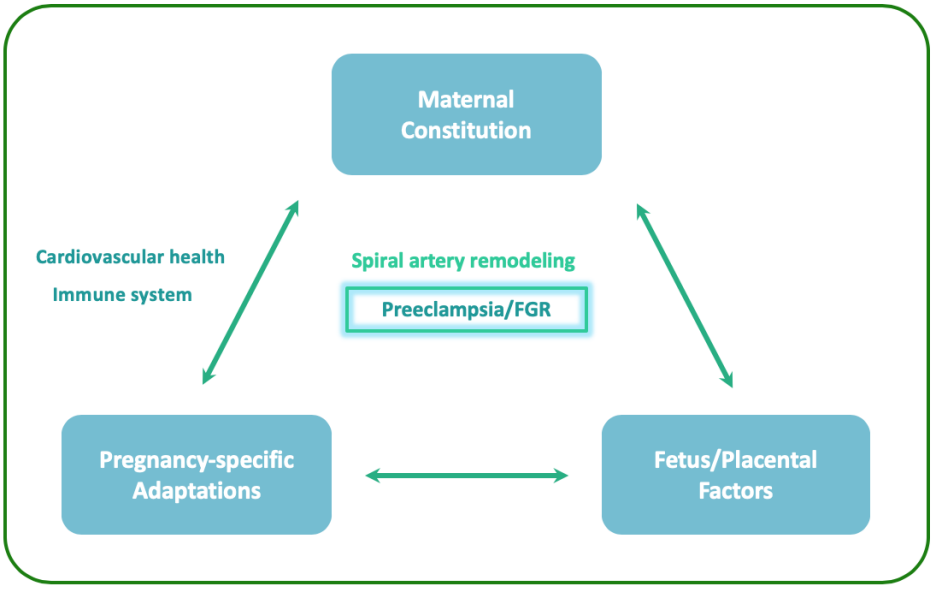

FIGURE 1. Heterogeneous and multifactorial pathophysiology of preeclampsia and fetal growth restriction. An unbalanced interplay between maternal constitution with pregnancy-specific adaptations and possible fetal or placental factors lead to a heterogeneous and interchangeable presentation of several key features. Impaired uterine vascular adaptation underlying the placenta (i.e. the placental bed), known as spiral artery remodeling, is thought to be the cornerstone in pathophysiology for a large proportion of these pregnancies and will be discussed at length in this thesis **(part I)**.

The underdeveloped placenta found in both disorders could be a direct result of ineffective remodeling of the underlying spiral arteries. Post-partum pathology review often shows histological features characteristic of severe dysfunction, including lesions associated with a prolonged state of chronic inflammation and ischemia, such as infarction, increased villous maturation and chronic villitis.[17] The fact that preeclampsia, FGR and other related complications present in different orders, combinations and at different gestational ages, make the interplay between the several pathways presented in Figure 1 likely to be variable for respective phenotypes. The hypothesis that a vulnerable maternal (cardiovascular and inflammatory) constitution and the need for pregnancy-specific adaptations may result in abnormal spiral artery remodeling and pathology is the basis for many of the studies discussed in this thesis and may need some further introduction.

Spiral artery remodeling and pathology

Spiral arteries underlie the placenta and emerge from the radial arteries that in turn come from the arcuate arteries lying deep within the myometrium of the uterus (Figure 2). During the first half of pregnancy several complex processes occur through which the mother transforms the decidual and myometrial segments of these tightly coiled arteries into 5-10 fold dilated blood vessels at the site of placental implantation. This allows for high volume blood flow to the growing and developing placenta which in turn exchanges nutrients, oxygen and other necessities with the fetus (Figure 3). Remodeling of spiral arteries is classically characterized by loss of smooth muscle cells and the elastic lamina from the vessel wall up to the inner one-third of the myometrium. Hypotheses on the physiology of this remodeling process are still up for debate.

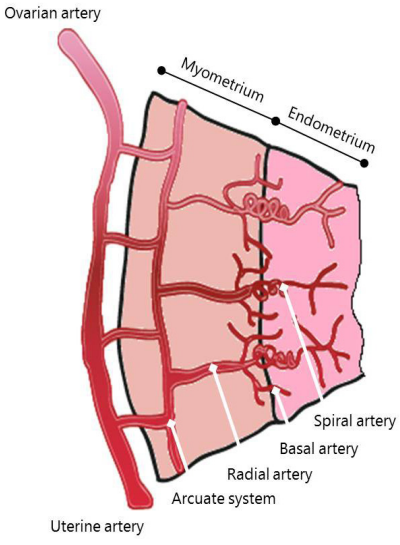

FIGURE 2. Topography of blood supply in a non-pregnant uterus.
Adopted from Robertson.[2]

A pioneering series of Caesarean hysterectomy samples due to preeclampsia and FGR in the early '60s and '70s first showed that "physiological" remodeling of these arterial segments was different when compared to healthy pregnancy specimens. This series showed that remodeling of spiral arteries in decidua was present, but did not cross the decidual-myometrial junction. Also, when present, remodeling was incomplete (i.e. not allowing the artery to expand as much as they noted in uncomplicated pregnancy). Similarly, invasion of extravillous trophoblast cells in to the uterine vascular wall was abnormally shallow.[18-23] Conceivably, this lead to the hypothesis that the lack of trophoblast invasion directly leads to failure of sufficient transformation of the spiral arteries needed for adequate uteroplacental circulation

that is necessary for sustaining a healthy pregnancy. Early gestation (pregnancy-terminating) hysterectomy samples within this series however, also showed spiral artery remodeling to have started before the arrival of trophoblast cells in the tissue. The exact role of trophoblast cells, even though believed crucial, is therefore still relatively unclear.[24]

In addition to this hypothesis, both the earlier mentioned series and several other groups have found lipid filled obstructive lesions ('acute atherosis') in spiral artery segments showing intriguing similarities with atherosclerosis (i.e. lipid-laden macrophages and leukocyte infiltration of the vascular wall) and argue this to be demonstrating early signs of maternal vascular damage.18-22 These lesions have led several research groups, including our own, to hypothesize that women who experience pregnancy complications associated with placental bed pathology, such as preeclampsia and FGR, have an increased constitutional risk for cardiovascular disease and events later in life. This risk merely manifests for the first time under the increased cardiovascular demands ("stress") of pregnancy. Our own hypotheses, which is shared by some other groups, poses a more multifactorial pathophysiology where vascular lesions and the incapability of functional remodeling is likely due to maternal vascular incompetence. In this theory, the role and function of the (incompatible) trophoblast and other mechanisms arising from the placenta and fetus play a secondary part (Figure 1). Since the 1970s, Caesarean hysterectomies have been replaced by other, less invasive, surgical techniques making it more difficult to explore specific vascular pathophysiology at the maternal-fetal interface. Some groups have studied vascular remodeling and pathology since then, including relatively small groups of biopsy samples ascertained from the placental bed.

As study set-ups diverge in selection of study population and sampling techniques a large variation in spiral artery remodeling problems is reported in the literature (normal pregnancy ranging 76-100%; preeclampsia ranging 3-41%).[25] Furthermore, the incidence of other vascular lesions, such as acute atherosis range even wider, with numbers published between 20-90%.[18,26] Conclusive studies on prevalence and depth of defective remodeling and vascular pathology in spiral arteries and the interplay with the maternal cardiovascular and inflammatory system have not sufficiently been performed.

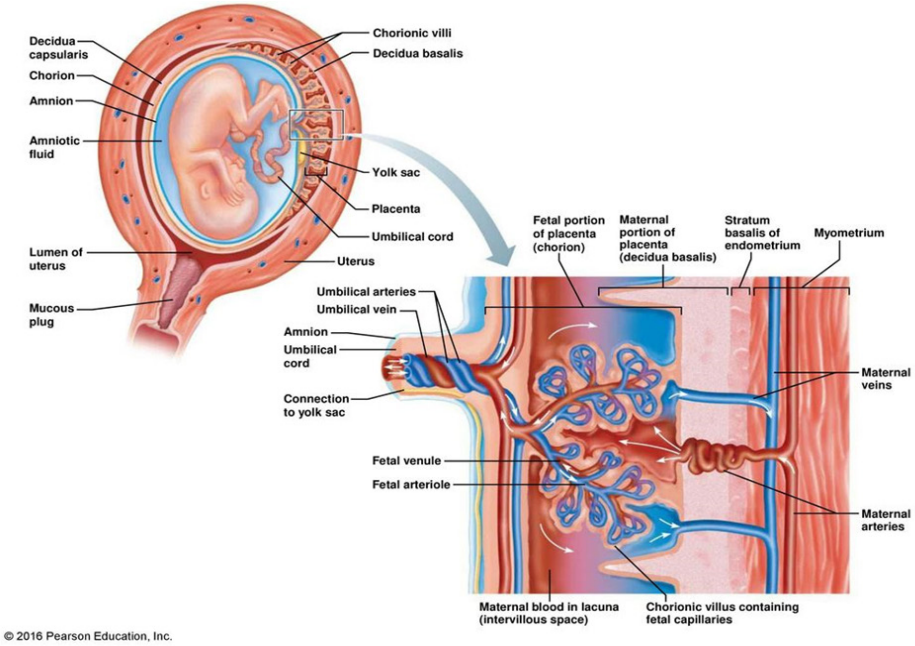

FIGURE 3. Topography of the placental bed in human pregnancy.
Adopted from human anatomy & physiology, copied with permission from the publisher.[1]

In **part I** of this thesis we will investigate immunovascular pathology in preeclampsia, fetal growth restriction and healthy pregnancy. In **chapter 2** we will use the biopsy technique previously established by our group to pilot a new scoring system investigating maternal immune cell presence and remodeling problems in preeclampsia and investigate whether specific lesions have a correlation to maternal cardiovascular risk parameters. In **chapter 3** we use this biopsy and scoring technique to establish important prevalence data on impaired spiral artery remodeling, pathological vascular lesions and local inflammation in a comprehensive prospective cohort of women with preeclampsia and/or FGR in comparison to healthy pregnancy. In **chapter 4** we aim to investigate if spiral artery remodeling can be used as a proxy for the intrauterine environment and study whether short term neonatal outcome after premature delivery (following preeclampsia and/or FGR) can be linked to impaired remodeling. In **chapter 5** we will use several state-of-the art techniques to investigate specific placental bed endothelial cell phenotype at the maternal –fetal interface and use transcriptomic profiling to identify differentially expressed genes and pathways. Additionally, we perform analysis of circulating markers of vascular, endothelial and inflammatory activation during preeclampsia and FGR. In **chapter 6** we investigate whether pre-gravid BMI, a strongly associated risk factor for pregnancy complications and CVD, can be associated with placental pathology when pregnancy is uncomplicated.

FEMALE-SPECIFIC CARDIOVASCULAR RISK FACTORS

With over a third of mortality being of cardiovascular nature, CVD is currently the most important cause of death in women.[27] The prevalence of CVD is generally associated with obesity, smoking, diabetes type II and is strongly associated with a prolonged unhealthy lifestyle.[28] It is believed that up to 90% of atherosclerotic CVD can be prevented by living a healthy life.[29,30] Over the last decades long-term population studies have produced evidence that other, non-traditional and non-modifiable, female specific risk factors affect the risk of CVD. Most importantly, preeclampsia and several other pregnancy complications are associated with an increased lifelong risk of CVD.[3,4,31,32]

Mechanisms

The etiological relationship between pregnancy complications with CVD is not fully clarified. The first and foremost hypothesis describes problems in pregnancy as a first event resulting from an underlying cardiovascular predisposition ('stress test concept'). Due to many shared risk factors (i.e. obesity, hypertension, kidney disease) and many disease markers being similar (i.e. inflammatory response, endothelial cell dysfunction) it is highly likely that cardiovascular susceptibility is unmasked in the short period of physical stress during pregnancy.

Alternatively, the increased risk for future CVD may result from actual damage occurring during hypertensive pregnancy ('causal relationship'). Despite many studies on this subject, a direct cause, or combinations of causes has not been identified. As the increase in cardiovascular risk is seen for various pregnancy complications this implies a pre-existent constitution to be more likely than a single, or a combination of, causal pathways.[33]

Although yet to be fully clarified, the importance of continuing endothelial dysfunction in causing additional cardiovascular risk, irrespective of interplay with maternal constitution has been shown.[34] It is possible that this damage may allow for a persistence of disrupted pathways such as increased inflammatory response and kidney dysfunction. A combination of both the causal relationship and the stress test theory is conceivable, with damage or persistence of detrimental pathways after pregnancy adding to an undesirable cardiovascular predisposition.

Timing and prevention

Several health organizations have acknowledged the increased risk for women have after pregnancy complications and have recommended screening and prevention. These guidelines however, still struggle to pinpoint both exact timing and which methods of screening and thresholds for treatment should be recommended in these women. As most women are younger than the available reference cohorts and the majority of current guidelines and risk reduction strategies are based on short-term risk assessment, they often do not reach the required thresholds for intervention (Figure 4).

In other words, current preventive strategies mainly focus on older individuals even though the development of CVD almost certainly starts many years earlier. Risk assessment and intervention during earlier life, soon after childbearing years, may form a substantial component of the global effort to reduce the burden of CVD worldwide. Although current primary care guidelines allow for obstetric history to be taken into account when indicating cardiovascular risk evaluation, this is often not executed in current usual care.

In **part II** of this thesis we aim to further uncover unknown information regarding whom and how to screen for CVD after a history of preeclampsia. In **chapter 7** we perform a systematic review and meta-analysis to investigate whether women having multiple pregnancies complicated by preeclampsia have an increased risk of CVD in comparison to having one complicated and subsequent uncomplicated pregnancies. In **chapter 8** we evaluate at what age coronary artery calcification develops in women with previous preeclampsia by coronary computed tomography (CCT) imaging. Last, but not least, we analyze if early and repetitive cardiovascular screening and lifestyle intervention can have a positive, both health and economic, impact on quality of life for women with a history of preeclampsia, using a model-based microsimulation in **chapter 9**.

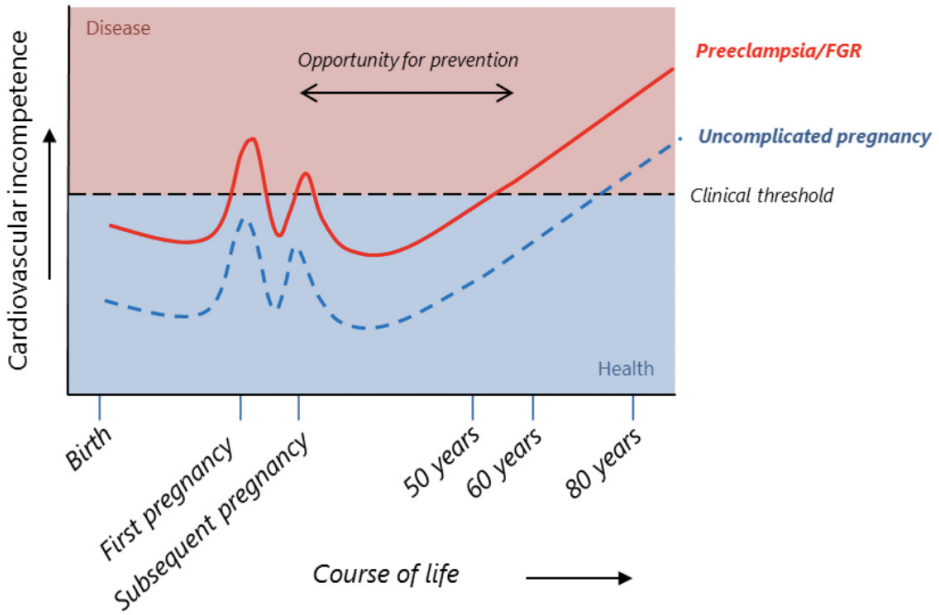

FIGURE 4. Concept of pregnancy as a "stress test", identifying women with an undesirable cardiovascular predisposition relatively early in life. *Adapted from Sattar & Greer, BMJ 2002*

AIMS OF THESIS

In this thesis we focus on spiral artery remodeling and pathology when pregnancy is complicated by preeclampsia and/or FGR. We investigate placental bed vascular health during pregnancy and assess possible links with perinatal (cardiovascular) health of both mother and child **(part I)**. Furthermore, we investigate for whom, when and how we should implement screening and preventive measures for cardiovascular disease in women with a history of preeclampsia **(part II)**.

Part I: Immunovascular pathology underlying preeclampsia, fetal growth restriction and healthy pregnancy

- To pilot a newly developed scoring system for vascular and inflammatory lesions within the placental bed of women with preeclampsia and to investigate the short term association of spiral artery pathology with maternal cardiovascular risk factors **(chapter 2)**.
- To describe data on prevalence of placental bed pathology (i.e. impaired spiral artery remodeling, acute atherosis, thrombosis, local inflammation) in pregnancy complicated by preeclampsia and/or fetal growth restriction and healthy pregnancy **(chapter 3)**.
- To assess whether babies born following pregnancies complicated by preeclampsia and fetal growth restriction associated with impaired spiral artery remodeling have worse short-term neonatal outcome, compared with pregnancies where spiral artery remodeling was normal **(chapter 4)**.
- To investigate specific maternal uterine endothelial cell transcriptomics and to investigate circulating markers of endothelial dysfunction and inflammation in women with early onset preeclampsia with fetal growth restriction in comparison to uncomplicated pregnancy **(chapter 5)**.
- To investigate whether maternal prepregnancy body mass index has an association with placental histopathological characteristics in uncomplicated pregnancy **(chapter 6).**

Part II: Maternal cardiovascular health after preeclampsia

- To provide a systematic overview of the literature available on whether recurrence of preeclampsia in more than one pregnancy leads to a further increase of the risk of future hypertension and cardiovascular disease **(chapter 7).**
- To evaluate at what age irreversible coronary artery calcification and atherosclerotic plaque develops in women with previous preeclampsia **(chapter 8).**
- To investigate if early screening and lifestyle interventions can prevent cardiovascular disease in women with history of preeclampsia and increase quality of life, using a model-based microsimulation based on short and long term cardiovascular follow-up data **(chapter 9).**

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PART I

Immunovascular pathology underlying preeclampsia, fetal growth restriction and healthy pregnancy

2

Spiral artery remodeling and maternal cardiovascular risk: the spiral artery remodeling (SPAR) study

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ABSTRACT

Background

Women with a history of placental bed disorders, including preeclampsia and intrauterine growth restriction have an increased long-term risk of cardiovascular disease (CVD). Further, similarities exist between atherosclerosis and abnormalities observed in placental bed spiral arteries in pregnancies affected by preeclampsia and intrauterine growth restriction, such as acute atherosis and defective remodeling. This suggests a common pathophysiological pathway underlying both disorders.

Objectives

The aim of this study was to investigate vascular and inflammatory lesions in the placental bed of women with preeclampsia and normal pregnancy using a systematic approach to characterize lesions of the placental bed, and relate spiral artery pathology to postpartum CVD risk assessment.

Methods

Placental bed punch biopsies were performed following Caesarean section and systematically studied to assess vascular pathology, arterial remodeling, and the presence of $CD3^+$, $CD56^+$ and $CD68^+$ cells. In addition, levels of modifiable CVD risk factors were assessed immediately postpartum.

Results

We found fewer spiral arteries with complete remodeling in women with preeclampsia than in the control group (21 vs. 68%; p=0.008). Further, women with preeclampsia showed less presence of $CD3^+$ cells in both the decidua and the myometrium. Preliminary findings of CVD risk factor assessment postpartum suggest a correlation between acute atherosis and higher triglyceride and low-density lipoprotein cholesterol levels.

Conclusion

Systematic study of vascular pathology in uterine spiral artery biopsy samples in relation to CVD risk factors provides valuable insight into the link between cardiovascular health and placental bed disorders.

INTRODUCTION

Preeclampsia is a common hypertensive disorder of pregnancy and is associated with several fetal and maternal complications, including eclamptic seizures, intrauterine growth restriction (IUGR) and intrauterine fetal death in some cases.[1] Our group and others have shown that these women show different cardiovascular disease (CVD) risk profiles postpartum as compared with women with uncomplicated pregnancies.[2-6] Similar, large observational studies have confirmed an increase in the long-term risk of CVD in women who experienced preeclampsia and/or other types of placental disorders associated with defective spiral artery remodeling, such as IUGR, placental abruption, and pregnancy-induced hypertension.[7,8] Characteristic vascular pathology of the spiral arteries in the placental bed is thought to be involved in the development of preeclampsia and other hypertensive disorders of pregnancy. Lack of adequate spiral artery remodeling, associated with incomplete trophoblast invasion, has been described as the key pathological defect in preeclampsia, and is present in about 52–90% of cases.[9] Impaired spiral artery remodeling, however, is not confined to preeclampsia and IUGR, but is also seen in 19% of pregnancies complicated by preterm birth and even in 4–7% of normal pregnancies.[9-11]

Apart from abnormal spiral artery remodeling, women with preeclampsia also show other characteristic placental vascular abnormalities described as 'acute atherosis'.[12-14] These lesions are more likely to occur in the decidual part of the placental bed and in vessels of the membranes, and are accompanied by inflammation and several features similar to vascular pathology found in early-stage atherosclerosis, including lipid accumulation in macrophages (foam cells).[15,16] It has been suggested that the presence of these lesions correlates with an increased risk of CVD,[17] which could be explained by a shared pathophysiological pathway underlying both conditions.[18] Similar to the pathophysiology of atherosclerosis, it is assumed that the inflammatory response (to the invading fetal trophoblast) has a central role in regulating spiral artery remodeling and may be an important culprit in the development of hypertensive disease in pregnancy.[19] Bio banking and evaluation of spiral artery pathology in complicated and uncomplicated pregnancies have been suggested to further clarify the relation between features of spiral artery pathology and underlying maternal CVD risk factors.[15,17] To shed more light on this relationship, we initiated the spiral artery remodeling (SPAR) study to systematically collect both spiral artery biopsy samples and perform subsequent CVD risk factor screening. Aims of this study were to: first, perform a feasibility study to test a recently developed scoring system for placental bed pathology; second to systematically assess lesions characteristic of placental bed pathology, using this scoring system; and third, to study the involvement of local immune cells in spiral artery remodeling in normal and in preeclamptic pregnancy; and to explore the correlation between levels of established CVD risk factors immediately postpartum and placental bed characteristics identified by the scoring system.

METHODS

Patient selection and definitions

This paper is the first report on the ongoing prospective cohort-control SPAR study conducted in a single tertiary perinatal center at the University Medical Center Utrecht (UMCU), the Netherlands. Patients were recruited from the antenatal clinic or maternity ward when consented for a Caesarean section because of preeclampsia and/or IUGR. Preeclampsia was defined as (new) onset of hypertension (≥140/90mmHg) and significant proteinuria (≥300mg/24 h) after 20 weeks of gestation, according to the 2001 criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP).[20] Of note, the ISSHP criteria have recently been updated (i.e., after initiation of our study), which did not lead to any reclassification in our cohort. Controls were women eligible for an elective Caesarean section after an uneventful pregnancy and without any major underlying pathology (mostly for breech presentation and women with previous Caesarean delivery declining a trial of labor).

IUGR was defined as an ultrasonographically estimated fetal weight below the 10th percentile, in the absence of major congenital and/or chromosomal abnormalities, or suspected causes of IUGR other than placental insufficiency.[21] Hemolysis, Elevated Liver enzymes, Low Platelets (HELLP) syndrome was defined by the following laboratory findings: aspartate aminotransferase more than 50 U/l or alanine aminotransferase more than 50 U/l, lactate dehydrogenase more than 600 U/l, platelet count less than 100 x 10^9/l, and/or clinical evidence of hemolysis.[22] All patients received study information and provided informed consent prior to participation. This study was reviewed and approved by the local Institutional Ethical Review Board of the University Medical Center Utrecht, protocol reference number: 11–503. The study adheres to the principles of the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of human Subjects, Revised 13 November 2001, effective 13 December 2001.

Placental bed biopsies and immunohistochemistry

Placental bed biopsies were obtained according to a previously published collection method.[23] In brief, at the time of Caesarean section, and after delivery of the neonate, the central part of the placental bed was manually located on the inner uterine myometrial wall. Four placental bed biopsies were obtained using cervical biopsy forceps (Aesculap ER063R, Braun, Germany). Samples were then fixed in 4% buffered paraformaldehyde for storage and transport. After dehydration, biopsies were embedded in paraffin wax for histological examination according in our routine diagnostic pathology laboratory, according to standard operating procedures. Series of sections were cut at 3mm thickness, mounted onto glass slides, and stained with hematoxylin and eosin, and periodic acid Schiff after diastase stain (using the DAKO coverstainer, Heverlee, Belgium) to enable basic orientation.

For this study, we used a recently published placental bed scoring system based on available literature describing spiral artery pathology and on a large study of systematic

scoring of atherosclerotic lesions.[23-25] Supplementary Table 1, shows the definitions used in the scoring system. All markers were scored as either absent or minimal, moderate, or heavy and/or clustering of cells, by two independent observers blinded for outcome (P.N. with J.V. or E.V.). When interpretations differed, a third observer was consulted (J.V. or E.V.).

As part of our scoring protocol, all sections (6–8/patient in total) were stained for specific cell types using a Ventana Benchmark Ultra (Roche, Basel, Switzerland). CK7 staining (clone OV-TL 12/3, Biogenex, Fremont, California, USA) was used to identify extravillous trophoblast cells. Immune cells were identified by CD56 (clone 123C3, Neomarkers, Fremont, California, USA) and CD3 (DAKO coverstainer) staining, to identify cell types likely to represent mostly natural killer cells and T cells, respectively. CD68 staining (clone KP1, Novocastra, UK) was used to identify the presence of the lipid scavenger receptor, and identifying macrophages and foam cells. Distinction was made in scoring the presence of inflammatory cells in decidua and the presence of inflammatory cells in the myometrium. Only the biopsies that sampled the 'true' placental bed (i.e. containing both myometrial and decidual tissue, as well as containing trophoblast cells), were used to score the number of immune cells. In those biopsies that also contained a spiral artery, the developmental state of spiral arteries, in the myometrium, was assessed, as well as the presence of acute atherosis – defined as aggregates of lipid laden macrophages in the artery wall – and the presence of thrombosis, as well as the degree of intima proliferation. Definitions of these specific lesions are shown in supplementary Table 1.

Postpartum cardiovascular biochemical parameters

Fasting blood samples were drawn coinciding with routine blood sampling after Caesarean section on the first day postpartum. All blood samples were analyzed by standard protocols used by the UMCU Laboratory of Clinical Chemistry and Hematology. Hemoglobin, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL), glucose, homocysteine, thyroid stimulating hormone and high sensitivity C-reactive protein (CRP) were measured using a Vitros analyzer (Ortho, Mulgrave, Australia) or DxC800 analyzer (Beckman Coulter, Brea, California, USA). Low-density lipoprotein cholesterol (LDL) was calculated using the Friedewald formula.[26] Within-run variation coefficients at the UMCU at time of this study were 1.5% for total cholesterol, 2.0% for HDL, 2.0% for triglycerides, 2.5% for CRP, and 2.4% for fasting glucose levels. In addition, several of these measurements were dichotomized to previously described (nonpregnancy specific) cutoff values.[2,3] Data on maternal characteristics (i.e., maternal age, history, BMI, and parity) were registered at the antenatal clinics during routine booking and follow-up visits throughout pregnancy. Data on neonatal outcomes, including fetal birth weight and birth weight percentile were recorded directly after birth.

TABLE 1. Maternal and pregnancy outcome characteristics in women with preeclampsia and controls.

	Control *n*=29	**Preeclampsia** *n*=29	***p*-value**
Maternal characteristics			
Age (years)	33.9 (30.2-36.6)	30.5 (27.9-36.4)	0.227
BMI (kg/m^2)	22.7 (21.4-26.3)	25.2 (21.4-27.3)	0.320
Caucasian (%)	28 (96.6%)	25 (86.2%)	0.352
Smoking (%)	0 (0%)	16 (55.2%)	0.105
Nulliparity (%)	0 (0%)	3 (10.3%)	0.001
Pregnancy outcome			
Gestational age at delivery (days)	275 (273-278)	212 (202-223)	<0.001
Birth weight (g)	3720 (3402-3958)	1135 (913-1395)	<0.001
IUGR (%)	0 (0%)	16 (55.2%)	<0.001
HELLP (%)	0 (0%)	11 (37.9%)	<0.001
SBP (mmHg)	125 (120-130)	180 (170-193)	<0.001
DBP (mmHg)	80 (70-80)	110 (105-116)	<0.001
General and obstetric history			
Preexistent hypertension	0 (0%)	5 (17.2%)	0.052
Preexistent diabetes mellitus	0 (0%)	1 (3.4%)	1.000
Hypertensive pregnancy complications	1 (5)	4 (80%)	0.002

Values are presented as medians (interquartile range) or as numbers (%). *P*-values were calculated by the Mann–Whitney U test for continuous variables and Fisher's exact test for categorical variables. *Abbreviations:* HELLP, hemolysis elevated liver enzymes low platelets syndrome; IUGR, intrauterine growth restriction; N/A, not applicable. Obstetric history is shown for multiparous women.

Statistical analysis

Baseline characteristics were presented as medians (interquartile ranges) or number (percentages) if variables were either continuous or categorical, respectively. P values to indicate differences between groups were calculated using Mann–Whitney U tests and χ^2 or Fisher's exact tests for continuous and categorical variables, respectively. CVD risk factors were compared by means of *t* test. Cutoff values deducted from clinical practice guidelines were used and compared between the groups.[2,3] CVD risk factors of women with acute atherosis were compared by means of *t* test. Statistical analyses were performed using SPSS (release 20.0; Chicago, Illinois, USA). P values less than 0.05 were considered statistically significant.

RESULTS

Baseline characteristics

In this first analysis, we present data of the first *n*=232 biopsy samples obtained from 29 women with preeclampsia and 29 normal pregnant controls included in the SPAR study. Maternal and neonatal baseline characteristics are presented in Table 1. The percentage of nulliparous women was higher in the preeclampsia group compared with controls (75.9 vs. 27.6%, respectively, p=0.001). In 11 cases (37.9%), preeclampsia was complicated by HELLP syndrome. As expected, gestational age and birth weight differed significantly between the preeclampsia and the control group (212 vs. 275 days and 1135 vs. 3730 g, respectively, p<0.001). Similar, maximum SBP and DBP levels in pregnancy were higher for preeclamptic women compared with women with a normal pregnancy outcome (180 vs. 125 and 110 vs. 80, respectively, p<0.001).

Postpartum cardiovascular disease and inflammatory markers

CVD and inflammatory markers, measured on the first day postpartum, are presented in Table 2. We found no between-group differences for hemoglobin, fasting blood glucose, thyroid stimulating hormone, total cholesterol, and CRP levels. Compared with controls, women with preeclampsia had higher creatinine and homocysteine levels (p<0.001 for both). Further, median triglyceride levels were slightly higher, and HDL levels lower in the preeclampsia group, but this was not statistically significant (p=0.077 and p=0.059, respectively). However, when using clinical cutoff points for CVD risk factor levels, we more often found low HDL levels in preeclampsia patients than in controls (48.3 vs. 20.7%, p=0.026). Similar, preeclampsia patients were more likely to have high homocysteine levels (65.5 vs. 17.2%, p<0.001). Adjustment for maternal age, BMI, and smoking by multivariable regression did not change the results (data not shown).

TABLE 2. Postpartum cardiovascular disease risk markers in women with preeclampsia and controls.

SPAR laboratory data	Control *n*=29	Preeclampsia *n*=29	*p*-value
Hemoglobin (mmol/l)	7.0 (6.3–7.7)	6.9 (6.4–7.6)	0.679
Fasting glucose (mmol/l)	5.4 (4.3–6.0)	5.1 (4.4–5.9)	0.867
Total cholesterol (mmol/l)	5.4 (4.9–6.4)	5.8 (4.6–6.6)	0.951
Triglycerides (mmol/l)	2.9 (2.2–3.3)	3.6 (2.4–4.1)	0.077
HDL (mmol/l)	1.6 (1.3–1.7)	1.3 (1.1–1.6)	0.059
LDL (mmol/l)	2.8 (1.9–3.3)	2.6 (2.2–3.5)	0.793
Creatinine (mmol/l)	51.5 (47.0–58.0)	65.0 (59.5–74.0)	<0.001
TSH (mIU/l)	2.5 (1.8–4.2)	2.6 (1.8–5.6)	0.283
HsCRP (mg/l)	56.1 (15.5–77.4)	36.5 (19.2–68.5)	0.241
Homocysteine (mmol/l)	4.5 (3.4–5.3)	7.0 (5.2–9.5)	<0.001
Cutoff values			
Glucose (>5.6 mmol/l)	12 (41.4%)	9 (31.0%)	0.582
Cholesterol (>6.2mmol/l)	8 (27.6%)	11 (37.9%)	0.573
Triglycerides (>1.7mmol/L)	29 (100%)	26 (89.7%)	0.491
HDL (<1.29 mmol/l)	6 (20.7%)	14 (48.3%)	0.026
LDL (>1.8 mmol/l)	22 (75.9%)	24 (82.8%)	0.469
Homocysteine (>12mmol/l)	5 (17.2%)	19 (65.5%)	<0.001

Values are presented as medians (interquartile range) or as number (%). *P*-values are calculated by the Mann–Whitney U test for continuous variables and Fisher's exact test for categorical variables. *Abbreviations:* HDL, high-density lipoprotein; HsCRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; SPAR, SPiral Artery Remodeling; TSH, thyroid stimulating hormone.

Placental bed biopsies

Table 3 shows characteristic findings of the placental bed biopsies containing both decidual and myometrial segments of spiral arteries. In the preeclampsia group, we excluded 12 samples where the biopsy sample appeared not to be obtained from the site of the placental bed, as no trophoblast and no spiral artery could be identified. A further three cases contained only trophoblast cells but no spiral artery sample. The remaining 14 (48.3%) cases in this group had biopsies containing both trophoblast cells and at least one spiral artery. In the control group, there were 10 cases without trophoblast or spiral artery. The remaining 19 (65.5%) cases showed both. This resulted in a total of 36 (62%) biopsies confirmed to be obtained from the placental bed, of which a total of 33 (57%) biopsies contained spiral arteries. The key placental bed features of these 36 biopsies are presented in Table 3. When investigating $CD3^+$ cell presence, we observed significantly more moderate to heavy staining of $CD3^+$ cells in the placental bed tissue of the control group compared with the preeclampsia group (p=0.041 and p=0.037, respectively).

TABLE 3. Characteristic pathology of the placental bed in women with preeclampsia and controls.

Placental bed characteristics	Control *n*=19	Preeclampsia *n*=17	*p*-value
CD3⁺ cell presence (decidua)			
Minimal	0 (0%)	0 (0%)	0.041
Moderate	9 (47.4%)	14 (82.4%)	
Heavy and/or clustering	10 (52.6%)	3 (17.6%)	
CD3⁺ cell presence (myometrium)			
Minimal	2 (10.5%)	6 (35.3%)	0.037
Moderate	7 (36.8%)	7 (41.2%)	
Heavy and/or clustering	10 (52.6%)	4 (23.5%)	
CD68⁺ cell presence (decidua)			
None to minimal	1 (5.3%)	1 (5.9%)	0.829
Moderate and/or clustering	11 (57.9%)	7 (41.2%)	
Heavy and/or clustering	7 (36.8%)	9 (52.9%)	
CD68⁺ cell presence (myometrium without spiral artery)			
None to minimal	3 (15.8%)	0 (0%)	0.052
Moderate and/or clustering	15 (78.9%)	12 (70.6%)	
Heavy and/or clustering	1 (5.3%)	5 (29.4%)	
CD68⁺ cell presence (myometrium with spiral artery)[a]			
None to minimal	9 (47.4%)	4 (28.6%)	0.160
Moderate and/or clustering	8 (42.1%)	8 (57.1%)	
Heavy and/or clustering	0 (0%)	2 (14.3%)	
N/A	2 (10.5%)	0 (0%)	
CD68⁺ cell presence (around spiral arteries)[a]			
None to minimal	7 (36.8%)	4 (28.6%)	0.741
Moderate and/or clustering	10 (52.6%)	6 (42.9%)	
Heavy and/or clustering	1 (5.3%)	2 (14.3%)	
N/A	1 (5.3%)	2 (14.3%)	
CD56⁺ cell presence (decidua)			
None	0 (0%)	0 (0%)	0.415
Minimal	1 (5.3%)	3 (17.6%)	
Moderate	11 (57.9%)	10 (58.8%)	
Heavy and/or clustering	7 (36.8%)	4 (23.5%)	
CD56⁺ cell presence (myometrium)			
None	0 (0%)	0 (0%)	0.032
Minimal	3 (50.8%)	1 (5.9%)	
Moderate	9 (47.4%)	15 (88.2%)	
Heavy and/or clustering	7 (36.8%)	1 (5.9%)	
Remodeling (myometrium)[a]			
None	0 (0%)	3 (21.4%)	0.008
Minimal	1 (5.3%)	4 (28.5%)	
Moderate	5 (26.3%)	4 (28.5%)	
Complete	13 (68.4%)	3 (21.4%)	
Thrombosis[a]	0 (0%)	1 (7.1%)	0.424
Atherosis[a]	1 (5.3%)	2 (14.2%)	0.561
Intima proliferation[a]	16 (84.2%)	7 (50%)	0.057

Data are presented as numbers (percentages), and *p*-values are calculated by Fisher's exact tests.

[a]Only in cases with biopsies containing one or more spiral arteries (control group: n=19 and preeclampsia group: n=14).

The number of $CD68^+$ cells in the decidua was similar for both groups. We found moderate to heavy presence of $CD68^+$ cells, in the myometrium of most patients in both the preeclampsia and control group, but with a trend toward more heavy clustering of $CD68^+$ cells in preeclampsia pregnancies (5/17 cases vs. 1/19 controls; p=0.052) with similar findings for $CD68^+$ cells surrounding spiral arteries. The incidence of $CD56^+$ cells in the decidua and myometrium showed no differences between women with preeclampsia and controls.

Incomplete spiral artery remodeling (myometrium) was more common in the preeclampsia group when compared with the control group with three (21.4%) vs. 13 (68.4%) cases, respectively (p=0.008). Presence of atherosis and thrombosis in, both decidual and myometrial, placental bed spiral arteries was similar for women with preeclampsia and controls. Intima proliferation was less often present in the preeclampsia group, however this difference was not statistically significant (50 vs. 84.2%, p=0.057).

Next, we explored associations between CVD risk factor levels assessed immediately postpartum and characteristics of placental bed and spiral artery pathology. Although our numbers are too small to draw any definitive conclusions, preliminary and exploratory analysis suggests a possible relation between higher levels of triglycerides and LDL cholesterol in women with acute atherosis lesions compared with women without these lesions (Table 4).

TABLE 4. Correlation between the presence of acute atherosis and postpartum cardiovascular disease risk markers

SPAR laboratory data	**Acute atherosis** *n=3*	**No acute atherosis** *n*=29	***p*-value**
Fasting glucose (mmol/l)	5.3 (0.97)	5.0 (0.91)	0.646
Total cholesterol (mmol/l)	7.1 (2.4)	5.6 (1.2)	0.079
Triglycerides (mmol/l)	4.5 (0.93)	2.9 (0.92)	0.010
HDL (mmol/l)	1.4 (0.45)	1.5 (0.33)	0.697
LDL (mmol/l)	3.8 (1.9)	2.3 (1.1)	0.043
Homocysteine (μmol/l)	6.8 (3.6)	5.9 (3.0)	0.642

Values are presented as means with SD. HDL, high-density lipoprotein; LDL, low-density lipoprotein; SPAR, SPiral Artery Remodeling.

DISCUSSION

In this study, we present the design and rationale for a new and original systematic effort to collect and biobank spiral artery biopsy samples with the purpose of studying spiral artery remodeling, vascular and inflammatory pathology of the placental bed in uncomplicated pregnancies and pregnancies complicated by preeclampsia, the SPAR biobank study. In addition, participants of the SPAR study undergo a detailed assessment of established CVD risk markers immediately postpartum, at 6–12 months and subsequent biennial CVD follow-up. In this first exploratory analysis, we present results of systematic evaluation of the placental bed biopsy samples using a scoring system with predefined pathological criteria, and show novel evidence for several differences in vascular and inflammatory characteristics of the placental bed in pregnancies affected by preeclampsia as compared with controls, including incomplete myometrial spiral artery remodeling and some striking alterations in the presence of a number of key maternal immune cells within the myometrium and decidua. This was most evident for $CD3^+$ cells that were present in lower numbers in women with preeclampsia. Preliminary and exploratory analysis of CVD risk factors, assessed immediately postpartum, revealed low HDL levels and high homocysteine levels in women with preeclampsia. Intriguingly, we found a possible association between high triglyceride and LDL levels and the presence of acute atherosis lesions in spiral arteries of the placental bed. This may suggest a relationship between maternal dyslipidemia and placental bed vascular pathology. However, these findings need to be further confirmed in a sufficient-sized study sample to include CVD risk factor assessment both immediately postpartum and at follow-up.

Abnormal vascular remodeling in both the decidual and myometrial part of the placental bed has been recognized as a characteristic pathological feature of preeclampsia.[27-29] The role of the maternal cardiovascular and inflammatory system in this process is unclear. In this cohort-control study, we aimed to combine data on the histopathology of the placental bed with subsequently obtained CVD risk factors measured directly postpartum. With this approach, the SPAR study has the potential to provide a unique insight into the role of underlying maternal CVD health in relation to normal and pathological spiral artery development.

The most striking pathological finding in this study is the lower presence of $CD3^+$ cells (a marker for T cells) in preeclampsia affected pregnancies. Although this needs to be explored in further experimental work, it is tempting to speculate that these T cells represent regulatory T cells (Treg), as recently has been demonstrated by Staff *et al*.[30] Tregs are known to act as a suppressor to several inflammatory cells that otherwise exacerbate the immune response. Therefore, Tregs may play an important role in preventing excessive immune reactions involved in inflammatory disease, including atherosclerosis.[31,32] In view of our findings, it is interesting to note the small study of Sasaki *et al*. who demonstrated that the population of decidual Tregs is lower in preeclampsia.[33] In addition, we observed

a trend toward an increased presence of $CD68^+$ cells. As $CD68^+$ is a marker for a lipid scavenger receptor, we hypothesize that this population of cells will most likely represent macrophages. This is interesting, as macrophages are thought to be directly involved in regulating trophoblast migration through regulating apoptosis, as well as can induce maternal tolerance to fetal antigens.[34] Accumulation of macrophages within the vascular wall is also a hallmark feature of atherosclerosis, and may contribute to endothelial damage by conversion of their immune response in response to lipids.[35] Whether or not macrophages fulfill similar roles in the generation of acute atherosis lesions found in spiral arteries remains to be established, and further studies are needed to characterize relevant subsets of macrophages and their interactions with other immune cells within the placental bed. More detailed characterization of subsets of immune cells (e.g. Tregs and macrophages) will be performed in the main SPAR study.

In addition to spiral artery sampling, we assessed circulating CVD risk markers in serum drawn on the first day postpartum and found higher homocysteine and creatinine levels in the preeclampsia group. Higher creatinine levels are likely explained by increased vascular permeability in patients with preeclampsia that also causes generalized peripheral edema, and leads to renal hyperfiltration and relative hemoconcentration, which is a common feature of this disease.[36] Homocysteine has been shown to be associated with maternal risk of hypertensive pregnancy complications in several previous studies and hyperhomocysteinemia may persist postpartum, although in most women homocysteine levels will return to normal over time.[37]

Adding to previously published data on the presence of acute atherosis lesions in spiral artery pathology, we were also able to demonstrate atherosis lesions in about 8% of the myometrial segments of spiral arteries, but with no statistical difference between preeclampsia cases and controls. Although our sample size may still be too small to draw firm conclusions, it may be that these vascular lesions primarily occur in the decidual part of the spiral arteries, which may explain why others did observe an association with preeclampsia history and perinatal outcomes.[12,17,38,39] It has been proposed that acute atherosis lesions, irrespective of pregnancy outcome, predispose to CVD later in life.[30] However, the relation between placental bed pathology, the presence or absence of underlying cardiovascular risk factors, and the risk of incident CVD events later in life is not clear. Further, it should be noted that the presence of acute atherosis lesions in the decidua is estimated to occur in about 11% uncomplicated pregnancies.[40] In the study by Harsem *et al.* [12] no associations were found between decidual atherosis lesions and antepartum lipid levels in *N*=17 preeclampsia patients. It is interesting to note that, despite the small sample size, our preliminary findings do suggest an association between atherosis lesions in the placental bed and subsequently measured postpartum triglycerides and LDL cholesterol levels. It will be interesting to see if these associations can be confirmed in the full SPAR cohort analysis, and whether or not acute atherosis lesions are related to lipids measured at 6–12 month postpartum follow-up.

Strengths of the SPAR study include detailed and reliable phenotyping of preeclampsia according to consensus criteria, use of a reliable and well tolerated protocol for biopsy sampling and predefined scoring system to investigate spiral artery lesions[20,23], and systematic follow-up postpartum with serial assessment of CVD health and risk factor status. With our approach, we successfully obtained a spiral artery in 57% of biopsies, reaching a relatively high success rate compared with previous studies (42–47%) of spiral artery biopsy studies in patients undergoing Caesarean section.[23] There were no adverse events, or complications related to the biopsy procedure. There were no discrepancies of judgment between pathology observers.

Some limitations of this study need to be addressed. Firstly, the sample size does not yet allow for detailed or stratified analysis. Secondly, CVD risk factor levels when assessed immediately postpartum, may have been influenced by stress following delivery and surgical Caesarean section trauma (e.g., an activated acute-phase response), which could have influenced CVD risk factor levels. It should be noted that clinical cutoff values used to for these risk markers may have been influenced by the short postpartum sampling interval. This should become evident as the results of the follow-up at 6 months postpartum become available, thereby reducing possible transient and/or pregnancy- related effects on CVD risk factor levels. Thirdly, it is relevant to note that – in this analysis – we only studied preeclampsia cases that had early-onset disease, that is, those patients who required a Caesarean delivery less than 34 weeks of gestation. Control women, however, were included after term elective Caesarean section. Also, there may be an effect of parity on the observed degree of spiral artery remodeling. In women with a previous pregnancy, the myometrial part of the spiral artery often remains partly remodeled, and has less smooth muscle cells and more connective tissue in the media of the vessel wall. To further explore this factor, we aim to perform a sub analysis among women of different parity (both primiparous and multiparous) in the full cohort study.

Perspectives

With this study, we established a new cohort and biomaterial resource that allows for systematic phenotyping of the placental bed in normal and complicated pregnancies, and relate findings to underlying maternal and fetal health. Findings presented in this paper, for example, the correlation between the pathological scoring of the placental bed and maternal CVD risk factors LDL and triglycerides, will then be verified in a larger sample size to further unravel the important research question as to how maternal health may influence spiral artery development. With the SPAR study, we expect our systematic evaluation of placental bed vascular and inflammatory lesions in women with a history of preeclampsia and/or fetal growth restriction to provide important new insights into associations between maternal cardiovascular health characteristics and the presence of pregnancy-specific disorders caused by spiral artery pathology, thereby providing an opportunity to identify potential modifiable targets to reduce susceptibility to both placental disease and CVD later in life.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE 1.

Scoring tool used to define maternal immune cell presence in placental bed biopsies.

Placental bed marker	Definition	FOV
CD3 + cell presence (decidua)		
Minimal	< 50 positive stained cells present	10x10 (2.5mm^2)
Moderate	≥ 50 cells but <100 cells present	10x20 (0.6mm^2)
Heavy and/or clustering	Diffuse presence (> 100 cells) and/or clustering (>50 cells).	
CD3 + cell presence (Myometrium)		
Minimal	< 50 positive stained cells present	10x10 (2.5mm^2)
Moderate	≥ 50 cells but <100 cells present	10x20 (0.6mm^2)
Heavy and/or clustering	Diffuse presence (> 100 cells) and/or clustering (>50 cells).	
CD68 + cell presence (decidua)		
None to minimal	< 50 positive stained cells present	10x10 (2.5mm^2)
Moderate and/or clustering	≥ 50 cells but <100 cells, or presence in small groups (<50 cells).	10x20 (0.6mm^2)
Heavy and/or clustering	Diffuse presence (> 100 cells) and/or clustering (>50 cells).	
CD68 + cell presence (myometrium without SA)		
None to minimal	< 50 positive stained cells present	10x20 (0.6mm^2)
Moderate and/or clustering	≥ 50 cells but <100 cells, or presence in small groups (<50 cells).	
Heavy and/or clustering	Diffuse presence (> 100 cells) and/or clustering (>50 cells).	
CD68 + cell presence (myometrium with SA)		
None to Minimal	< 50 positive stained cells present	10x20 (0.6mm^2)
Moderate and/or clustering	≥ 50 cells but <100 cells, or presence in small groups (<50 cells).	
Heavy and/or clustering	Diffuse presence (> 100 cells) and/or clustering (>50 cells).	
N/A	Not applicable, when a spiral artery was not visible in this specific stained section of the biopsy.	N/A
CD68 + cell presence (around spiral arteries)		
None to Minimal	< 50 positive stained cells present	10x20 (0.6mm^2)
Moderate and/or clustering	≥ 50 cells but <100 cells, or presence in small groups (<50 cells).	
Heavy and/or clustering	Diffuse presence (> 100 cells) and/or clustering (>50 cells).	
N/A	Not applicable, when a spiral artery was not visible in this specific stained section of the biopsy.	N/A
CD56 + cell presence (decidua)		
None	No positive stained cells present.	10x10 (2.5mm^2)
minimal	< 50 positive cells present	
Moderate	≥ 50 cells but <100 cells, or presence in small groups (<50 cells).	
Heavy and/or clustering	Diffuse presence (> 50 cells) and/or clustering (>50 cells).	10x20 (0.6mm^2)

SUPPLEMENTARY TABLE 1 CONTINUED.

Placental bed marker	Definition	FOV
CD56 + cell presence (myometrium)		
None	No positive stained cells present.	10x10 (2.5mm^2)
Minimal	< 50 positive cells present	
Moderate	≥ 50 cells but <100 cells, or presence in small groups (<50 cells).	
Heavy and/or clustering	Diffuse presence (> 50 cells) and/or clustering (>50 cells).	10x20 (0.6mm^2)
Remodeling present (myometrium)		
None	No (physiological) PAS-D positive degeneration of the vessel wall and change of the SA circumference	N/A
Minimal	Focal PAS-D degeneration of <1/3 of the SA wall circumference, some arterial muscle still present.	
Moderate	Focal PAS-D degeneration of >1/3 but <2/3 of the SA wall circumference, some arterial muscle still present.	
Complete	Complete circular PAS-D degeneration of the SA wall.	
Thrombosis	Presence of organized thrombus formation, attached to the arterial wall.	N/A
Atherosis	Aggregates of lipid laden foam cells (CD68+) in the artery wall.	
Intima proliferation	Intraluminal fibrosis	

FOV indicates Field of view as used by the pathologist.

3

Prevalence of impaired placental bed spiral artery remodeling in preeclampsia and fetal growth restriction

Submitted

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ABSTRACT

Importance

Preeclampsia and fetal growth restriction (PEFGR) are pregnancy complications associated with unfavorable maternal cardiovascular health that may affect “physiological” remodeling of the spiral arteries underlying the placenta (i.e. the placental bed). The prevalence of impaired spiral artery remodeling and vascular lesions associated with PEFGR and normal pregnancy outcome has not been clearly established.

Objectives

To establish the prevalence of predefined histopathological lesions associated with abnormal development of spiral arteries in PEFGR and normal pregnancy.

Design

In the SPiral Artery Remodeling (SPAR) study, placental bed biopsies were obtained from women eligible for Caesarean section between May 2015 and February 2018.

Setting

Prospective, multi-center, observational cohort study.

Participants

Out of 1267 Caesarean sections, 394 women consented to undergo placental bed biopsy. Of these women, we compared outcomes for 121 women with PEFGR and 149 healthy controls in whom correct localization of the placental bed was confirmed by the presence of trophoblast cells in the biopsy sample.

Main outcome measures

Prevalence of placental bed lesions, including; impaired spiral artery remodeling, absence of intramural trophoblast, atherosclerosis-like lesions of the vascular wall (‘acute atherosis’), thrombosis, and the presence of (perivascular) inflammatory cells (macrophages, T-cells, NK-cells).

Results

PEFGR was associated with a high prevalence of impaired arterial remodeling compared with controls (63.6 vs 10.1%, $p<0.001$), and a higher prevalence of non-remodeled spiral arteries without the presence of intramural trophoblast (45.5 vs 6.7%, $p<0.001$), despite abundant interstitial trophoblast invasion. Acute atherosis (28.9 vs 3.4%, $p<0.001$) and thrombosis (16.5 vs 5.4%, $p=0.003$) lesions were significantly more prevalent in PEFGR. Impaired remodeling, acute atherosis and thrombosis lesions were equally present in both the decidual and the myometrial segments of the spiral arteries in both groups. PEFGR was associated with differences in the presence of inflammatory cells in the placental bed, with more macrophages and lower numbers of NK-cells and T-cells.

Conclusion

PEFGR is associated with a high prevalence of impaired physiological remodeling and vascular lesions to the uterine spiral arteries in the placental bed. These findings suggest that impaired health of the maternal arteries supplying the placenta is a key contributor to an unsuccessful pregnancy outcome.

INTRODUCTION

Preeclampsia and fetal growth restriction (PEFGR) are related pregnancy complications associated with poor placental function and excessive risk of cardiovascular disease (CVD) in the mother, as well as long-term developmental consequences for their children.[1-4] Ten million women develop PEFGR worldwide each year and approximately 76.000 mothers and 500.000 babies annually die as a direct consequence.[5] Although each condition can occur independently, preeclampsia and FGR are often combined, in particular in those where the disorder presents itself early in pregnancy.[6] For the mother, the clinical presentation is characterized by severe hypertension accompanied by proteinuria, as a sign of generalized endothelial dysfunction with accompanied circulatory compromise and potential end-organ damage.[6,7] For the baby, this disorder is characterized by impaired fetal growth leading to fetus not reaching their genetically determined growth potential, vascular adaptions to maintain cerebral development, risk of stillbirth and preterm birth, and long-term effects on vulnerability to metabolic and cardiac disease.[3,8,9]

It is generally hypothesized that PEFGR both have its origin in abnormal transformation of the spiral arteries underlying the placenta in response to the migrating trophoblast cells invading the wall of the uterus. In a number of classic papers, describing small series of placental bed (biopsy) samples, the observation was made that this so-called “physiologic” remodeling of uterine spiral arteries appears abnormal in PEFGR compared with healthy pregnancies.[10-13] In addition to incomplete or absent remodeling, atherosclerosis-like lesions have been described, in particular in preeclampsia. This is normally referred to as ‘acute atherosis’ and is characterized by infiltration of macrophages, foam cells and thickening of the intimal and medial layer of the spiral arteries. These lesions have been interpreted as possible signs of vascular damage and speculated to be indicators of underlying maternal risk for future CVD.[14,15]

Despite stating the importance of spiral arteries lesions as a hallmark feature of placental disorders and adverse pregnancy outcomes, data on the prevalence in normal and PEFGR complicated pregnancy has not been clearly established in sufficiently powered prospective studies.[12,16]

The aim of this study was to determine the prevalence of spiral artery lesions in a prospective cohort of women by studying placental bed biopsies taken at Caesarean section, including different stages of impaired remodeling, absence of intramural trophoblast cells, damage to the vascular wall (i.e. acute atherosis and thrombosis) and presence of inflammatory cells in the placental bed in women with PEFGR compared to women with normal pregnancy outcome.

METHODS

Patient selection and definitions

The SPiral Artery Remodeling (SPAR) study allows for an on-going systematic and prospective collection of placental bed biopsy samples in women undergoing Caesarean section for PEFGR, and women with uncomplicated pregnancy as a reference group. Recruitment is set up as a multicenter cohort study in one tertiary (University Medical Center Utrecht) and one secondary (Diakonessenhuis Utrecht) perinatal birth center in Utrecht, the Netherlands. Detailed description of the study design and protocol was previously published.[17] In short, all women undergoing Caesarean section between May 2015 and February 2018 were invited to participate in the study. Women who participated in the pilot study were included in this analysis.[17] We defined preeclampsia, according to the latest International Society for the Study of Hypertension in Pregnancy (ISSHP) guideline, as new-onset hypertension (≥140/90 mmHg) after 20 weeks of gestation in combination with; significant proteinuria (≥300 mg/24 h or protein/creatinine ratio ≥0.3mg/mmol) or maternal organ dysfunction (i.e. renal insufficiency, liver involvement, neurological or hematological complications).[18,19] Hemolysis, elevated liver enzymes and low platelet (HELLP) syndrome was diagnosed following the same guidelines.[18] FGR was defined according to the Dutch national guidelines, as an ultrasonographical estimated fetal weight or abdominal circumference below the tenth percentile or a reduction in the standardized growth curves of ≥20 percentiles.[20,21] Early onset was defined as PEFGR occurring before 34 weeks of gestation.[22] Poor placental function had to be the suspected cause of PEFGR and exclusion criteria entailed: fetal congenital or chromosomal abnormalities, major operative complications, multiple pregnancy, (current/ previous) placental abruption or preterm birth (when included in the control group). Data on maternal characteristics and neonatal outcomes were extracted from hospital records. All patients received study information and provided informed consent prior to participation. This study was reviewed and approved by the local Institutional Ethical Review Board of the University Medical Center Utrecht, protocol reference number: 16-198.

Placental bed sampling and histopathological characterization

Placental bed biopsies were obtained by the clinician performing the Caesarean section as previously published.[17,23] In brief, after delivery of the baby and the placenta, four biopsy samples were obtained from the central part of the placental bed, using a cervical biopsy forceps. Samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin wax for histological examination. Women with immunohistochemical confirmed presence of trophoblast cells (using antibodies against keratin (CK AE1/AE3, Invitrogen, MA-13156) in one or more biopsy specimen, confirming localization of the placental bed, were included in the analysis of the study. A periodic Acid Schiff after diastase (PAS-D) stain was used to localize remodeled spiral arteries. A detailed description of the scoring system can be found in Supplementary Table 1. In brief, spiral artery remodeling degree was scored as none,

minimal, moderate or complete when intramural trophoblast was present, depending on the mean external diameter and on the extent of physiological changes throughout the biopsy. We defined impaired remodeling as absence of intramural trophoblast (and presence of perivascular trophoblasts) or remodeling lower than moderate when intramural trophoblasts were present. An example of all degrees of remodeling can be found in Supplementary Figures 1 and 2. Acute atherosis was defined as presence of lipid laden foam cells (vacuolated CD68+ macrophages) in the arterial wall. Thrombosis was defined as presence of an organized thrombus within the artery, attached to the vascular wall. The presence of inflammatory cells in the placental bed was investigated by immunohistochemical staining for T cells (CD3+), NK-cells (CD56+) and macrophages (CD68+). Description of immunohistochemical staining methods can be found in a previous publication.[17] All biopsies were scored by two independent observers blinded for outcome (PN with SG or LB). When interpretations differed, a third observer was consulted (SG or LB). No serious adverse events occurred during the study period and no re-surgeries were performed.

Statistical analysis

Comparisons were made for the degree of spiral artery remodeling (impaired, and categorical; none, minimal, moderate, complete), the presence of intramural trophoblast cells (yes/no), the presence of thrombosis (yes/no), acute atherosis (yes/no) and the degree of (perivascular) immune cell presence. Groups were defined as participants having either preeclampsia, and/or FGR (PEFGR) or being part of the reference group of uneventful pregnancies (controls). Data are presented as means (standard deviations) or number (percentages) if variables were either continuous or categorical. *P*-values to indicate differences between groups were calculated using the Fisher's exact test for continuous variables and $\chi 2$ for categorical variables. Statistical analyses were performed using SPSS (release 25.0; Chicago, Illinois, USA). *P*-values less than 0.05 were considered statistically significant.

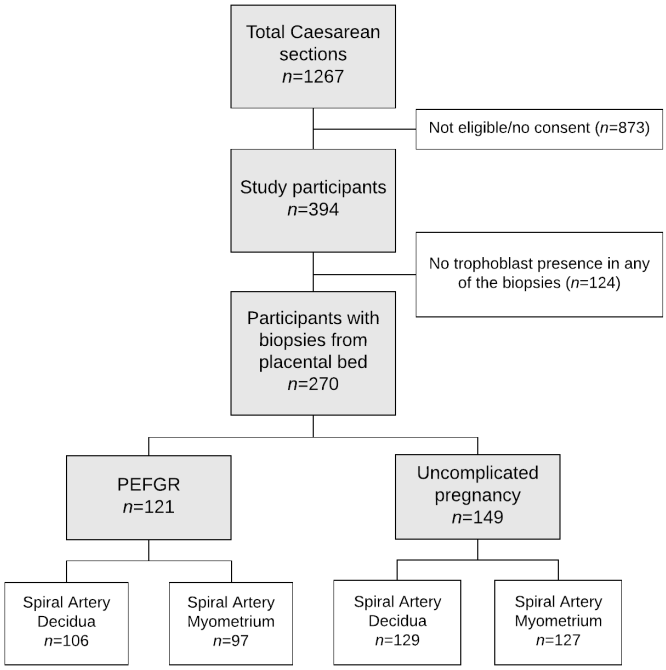

FIGURE 1. Inclusions in the SPAR study.

Abbreviations: PEFGR, preeclampsia and/or fetal growth restriction.

RESULTS

Baseline characteristics

Within the study period 394 out of 1267 women undergoing Caesarean section were included in the study. Figure 1 represents the recruitment process, number of biopsies correctly obtained from the placental bed and the number of participants with biopsy material representative of both the myometrial and decidual parts of the placental bed. Correct localization of the placental bed (presence of keratin positive trophoblast cells in ≥1 biopsy sample) was confirmed in 270 of 394 patients (68.5%). Clinical and patient characteristics are presented in Table 1. As expected, women with PEFGR were younger, more often nulliparous, were less often of white European origin, and were more likely to have pre-existent hypertension, a previous hypertensive pregnancy or a previous pregnancy complicated by FGR. Consistent with the diagnosis of PEFGR, gestational age (223 vs 274 days, $p<0.001$) and birthweight (1421 vs 3576 grams, $p<0.001$) were both lower than in women with normal pregnancy outcome.

Placental bed and spiral artery characteristics

Data on spiral artery remodeling and associated vascular lesions can be found in Table 2. Impaired remodeling was significantly more present throughout the placental bed in PEFGR (63.6 vs 10.1%, $p<0.001$), with similar prevalence in decidua (42.5 vs 8.5%, $p<0.001$) and myometrium (54.6 vs 4.7%, $p<0.001$). Complete remodeling was significantly less prevalent in PEFGR, in the placental bed (15.7 vs 71.1%, $p<0.001$) and for both separate compartments of the placental bed (17.0 vs 76.0%, $p<0.001$ in the decidua and 20.6 vs 77.2%, $p<0.001$ in the myometrium). In almost half of biopsies obtained from women with PEFGR we observed non-remodeled spiral arteries with abundant presence of interstitial trophoblast, but without intramural invasion (45.5 vs 6.7%, $p<0.001$). Overall, the degree of remodeling in segments of spiral arteries located in the decidua was comparable for segments within the myometrium for both groups (controls; 75-100% and PEFGR 64.7-75.0%, $p<0.001$).

Acute atherosis was prevalent in 28.9% of PEFGR compared to 3.4% of controls ($p<0.001$), and in similar amounts in the decidual (16.3%) and myometrial (16.5%) spiral artery segments in women with PEFGR. Interestingly none of the biopsies from the control group showed signs of acute atherosis in the myometrial spiral arteries. Thrombosis was less prevalent but occurred significantly more often in PEFGR when looking at the whole biopsy (16.5 vs 5.4%, $p=0.003$), with an even distribution among compartments of the placental bed (8.7% vs 2.4%, $p=0.033$ in decidua and 8.2 vs 3.1%, $p=0.100$ in myometrium). Intima proliferation was significantly associated with uncomplicated pregnancy (97.3 vs 85.0%, $p=0.003$ in decidua and 100 vs 90.9%, $p=0.001$ in myometrium). Interstitial (decidual and myometrial) and myometrial perivascular presence of inflammatory cells can be found in Supplementary Table 2. Women with PEFGR more often showed high numbers of myometrial perivascular macrophages (16.7 vs 5.2%, $p=0.014$), the same trend was visible

TABLE 1. Maternal and pregnancy baseline characteristics in women with PEFGR and healthy pregnancy.

	Control *n*=149	**PEFGR** *n*=121	***p*-value**
General characteristics			
Age (years)	33.9 (3.9)	31.4 (5.3)	<0.001
Caucasian (%)	142 (95.3%)	94 (78.3%)	<0.001
BMI (kg/m^2)	24.6 (5.1)	26.5 (6.1)	0.009
Obesity (%)	23 (15.5%)	23 (20.5%)	0.296
Smoking (%)	5 (3.4%)	13 (10.7%)	0.016
General and obstetric history			
Pre-existent hypertension (%)	1 90.7%)	18 (14.9%)	<0.001
Pre-existent kidney disease (%)	1 (0.7%)	4 (3.3%)	0.110
Pre-existent Diabetes (%)	2 (1.3%)	4 (3.3%)	0.276
Nulliparity (%)	37 (24.8%)	82 (67.8%)	<0.001
CS in history (%)*	91 (81.3%)	26 (66.7%)	0.060
HDP in history (%)*	7 (6.3%)	20 (51.3%)	<0.001
FGR in history (%)*	15 (13.4%)	21 (53.8%)	<0.001
Maternal characteristics			
Systolic blood pressure (mmHg)	124.0 (12.2)	163.9 (23.2)	<0.001
Diastolic blood pressure (mmHg)	75.7 (8.0)	103.1 (15.0)	<0.001
Antihypertensive treatment oral (%)	2 (1.3%)	89 (73.6%)	<0.001
Antihypertensive treatment iv (%)	0 (0.0%)	48 (39.7%)	<0.001
Antepartum MgSO4 iv (%)	0 (0.0%)	86 (71.1%)	<0.001
Antepartum CCS (%)	2 (1.3%)	93 (76.9%)	<0.001
Pregnancy characteristics			
PIH (%)	9 (6.0%)	6 (5.0%)	0.700
Preeclampsia (%)	n/a	100 (82.6%)	n/a
FGR (%)	n/a	90 (74.4%)	n/a
Preeclampsia with FGR (%)	n/a	69 (57.0%)	n/a
HELLP (%)	n/a	36 (29.8%)	n/a
HPP (%)	7 (4.7%)	2 (1.7%)	0.166
GDM with insulin use (%)	2 (1.3%)	2 (1.7%)	0.834
GA at delivery (days)	274 (5)	223 (25)	<0.001
Delivery<34 weeks GA (%)	0 (0.0%)	87 (71.9%)	<0.001
Birthweight (grams)	3576 (499)	1421 (737)	<0.001
Fetal sex, male (%)	63 (43.6%)	64 (52.9%)	0.129

Baseline characteristics for all women with extravillous trophoblast cell presence (indicating true placental bed) in ≥1 biopsy. Values are presented as means (standard deviations) or as numbers (%). *P*-values were calculated by the Fisher's exact test for continuous variables and $\chi 2$ test for categorical variables. *Obstetric history is calculated amongst multiparous patients only. *Abbreviations*: PEFGR, preeclampsia and/or fetal growth restriction; BMI, Body Mass Index; Obesity was defined as an BMI >30kg/m2; CS, Caesarean section; HDP, hypertensive disease of pregnancy; FGR, fetal growth restriction; iv, intravenous; MgSO4, magnesiumsulphate; CCS, corticosteroid treatment; GDM, gestational diabetes mellitus, PIH, pregnancy induced hypertension; HELLP, Hemolysis Elevated Liver enzymes Low Platelets syndrome; HPP, hemorrhage post partum; GA, gestational age; n/a; not applicable.

for myometrial macrophages, but not statistically significant (23.1 vs 14.2%, *p*=0.113). Women with PEFGR more often had lower numbers of perivascular NK- and T-cells compared to controls (NK-cells; 7.4 vs 0.0%, *p*=0.012 and T-cells; 49.1 vs 37.6%, *p*=0.001).

When stratifying the data for women having only preeclampsia, only FGR or having both, prevalence of impaired remodeling was highest when both conditions were present (72.5% for both vs 66.7% for FGR only, and 41.9% for preeclampsia only, Supplementary Table 3). Up to 55.1% showed absence of intramural trophoblast when preeclampsia was concurrent with FGR. The group with only preeclampsia showed the highest prevalence of complete remodeling (25.8%), which significantly differed when compared with women with preeclampsia and FGR combined (*p*=0.039). The percentage of women with preeclampsia without FGR who showed impaired remodeling was significantly lower than women with both preeclampsia and FGR combined (41.9% vs 72.5%, *p*=0.003). Similarly, women with only FGR less often showed absence of intramural trophoblast compared with women with both conditions, but had a similar frequency of impaired remodeling (66.7 vs 72.5%). Prevalence of acute atherosis was highest in women with preeclampsia, with or without FGR (31.9% vs 29.0%, respectively, compared to 19.0% in only FGR). Thrombosis was most prevalent in women with both conditions (20.3%) or only FGR (14.3%), compared to only preeclampsia (9.7%).

TABLE 2. Prevalence and degree of impaired spiral artery remodeling and associated vascular lesions in women with PEFGR and and healthy pregnancy.

	Control (*n*=149)	**PEFGR** (*n*=121)	***p*-value**
Placental bed			
No intramural trophoblast (%)	10 (6.7%)	55 (45.5%)	<0.001
Remodeling*			<0.001
None (%)	0 (0.0%)	2 (1.7%)	
Minimal (%)	5 (3.4%)	20 (16.5%)	
Moderate (%)	28 (18.8%)	25 (20.7%)	
Complete (%)	106 (71.1%)	19 (15.7%)	
Impaired remodeling (%)‡	15 (10.1%)	77 (63.6%)	<0.001
Acute atherosis (%)	5 (3.4%)	35 (28.9%)	<0.001
Thrombosis (%)	8 (5.4%)	20 (16.5%)	0.003
Decidua			
Spiral artery in biopsy (%)	129 (86.6%)	106 (87.6%)	0.803
Intima proliferation† (%)	107 (97.3%)	51 (85.0%)	0.003
No intramural trophoblast (%)	9 (7.0%)	41 (38.7%)	<0.001
Remodeling*			<0.001
None (%)	0 (0.0%)	1 (0.9%)	
Minimal (%)	2 (1.6%)	19 (17.9%)	
Moderate (%)	20 (15.5%)	27 (25.5%)	
Complete (%)	98 (76.0%)	18 (17.0%)	
Impaired remodeling (%)‡	11 (8.5%)	45 (42.5%)	<0.001
Acute atherosis (%)	4 (3.2%)	17 (16.3%)	0.001
Thrombosis (%)	3 (2.4%)	9 (8.7%)	0.033
Myometrium			
Spiral artery in biopsy (%)	127 (85.2%)	97 (80.2%)	0.736
Intima proliferation† (%)	126 (100.0%)	60 (90.9%)	0.001
No intramural trophoblast (%)	1 (0.8%)	31 (32.0%)	<0.001
Remodeling*			<0.001
None (%)	0 (0.0%)	3 (3.1%)	
Minimal (%)	5 (3.9%)	19 (19.6%)	
Moderate (%)	23 (18.1%)	24 (24.7%)	
Complete (%)	98 (77.2%)	20 (20.6%)	
Impaired remodeling (%)‡	6 (4.7%)	53 (54.6%)	<0.001
Acute atherosis (%)	0 (0.0%)	16 (16.5%)	<0.001
Thrombosis (%)	4 (3.1%)	8 (8.2%)	0.100

Spiral artery remodeling characteristics within biopsies from the placental bed (throphoblast cell presence), separated in distinct compartments; decidua, myometrium and overall placental bed. *P*-values were calculated by $\chi 2$ test. *Remodeling assessed in (spiral) arteries with intramural trophoblast cells. † Intima proliferation was assessed only arteries with intramural trophoblast cells. ‡Impaired remodeling was defined as arteries without intramural trophoblast cells or when remodeling was less than moderate. *Abbreviations*: PEFGR, preeclampsia and/or fetal growth restriction.

DISCUSSION

In this study we show that pregnancies complicated by preeclampsia or fetal growth restriction, or both, here grouped together as PEFGR, are associated with a high prevalence of impaired vascular remodeling, atherosclerosis-like lesions and differences in immune cell composition of the placental bed. These lesions are not confined to the superficial decidual layer of the uterus, but appear throughout the inner myometrial segments deep within the placental bed. To our knowledge, our study represents the first prospectively collected adequately-sized cohort to systematically study and quantify the prevalence of vascular pathology lesions of the placental bed in women undergoing Caesarean section. Findings from our study imply a strong association between impaired, or abnormal, spiral artery remodeling and inappropriate vascular adaptation of the placental bed and adverse pregnancy outcomes traditionally attributed to poor placental function.

The most striking feature of spiral artery pathology in women with PEFGR in our study is the impaired, and often entirely absent trophoblast-induced remodeling response needed to accommodate the vast blood supply to the developing placenta. This "physiological remodeling" response was first described by Brosens *et al*. in the 1960s on the basis of a small case series of Caesarean hysterectomy samples, and is thought to occur between 8-16 weeks of pregnancy.[24-26] This widely accepted hypothesis proposes that spiral artery remodeling is dependent on a complicated and delicate interplay between the fetal extravillous trophoblast cells, the maternal immune system and components of the arterial wall including smooth muscle cells, extracellular matrix and endothelial cells.[13,27-36] Since these early observations in small case series, to our knowledge, no previous study has aimed to quantify the prevalence of lesions resulting from inadequate transformation and remodeling of these arteries. In our cohort, insufficient remodeling of spiral arteries was observed in the majority of cases, and was only found in a very small proportion of uneventful pregnancies. We believe this finding makes a strong case for potential involvement of maternal vascular incompetence in the pathology of PEFGR, although our study does not necessarily imply a cause-effect relation of the first with the latter. Of importance is the observation that in almost half of pregnancies with PEFGR the spiral arteries vascular wall appeared not to be invaded by trophoblast cells at all although interstitial trophoblast is abundantly present in the surrounding decidua or myometrium. Consistent with some of the earlier observations in small series of placental bed biopsy studies, arterial branches whose vessel wall has not been disrupted by intramural trophoblast consistently lack signs of adequate remodeling.[12,16]

In the initial papers reporting on small series of spiral artery biopsy samples, emphasis was placed on the depth of the trophoblast cells migrating from the placenta towards the myometrial inner parts of the uterus.[10,24-26] Some papers have suggested 'shallow' invasion into the wall of the uterus, leads to lower presence of trophoblast cells at the proximal part of the spiral arteries and consequently a lower invasion into the arterial wall.[37] However, more recent

work has demonstrated the opposite, with more abundant trophoblast cells in perivascular tissue in pregnancies with FGR.[16] In our study, biopsy samples of the myometrial parts of the placental bed often showed abundant presence of trophoblast cells, consistent with the findings of Lyall *et al*., pointing towards other mechanisms responsible for failure of remodeling than 'less invasive' trophoblast.[16] Similarly, we found associated maternal inflammatory cell presence differences. In particular, we found increased numbers of macrophages in the placental bed of women with PEFGR, which has previously been associated with poor intravascular trophoblast invasion resulting in abnormal adaptation of the placental bed arterial vasculature.[38,39] In addition, other lesions of the vascular wall of the spiral arteries were common in PEFGR, including lipid accumulation, presence of foamy macrophages and fibrin deposition ('acute atherosis'), thrombotic lesions and invasion of foam cells.[14,40,41] Again, these lesions are rarely present in women with normal pregnancy outcome.

Taken together, these observations suggest an association between ineffective remodeling, in combination with acute atherosis lesions thickening the vascular wall, and presence of (peri)vascular immune cells, leading to inappropriate vascular adaption of the placental bed. The result of this inadequate transformation of spiral arteries on blood flow to the placenta has been debated by several groups. Some have argued that the narrowing of the luminal diameter as a result of either incomplete outward remodeling, thickening of the intima, or both, will lead to alterations in blood flow and possible ischemia and/or increased turbulence at the surface of the placenta, damaging the placental villous structure.[42] It is not entirely clear how these changes lead to the chain of events resulting in restricted fetal growth and preeclampsia (or both), although some evidence points towards changes in nutrient transport, chronic hypoxia, release of free radicals and imbalance of angiogenic factors.[43-46]

Little is known on how maternal cardiovascular constitution interacts with spiral artery remodeling and contributes to the intimal lesions observed in women with PEFGR. There are some suggestions of associations with the presence of chronic hypertension and dyslipidemia.[12,17,47] Recent studies on long-term health of women with pregnancies affected by PEFGR demonstrate a consistent association with accelerated development of hypertension, atherosclerotic lesions of coronary arteries and subsequent ischemic heart disease on average 8-10 years earlier than women without such pregnancy complications.[4,48-50] It is not clear which factors contribute to arterial disease in these women, although both traditional risk factors, e.g. lipids and hypertension, and non-traditional risk factors including inflammation and endothelial erosion appear to be involved.[51-54] Based on our findings, we speculate that similar mechanisms are likely to contribute to the susceptibility of impaired remodeling and disruption of the vascular wall with its characteristic 'acute atherosis' lesions in women affected by these same disorders revealing underlying shared cardiovascular risk. Of note, spiral artery lesions within the placental bed are not restricted to pregnancies complicated by PEFGR, and occur in other conditions associated with future cardiovascular risk, such as preterm birth, placental abruption and stillbirth through a similar mechanism of

shared susceptibility.[55-57]

Some strengths and limitations of our study should be mentioned. A strength of the present approach is the possibility to systematically assess pathology and evaluation of the placental bed using a biopsy technique that includes sampling of the myometrial segments of the spiral arteries. Previous groups used a suction methods to acquire only the superficial decidual segments of the spiral arteries, or even the distal fragments of the spiral arteries attached to the delivered placenta. Myometrial segments of spiral arteries are known to retain more of their original structure, indicating the maternal vascular system to adapt differently deeper in the placental bed. The discussion regarding 'depth' of invasion and exact pathophysiology of defective remodeling cannot be answered when solely reviewing the decidual segments. Additionally, the inclusion of a large number of consecutive patients means this is, to the best of our knowledge, the first adequately powered study on this subject.

Limitations of the present study include the difference in gestational age at delivery associated with the higher rate of indicated preterm birth in women with PEFGR. Although spiral artery remodeling itself occurs much earlier in pregnancy and the state of spiral artery pathology at the cross-sectional sampling moment of our study probably represents the end-point of the remodeling response for both groups, some studies have suggested that remodeling and perivascular inflammation may change further as pregnancy progresses, which needs to be taken into consideration when interpreting the results.[58-60] For this reason, some groups have chosen to compare their findings to women with spontaneous preterm birth. However, we find that this comparison may not be the most meaningful approach, as preterm birth itself is also associated with both spiral artery lesions and predisposition to CVD compared with women with healthy pregnancy outcome.[55,61,62] Lastly, biopsy specimens provide information in a small portion of the placental bed. It is possible that the area next to the biopsy has a different degree of trophoblast invasion and/or vascular remodeling. Therefore we cannot rule out the possibility of some degree of sampling bias. Although this may underestimate the difference in prevalence of impaired remodeling, we pose that this did not have major impact on our findings given the large differences between cases and controls.

Future perspectives

This study establishes an unequivocal association of impaired remodeling and vascular pathology in the placental bed of women with PEFGR. This does not, however, clarify the intricate (patho)physiology of remodeling itself. An important gap in current knowledge is the question to what extent the maternal cardiovascular health contributes to the abnormal remodeling process. In addition, despite several decades of research, the interaction between maternal and pregnancy-specific factors and trophoblast cells resulting in the spiral artery remodeling response essential for normal pregnancy outcome, remains an enigma.[63] Although the use of current biopsy techniques are a significant challenge, more

research in human uterine tissue is needed. Future research should focus on identifying the interplay between the maternal immune system, components of the vascular wall (i.e. endothelial cells, smooth muscle cells), and interaction with trophoblast. Not only may this impact health of mother and child in the perinatal period, but may lead to a better understanding, and preventive possibilities, for future cardiovascular risk in the mother. As it is suspected that a hostile in-utero environment (i.e. chronic hypoxia) can 'program' detrimental mechanisms in the fetus, impact on health and disease later in life of the offspring needs to be investigated.[1,64]

Conclusion

In a high proportion of cases preeclampsia and fetal growth restriction is associated with maternal remodeling problems and early signs of uterine vascular damage in association with absent intramural trophoblast cells in the arteries underlying the placenta. Since perivascular trophoblast cells were abundantly present, this indicates that the maternal vascular composition may hamper intramural invasion and subsequent pregnancy specific adaptations necessary for healthy pregnancy. Although placental bed biopsy sampling poses research challenges (i.e. invasive technique, necessity for long study period and follow-up), gathering of additional adequately-powered cohorts is needed to assess how health of the mother influences this process and what impact this may have on future cardiovascular health.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE 1. Histopathological characteristics assessed in placental bed biopsies.

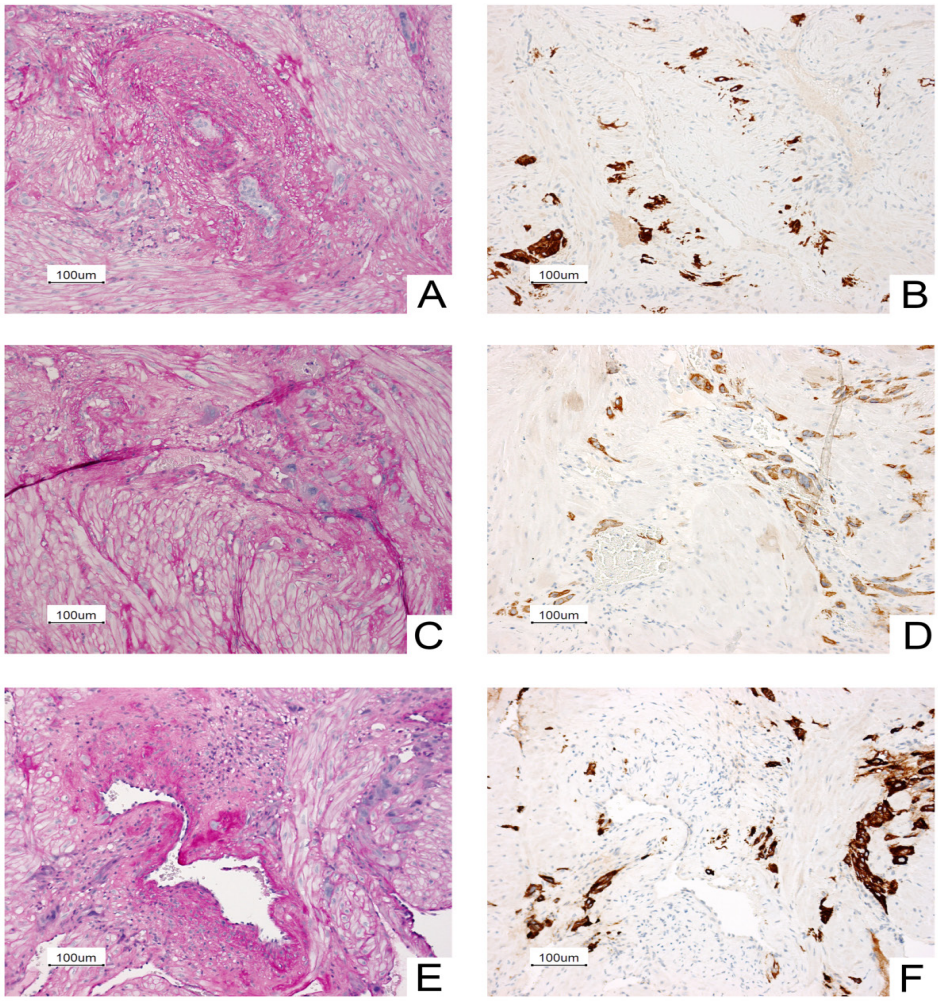
Placental bed characteristic	Classification	Definition	FOV*
Trophoblast presence (CK AE1/3 positive)	Yes/no	Confirmation of placental bed localisation when CKAE1/2 positive trophoblasts are present.	N/A
Degree of remodeling of spiral arteries†	Non invaded	Three-layered vessels with a tunica intima, media and adventitia. The tunica media is composed of a rather thick layer of smooth muscle cells. No intramural trophoblast presence, while interstitial trophoblasts are present.	N/A
	None	No (physiological) PAS-D positive degeneration of the vessel wall and change of artery circumference. Presence of ≥ 1 intramural trophoblast.	N/A
	Minimal	Focal PAS-D degeneration of < 1/3 of the artery wall circumference, some arterial muscle still present, small artery diameter. Presence of intramural trophoblasts.	N/A
	Moderate	Focal PAS-D degeneration of > 1/3 but < 2/3 of the artery wall circumference, some arterial muscle still present, medium artery diameter. Presence of intramural trophoblasts.	N/A
	Complete	Complete circular PAS-D degeneration of the artery wall, large artery diameter. Presence of intramural trophoblasts.	N/A
Impaired remodeling†	yes/no	Non-invaded arteries (presence of interstitial trophoblasts without presence of intramural trophoblasts) or a degree of remodeling as defined above less than moderate (none or minimal) when intramural trophoblasts are present.	
Acute atherosis†	Yes/no	Presence of lipid laden foam cells (vacuolated CD68⁺ macrophages) in the artery wall.	N/A
Thrombosis†	Yes/no	Presence of an organized thrombus, attached to the artery wall.	N/A
Intima proliferation	Yes/no	Presence of intraluminal fibrosis.	N/A
CD68⁺, CD56⁺ and CD3⁺ cell presence‡	None	No positive stained cells present.	N/A
	Minimal	< 50 positive stained cells present.	10x10 (2.5mm^2)
	Moderate	≥ 50 but <100 positive stained cells present, or presence in small groups (< 50 cells).	10x10 2.5mm^2)
	Heavy	Diffuse cell presence (≥ 100 cells).	10x10 (2.5mm^2)
	Clustering	Aggregrates of ≥ 50 positive cells.	N/A
CD68⁺, CD56⁺ and CD3⁺ cell presence in spiral arteries◊	None	No positive stained cells present.	N/A
	Minimal	< 20 positive stained cells present.	10x20 (0.6mm^2)
	Moderate	≥ 20 but <50 positive stained cells present, or presence in small groups (< 50 cells).	10x20 (0.6mm^2)
	Heavy	Diffuse cell presence (≥ 50 cells).	10x20 (0.6mm^2)
	Clustering	Aggregrates of ≥ 50 positive cells.	N/A

Histological parameters are based on the Athero-express scoring system and adjusted for use on placental bed biopsies as previously reported by our group.[17] *FOV indicates Field of View as used by the pathologist. †Assessed for the whole placental bed biopsy and for decidua and myometrium separately. ‡Scored separately for decidua and myometrium. ◊Scored in myometrial spiral arteries with intramural trophoblasts. *Abbreviations*: CK, cytokeratin; PAS-D, periodic acid-Schiff-diastase; N/A, not applicable; CD, cluster of differentiation.

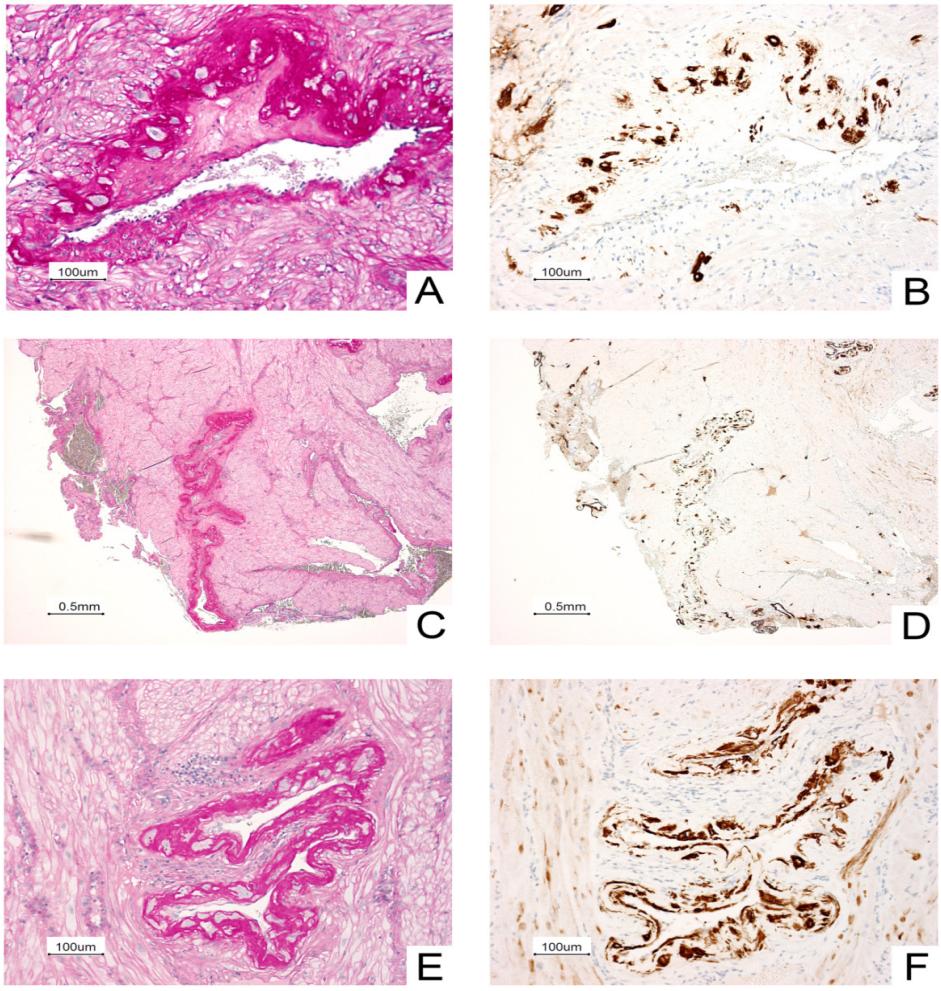
SUPPLEMENTARY TABLE 2. Local and perivascular immune cell presence within the placental bed from women with PEFGR and healthy pregnancy.

	Control *n*=142	**PEFGR** *n*=108	***p*-value**
Macrophages (CD68+)			
Decidua			0.228
None/minimal	12 (8.5%)	6 (5.6%)	
Moderate	95 (67.4%)	66 (61.1%)	
Heavy	34 (24.1%)	36 (33.3%)	
Myometrium			0.113
None/minimal	34 (24.1%)	18 (16.7%)	
Moderate	87 (61.7%)	65 (60.2%)	
Heavy	20 (14.2%)	25 (23.1%)	
Myometrial perivascular presence*			0.014
None/minimal	52 (45.2%)	28 (51.9%)	
Moderate	57 (49.6%)	17 (31.5%)	
Heavy	6 (5.2%)	9 (16.7%)	
NK cells (CD56+)			
Decidua			0.190
None	0 (0.0%)	0 (0.0%)	
Minimal	41 (29.1%)	27 (25.0%)	
Moderate	81 (57.4%)	57 (52.8%)	
Heavy	19 (13.5%)	24 (22.2%)	
Myometrium			0.267
None	0 (0.0%)	1 (0.9%)	
minimal	122 (86.5%)	86 (79.6%)	
Moderate	19 (13.5%)	20 (18.5%)	
Heavy	0 (0.0%)	1 (0.9%)	
Myometrial perivascular presence*			0.012
None	0 (0.0%)	4 (7.4%)	
minimal	106 (92.2%)	47 (87.0%)	
Moderate	9 (7.8%)	3 (5.6%)	
Heavy	0 (0.0%)	0 (0.0%)	
T cells (CD3+)			
Decidua			0.172
None/minimal	15 (10.6%)	14 (13.0%)	
Moderate	105 (74.5%)	69 (63.9%)	
Heavy	21 (14.9%)	25 (23.1%)	
Myometrium			0.139
None/minimal	45 (39.5)	29 (53.7%)	
Moderate	51 (44.7%)	21 (38.9%)	
Heavy	18 (15.8%)	4 (7.4%)	
Myometrial perivascular presence*			0.001
None/minimal	53 (37.6%)	53 (49.1%)	
Moderate	84 (59.6%)	43 (39.8%)	
Heavy	4 (2.8%)	12 (11.1%)	

Immune cell presence are given for decidua, myometrium and (peri)vascular spiral ateries presence seperately. Results are shown for women with a primary Caesarean section, i.e. no contractions or rupture of membranes. Numbers may be lower for cell type seperately as some stainings were not available for each patient. *Scored for patients with spiral arteries with intramural trophoblast. *Abbreviations*: PEFGR, preeclampsia and/or fetal growth restriction, CD, cluster of differentiation.



SUPPLEMENTARY FIGURE 1. Impaired remodeling in (myometrial) spiral artery segments. (**A, B**) No invasion: spiral artery without any physiological change of the artery wall. Parallel CK AE1/3 staining shows presence of multiple interstitial trophoblasts surrounding the spiral artery, but no intramural trophoblasts. (**C, D**) No remodeling: spiral artery without physiological PAS-D positive degeneration of the artery wall, the original arterial muscle is present. Parallel CK AE1/3 staining shows the presence of a few intramural trophoblasts, surrounding tissue is invaded with trophoblasts. (**E, F**) Minimal remodeling: spiral artery with focal PAS-D positive degeneration of < 1/3 of the spiral artery wall circumference, most of the original arterial muscle is still present. Parallel CK AE1/3 staining shows the presence of a few intramural trophoblasts, although perivascular invasion with interstitial trophoblasts is clearly present.



SUPPLEMENTARY FIGURE 2. Moderate and complete remodeling of (myometrial) spiral artery segments. (**A, B**) Moderate remodeling: spiral artery with focal PAS-D positive degeneration of > 1/3 but <2/3 of the spiral artery wall circumference, some of the original arterial muscle is still present. Parallel CK AE1/3 staining shows the presence of multiple intramural trophoblasts. (**C, E**) PAS-D staining shows a myometrial spiral artery segment with complete circular PAS-D positive degeneration of the spiral artery wall, without presence of the original arterial muscle. (**D, F**) Parallel CK AE1/3 staining shows presence of multiple intramural trophoblasts.

SUPPLEMENTARY TABLE 3. Sub stratified data of impaired spiral artery remodeling and associated vascular lesions amongst women either only preeclampsia, only fetal growth restriction or both conditions combined.

	Preeclampsia only *n*=31	**FGR only** *n* = 21	**Preeclampsia and FGR** *n* =69
Placental bed	n=237	n=157	
No intramural trophoblast (%)	8 (25.8%)	9 (42.9%)	38 (55.1%)
Remodeling*§			
None (%)	0 (0.0%)	1 (4.8%)	1 (1.4%)
Minimal (%)	5 (16.1%)	4 (19.0%)	11 (15.9%)
Moderate (%)	10 (32.3%)	3 (143%)	12 (17.4%)
Complete (%)	8 (25.8%)	4 (19.0%)	7 (10.1%)
Impaired remodeling (%)‡§	13 (41.9%)	14 (66.7%)	50 (72.5%)
Acute atherosis (%)	9 (29.0%)	4 (19.0%)	22 (31.9%)
Thrombosis (%)	3 (9.7%)	3 (14.3%)	14 (20.3%)
Decidua			
Spiral artery in biopsy (%)§	23 (74.2%)	20 (95.2%)	63 (91.3%)
Intima proliferation (%)†	14 (82.4%)	11 (91.7%)	28 (84.8%)
No intramural trophoblast (%)	6 (26.1%)	7 (35.0%)	28 (44.4%)
Remodeling*§			
None (%)	0 (0.0%)	1 (5.0%)	0 (0.0%)
Minimal (%)	3 (13.0%)	4 (20.0%)	12 (19.0%)
Moderate (%)	6 (26.1%)	4 (20.0%)	17 (27.0%)
Complete (%)	8 (34.8%)	4 (20.0%)	6 (9.5%)
Impaired remodeling (%)‡§	9 (39.1%)	12 (60.0%)	40 (63.5%)
Acute atherosis (%)	5 (21.7%)	2 (10.0%)	10 (15.9%)
Thrombosis (%)	1 (4.3%)	2 (10.0%)	6 (9.5%)
Myometrium			
Spiral artery in biopsy (%)	25 (86.2%)	14 (77.8%)	58 (84.1%)
Intima proliferation (%)†#	20 (100%)	8 (72.7%)	33 (91.7%)
No intramural trophoblast (%)	5 (20.0%)	3 (21.4%)	23 (39.7%)
Remodeling*			
None (%)	0 (0.0%)	0 (0.0%)	3 (5.2%)
Minimal (%)	4 (16.0%)	4 (28.6%)	11 (19.0%)
Moderate (%)	7 (28.0%)	3 (21.4%)	14 (24.1%)
Complete (%)	9 (36.0%)	4 (28.6%)	7 (12.1%)
Impaired remodeling (%)‡§	9 (36.0%)	7 (50.0%)	37 (63.8%)
Acute atherosis (%)	5 (19.2%)	1 (7.1%)	10 (17.9%)
Thrombosis (%)	1 (3.8%)	2 (14.3%)	5 (8.9%)

Spiral artery remodeling characteristics within biopsies from the placental bed (throphoblast cell presence), separated in distinct compartments; decidua, myometrium and overall placental bed. *P*-values were calculated by $\chi 2$ test. Sub stratification was performed for specific pregnancy complications in either only preeclampsia, only fetal growth restriction (FGR) or when both were present. * Remodeling degree in (spiral) arteries with intramural throphoblast cells. ‡ Impaired remodeling was scored when the placental bed (or each separate compartment) showed arteries without intramural throphoblast cells or when remodeling was less than moderate. †Intima proliferation was scored in arteries with intramural throphoblast cells. §Indicates a *p*-value <0.05 when comparing patients with only preeclampsia to preeclampsa with FGR, # indicates a *p*-value <0.05 when comparing patients with only FGR to only preeclampsa. *Abbreviations*: FGR, fetal growth restriction.

4

Spiral artery remodeling in relation to preterm birth and associated adverse neonatal outcome in preeclampsia and fetal growth restriction

In preparation

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ABSTRACT

Objectives

To investigate the relationship between placental bed spiral artery remodeling in preeclampsia and fetal growth restriction (FGR) with adverse neonatal outcome.

Study design

Prospective cohort of women delivering preterm by Caesarean section due to preeclampsia and/or FGR, undergoing placental bed biopsy sampling (*n*=100, gestational age [GA] 215 ±18 days, birthweight [BW] 1172 ±400 grams). The degree of spiral artery remodeling was scored as impaired (none to minimal) or normal (moderate to complete), and compared with neonatal outcome parameters by Mann-Whitney U, $\chi 2$ or Fisher's exact tests were appropriate. Linear regression was used to adjust for GA.

Results

Impaired spiral artery remodeling was associated with significantly lower mean GA at birth (212 ±17 vs 223 ±19 days, p=0.004), lower BW (1078 ±352 vs 1393 ±424 grams, p<0.001) and lower BW percentile compared to normal remodeling. Neonates born following impaired remodeling more often needed neonatal interventions than neonates in the normal remodeling group. Higher adverse neonatal outcome scores (median 2 (IQR 1-4) vs 1 (IQR 0-3), p=0.003) and higher CRIB II scores (median 7, IQR 5-9 vs 4, IQR 3-7, p=0.008) were found when remodeling was impaired. When adjusted for GA, BW and BW percentile differences remained statistically significant.

Conclusions

Neonates born after preeclampsia and/or FGR with impaired spiral artery remodeling in placental bed biopsy samples show increased neonatal morbidity, partially explained by a lower gestational age and birthweight at delivery. These findings suggest that the quality and extent of maternal spiral artery remodeling in the first half of pregnancy is associated with the outcome of the neonate.

INTRODUCTION

Preeclampsia and fetal growth restriction (FGR) are major pregnancy complications associated with high risk of neonatal morbidity, increased mortality, and long-term effects including impaired cognitive function and increased cardiovascular and metabolic risk later in life.[1-7] Preeclampsia and FGR consist of different maternal and fetal symptoms which often co-occur and are considered to share a complex multifactorial pathophysiology.[8] Due to heterogeneity in the presentation of these pregnancy complications it is possible that different causes of either placental, fetal or maternal origin, ultimately lead to the same key symptoms. However, when preeclampsia and FGR co-occur early in pregnancy, the most important cause of these pregnancy complications is thought to be insufficient remodeling of the spiral arteries in the tissue underlying the placenta (i.e. the placental bed).[9-13] An unbalanced interplay between pre-existent maternal cardiovascular and immunological constitution with the developing placenta fail to make pregnancy specific adaptations necessary for healthy pregnancy. The exact balance between each of these aspects and what exactly drives the fetus, the mother, or both to experience difficulties, is unclear. Consequently, the clinical management of preeclampsia and FGR are challenging as no curative treatment exists. When the risk of complications for either mother or fetus become significant, delivery of both the baby and the placenta is the only definitive way to cure the mother and to rescue the fetus out of its precarious situation. Consequently, the infant is often confronted with the challenges of preterm birth. Studying neonatal outcome in association with spiral artery pathology may provide insight into underlying pathophysiology and short term neonatal morbidity and mortality. In this study we aim to investigate the contribution of impaired spiral artery remodeling to neonatal outcome in preterm babies born after preeclampsia and/or FGR.

METHODS

Patient selection and definitions

The SPiral Artery Remodeling (SPAR) study, is a prospective cohort study investigating placental bed biopsies collected at Caesarean section to examine spiral artery remodeling in pregnancies complicated by preeclampsia and/or FGR. Study set up and protocol can be found in a previous publication.[14] For the present analysis we included all neonates from mothers who participated in the SPAR study born before 37 completed weeks of gestation after preeclampsia and/or FGR admitted to a level II or III neonatal unit between April 2015 and January 2018. In addition, correct localization of the biopsies within the placental bed (i.e. presence of keratin positive extravillous trophoblast cells) and degree of spiral artery remodeling (i.e. presence of decidual and myometrial spiral arteries) needed to be ascertained at histopathological review. The selection process can be found in Supplementary Figure 1. FGR was defined as an ultrasonographically estimated fetal weight (EFW) or abdominal circumference (AC) below the tenth percentile or a reduction in the standardized growth curves of ≥20 percentiles.[15-17] We defined preeclampsia, as new-onset hypertension (≥140/90mmHg) after 20 weeks of gestation in combination with either significant proteinuria (≥300mg/24 h or protein/creatinine ratio ≥0.3 mg/mmol) or maternal organ dysfunction (i.e. renal insufficiency, liver involvement, neurological or hematological complications).[18] Placental insufficiency had to be the suspected cause of preeclampsia and/or FGR and exclusion criteria entailed; congenital or chromosomal abnormalities, major operative complications or multiple pregnancy.

Maternal and neonatal charts were reviewed for demographic and perinatal characteristics. Birthweight (BW), BW percentiles and Z-score were computed according to the Dutch Perinatal registry reference data.[19] Low APGAR scores were defined as <7 at five minutes after birth. Postnatal events that were considered included respiratory distress syndrome (RDS), intraventricular hemorrhage (IVH), patent ductus arteriosus (PDA), culture proven sepsis, necrotizing enterocolitis (NEC), necessity for mechanical ventilation, surfactant administration, postnatal corticosteroid treatment, blood transfusion, inotropic support, parenteral nutrition, phototherapy, hypoglycemia, or neonatal seizures. Mortality was defined as in hospital death, since it covers mortality during NICU admission. Score for Neonatal Acute Physiology Perinatal Extension II (SNAPPE-II) and Clinical Risk Index for Babies II (CRIB II) scores were computed as described recently by our group.[20,21] An additional neonatal adverse outcome sum score was calculated with a point being awarded when common neonatal complications were diagnosed, including mortality (double points), RDS, IVH, PDA, sepsis, NEC, inotropic support, hyperbilirubinemia, blood transfusion and hypoglycemia. Cut off value for hematological measurements within 48 hours after birth were based on values predicting poor fetal outcome in low birth weight infants.[22,23]

All mothers received study information and provided informed consent prior to participation. This study was reviewed and approved by the local Institutional Ethical Review Board of the University Medical Center Utrecht, protocol reference number: 16-198.

Placental bed biopsies

A detailed description of the tissue sampling and remodeling scoring can be found in previous publication.[14] In short, at Caesarean section, after delivery of the baby and the placenta, the central part of the placental bed was manually located on the inner uterine myometrial wall and four placental bed biopsies were obtained using cervical biopsy forceps. When immunohistochemical assessment showed presence of trophoblast cells, the degree of spiral artery remodeling was scored as none (perivascular or intramural trophoblast presence, but no remodeling), minimal (intramural trophoblast presence, remodeling <1/3 of the artery wall circumference), moderate (intramural trophoblast presence, remodeling >1/3 but <2/3 of the artery wall circumference) or complete (intramural trophoblast presence, complete circular remodeling), depending on the mean external diameter and on the extent of physiological changes throughout the biopsy. Spiral artery remodeling was dichotomized as impaired (none to minimal) or normal (moderate to complete) for further analysis due to estimated clinical effect and power of the study. All biopsies were scored by two independent observers blinded for outcome (PN with SG or LB). When interpretations differed, a third observer was consulted (SG or LB).

Statistical analysis

We investigated whether dichotomized (impaired vs. normal) spiral artery remodeling was associated with adverse neonatal outcome, including adjusted analyses for GA. Data is presented as means with standard deviations or number (percentages) if variables were either continuous or categorical with normal distribution and as medians with an interquartile range for the parameters with skewed distribution. *P*-values to indicate differences between groups were calculated using χ^2 or Mann-Whitney U test for categorical variables or Fisher's exact tests for continuous variables where appropriate. Linear regression was used to adjust for GA. Statistical analyses were performed using SPSS (release 25.0; Chicago, Illinois, USA). Figures were made using GraphPad Prism (version 7.04). A p-value<0.05 was considered statistically significant. With a sample size of 70 patients with impaired remodeling and 30 patients with normal remodeling, the power to detect a difference in adverse neonatal outcome score of more than 0.8 points with p<0.05 was 0.80.

RESULTS

Patient characteristics

Baseline characteristics of the 100 included neonates with data on spiral artery remodeling and neonatal outcome are presented in Table 1. Mothers were most often of white European descent and in the third decade of life. Of note, women often received antepartum oral (78%) or intravenous (46%) antihypertensive treatment, intravenous magnesium sulfate (78%) to prevent seizures and stroke due to preeclampsia, or antepartum corticosteroids to enhance fetal lung maturation in anticipation to preterm delivery (85%). Women who had normal remodeling more often only had experienced preeclampsia without concurring FGR compared to when remodeling was impaired (33.3 vs 12.9%, p=0.017). No other baseline characteristics significantly differed between the groups.

Neonatal outcome

Neonatal outcome in association with spiral artery remodeling are presented in Table 2 and Figure 1. When comparing the groups, impaired spiral artery remodeling was associated with significantly lower GA (212 vs 223 days, p=0.004) and with lower BW (1078 vs 1393 grams, p<0.001) compared to normal remodeling.

TABLE 1. Baseline characteristics of mothers, pregnancies and neonates included in the study.

	Total population *n*=100	**Impaired remodeling** *n*= 70	**Normal remodeling** *n*= 30	***p*-value**
Maternal characteristics				
Age (years)	30.9 (5.2)	31.0 (5.4)	30.5 (4.7)	0.643
White European decent (%)	78 (78.0%)	53 (75.7%)	25 (86.2%)	0.245
Obesity* (%)	20 (20.0%)	13 (20.3%)	7 (25.0%)	0.616
Obstetric characteristics				
Nulliparity (%)	68 (68.0%)	48 (68.6%)	20 (66.7%)	0.852
Smoking during pregnancy (%)	12 (12.0%)	8 (11.4%)	4 (13.3%)	0.788
Preeclampsia only (%)	19 (19.0%)	9 (12.9%)	10 (33.3%)	0.017
FGR only (%)	15 (15.0%)	12 (17.1%)	3 (10.0%)	0.359
Preeclampsia and FGR (%)	66 (66.0%)	49 (70.0%)	17 (56.7%)	0.197
GDM with insulin use (%)	1 (1.0%)	1 (1.4%)	0 (0.0%)	0.511
Oral antihypertensive treatment (%)	78 (78.0%)	53 (75.7%)	25 (83.3%)	0.399
Antihypertensive treatment iv (%)	46 (46.0%)	31 (44.3%)	15 (50.0%)	0.599
Antepartum $MgSO_4$ (%)	78 (78.0%)	52 (74.3%)	26 (86.7%)	0.171
Antepartum CCS (%)	85 (85.0%)	64 (91.4%)	26 (86.7%)	0.467
Neonatal sex, boys (%)	52 (52%)	36 (51.4%)	16 (53.3%)	0.861

Baseline characteristics for all babies born after preeclampsia and/or fetal growth restriction before 37 completed weeks of gestation in whom spiral artery remodeling score was available and who were admitted to level III NICU or level II MC. Values are presented as means (standard deviations) or as numbers (%). *P*-values were calculated by the Fisher's exact test for continuous variables and χ2 test for categorical variables. *Obesity was defined as an body mass index >30kg/m2. *Abbreviations*: FGR, Fetal Growth Restriction; GDM, gestational diabetes mellitus; iv, intravenous; MgSO4, magnesium sulphate; CCS, (antepartum) corticosteroid treatment.

TABLE 2. Obstetric and neonatal outcome in association to spiral artery remodeling.

	Impaired remodeling *n*= 70	**Normal remodeling** *n*= 30	***p*-value**	**Adjusted *p*-value**[§]
Obstetric outcome				
GA (days)	212 (17)	223 (19)	0.004	n/a
BW (grams)	1078 (352)	1393 (424)	<0.001	0.022
BW percentile <p3 (%)	52 (74.3%)	14 (48.3%)	0.012	0.008
BW Z-score	-2.52	-2.08	0.081	0.035
Head circumference at birth (cm)	26.0 (2.3)	28.5 (3.0)	<0.001	0.004
Admission level III NICU (%)	62 (88.6%)	19 (63.3%)	0.003	0.192
Apgar score <7 at 5 min (%)	9 (12.9%)	3 (10.0%)	0.687	0.870
Adverse neonatal outcome*				
Neonatal mortality (%)[†]	3 (4.3%)	0 (0.0%)	0.250	0.712
RDS (%)	37 (52.9%)	10 (33.3%)	0.073	0.917
Surfactant (%)	29 (41.4%)	9 (30.0%)	0.281	0.446
Mechanically assisted ventilation (%)	30 (42.9%)	9 (30.0%)	0.227	0.363
Inotropic support (%)	14 (20.0%)	1 (3.3%)	0.032	0.271
PDA with treatment (%)	16 (22.9%)	1 (3.3%)	0.017	0.154
IVH ≥ grade II[24] (%)	6 (8.6%)	0 (0.0%)	0.098	0.287
Convulsions (%)	1 (1.5%)	0 (0.0%)	0.512	0.648
Hyperbilirubinemia (%)	58 (82.9%)	19 (63.3%)	0.034	0.727
Hypoglycemia (%)	17 (24.3%)	4 (13.3%)	0.218	0.176
Blood transfusion (%)	32 (45.7%)	7 (23.3%)	0.035	0.632
NEC, Bell stage ≥ II[25] (%)	2 (2.9%)	0 (0.0%)	0.350	0.476
Post-partum CCS (%)	9 (12.9%)	2 (6.7%)	0.365	0.785
Culture proven sepsis (%)	13 (18.6%)	4 (13.3%)	0.523	0.866
Additional neonatal outcome				
Total admission (days)	28.7 (28.6)	23.7 (27.3)	0.412	0.367
Parenteral feeding (%)	67 (95.7%)	23 (76.7%)	0.004	0.118
Hematological measurements[◊]				
Hemoglobin <8 mmol/L (%)	9 (12.9%)	1 (3.3%)	0.146	0.565
Thrombocyte count <150x10^9/L (%)	34 (49.3%)	11 (36.7%)	0.247	0.680
NRBC >2x10^9/L (%)	38 (57.6%)	15 (50.0%)	0.489	0.909

Obstetric and neonatal outcome in association to the extent of spiral artery remodeling. Values are presented as means (standard deviations) or as numbers (%). *P*-values were calculated by the Fisher's exact test for continuous variables and $\chi 2$ test for categorical variables. §Adjusted for gestational age by linear regression. *Within the first 60 days of life. †In hospital mortality. ‡Sum score with point awarded for: mortality (2 pts), RDS (1 pt), IVH (1 pt), PDA (1 pt), IVH (1 pt), sepsis (1 pt), NEC (1 pt), inotropic support (1 pt), convulsions (1 pt), hyperbilirubinemia (1 pt), blood transfusion (1 pt), hypoglycemia (1 pt), CCS (1 pt). ◊ within 48 hours after birth. *Abbreviations*: GA, gestational age (days); BW, birthweight; NICU, level III neonatal intensive care unit; RDS, respiratory distress syndrome; PDA, patent ductus arteriosus; IVH, Intraventricular hemorrhage; NEC, necrotizing enterocolitis; CCS, corticosteroid treatment post-partum, NRBC, nucleated red blood cell count.

Interestingly, the neonates born following impaired remodeling had a BW below the third percentile more often (p=0.012), were more often admitted to the level III NICU (p=0.003), received more often parenteral nutrition (p=0.004) and were treated significantly more frequently with inotropic medication (p=0.032), blood transfusions (p=0.035), phototherapy (p=0.034) and needed treatment for PDA more often than neonates in the normal remodeling group (p=0.017). We found a trend toward more RDS (52.9 vs 33.3%, p=0.073) and IVH (9.8 vs 0.0%, p=0.098) in the impaired remodeling group, but were not statistically significant, likely due to low numbers.

We found higher adverse neonatal outcome scores (2, IQR 1-4 vs 1, IQR 1-3, p=0.001, Figure 1A) and higher CRIB II scores (7, IQR 5-9 vs 4, IQR 2-7, p=0.003, Figure 1B) in the impaired remodeling group, SNAPPE-II score did not vary significantly amongst the groups (Figure 1C). When we performed analysis with the neonatal outcome-, SNAPPE-II- and CRIB II scores with the original spiral artery remodeling score (i.e. none, minimal, moderate, complete) we found a trend for worse neonatal outcome when remodeling was more impaired (Supplementary Figure 2).

Next, we adjusted the above mentioned comparison of neonatal outcome by spiral artery remodeling for the GA at which the babies were born. BW, BW percentile, Z-score and head circumference at birth remained significantly lower in pregnancies with impaired remodeling. When stratifying data for neonates born before and after 32 weeks (n=57 vs 16), GA was no longer different between the groups and we confirmed significant differences for BW, BW Z-score, head circumference at birth and CRIB II score. Again, only trends were seen for neonatal outcome scores, PDA, inotropic support and hypoglycemia. Stratified data can be found in Supplementary Table 1.

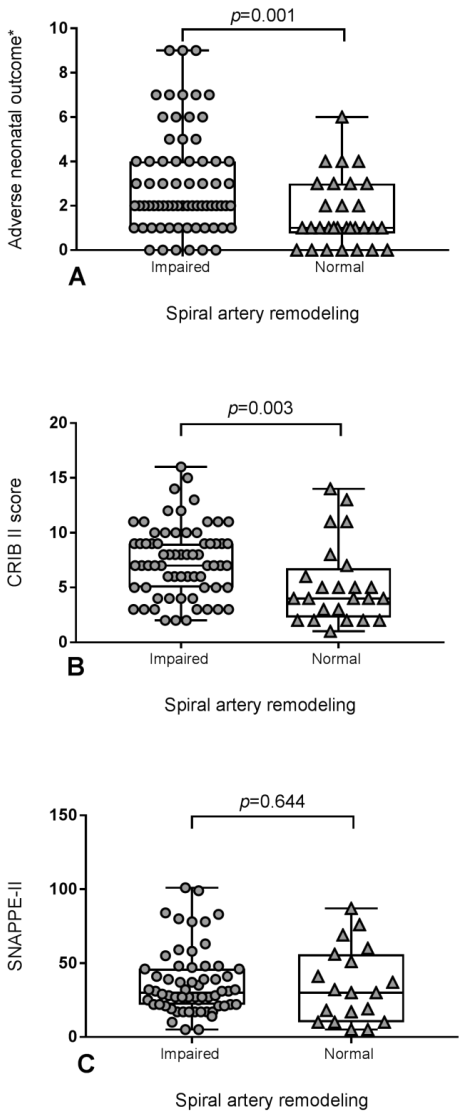

FIGURE 1. Spiral artery remodeling in association to adverse neonatal outcome score (A), CRIB II (B), and SNAPPE-II (C) scores in neonates born before 37 weeks of gestation. Boxes indicate medians with interquartile ranges and lines depict the full range, *p*-values are calculate by Mann-Whitney U test.

DISCUSSION

In this study we related adverse neonatal outcome from neonates born prematurely after preeclampsia and/or FGR to the extent of physiologic spiral artery remodeling in the maternal placental bed. We found that when spiral artery remodeling was impaired, neonates were delivered almost two weeks earlier and had lower (relative) BW compared to when remodeling was normal and more often experienced adverse neonatal events. After correction for gestational age the association of impaired spiral artery remodeling with lower GA and lower BW, BW percentile remained a statistically robust finding but lost power to find an association with other neonatal adverse outcome.

Preeclampsia and FGR are common pregnancy complications associated with poor neonatal outcome.[1,3] With the lack of effective prevention and treatment options, the health of both the mother and the fetus must be balanced when deciding on timing of delivery, resulting in many babies being born prematurely in these pregnancies. We hypothesized that babies who are born prematurely following ineffectively remodeled spiral arteries are more deprived in-utero and may therefore be more susceptible to adverse neonatal outcome. We found that complicated pregnancies following impaired remodeling were ended almost two complete weeks earlier than pregnancies who experienced preeclampsia and/or FGR without remodeling problems, even though a fetal indication for Caesarean section was evenly distributed amongst the groups (38.6 vs 36.7%, p=0.410). Babies had both lower BW and BW Z-scores when born after inadequate spiral artery remodeling. As expected more neonates showed complications and adverse outcome in this group of younger and smaller neonates. This association was however likely due to the lower GA at which these babies were born, as shown in our adjusted and stratified data. Unfortunately our study was not powered enough to use only GA stratified data for the analyses. We can therefore not conclusively say whether insufficient remodeling has a direct effect on neonatal outcome (i.e. through chronic hypoxia in utero), whether it is solely based on reduction of the GA at birth (i.e. prematurity) or whether it might be the combination of both. We speculate that if numbers had been higher we might have seen statistical significant associations with neonatal outcome based on the trends we have observed when correcting for GA.

It is apparent that preeclampsia and FGR pose severe risks for babies to be born preterm and with low birth weight. The long term health of these children should be followed-up on to find out whether we can find associations between pathophysiological mechanisms and the ability to develop normal motor, neurological and cognitive function as a child.[4,26,27] Similarly, it is suspected that a hostile in-utero environment may 'program' mechanisms in the fetus, possibly through poor placental function or perhaps through transport of detrimental factors across it, and may have a bad impact on health and disease in later life.[1,28] Next to fetal programming, it is believed that bigger post-natal catch up and perhaps even the carry-over of genetic predisposition play a role in the ultimately worse outcome for these babies.[29,30] Follow up should not only focus on making these distinct associations apparent, but also to

identify whether these babies benefit from early intervention or screening in regard to their increased risk for cardiovascular disease and associated problems. Perhaps the unhealthy environment in utero is doing more harm than a consequently premature birth would do.

Ultimately, we found that when the mother's vascular system 'failed' to make the necessary pregnancy-specific adaptations during pregnancy, there is a big impact on obstetric and neonatal outcome and perhaps ultimately on the lifelong health of her child. As some risk factors known for preeclampsia and FGR are modifiable (i.e. obesity and pre-existent hypertension), a case can be made for the importance of a healthy lifestyle and physique before getting pregnant. For the women in whom these modifiable risk factors are not (noticeably) present, prevention and early treatment is a greater challenge. Comparison of these results with other studies is difficult, as to our knowledge, this study is the first to investigate the relationship between defective spiral artery remodeling and neonatal outcome.

Strengths and limitations

Strengths include the prospective nature allowing for the collection of extensive and detailed neonatal outcome data and the intention for long-term follow up of both mother and child. To the best of our knowledge, the SPAR study entails the largest systematically collected series of placental bed biopsies with extensive remodeling scoring, to date, and is the first study comparing remodeling problems to (adverse) neonatal outcome.

Limitations include the fact that remodeling of spiral arteries is not an all-or-none phenomenon. Remodeling varies between spiral arteries within a patient and a tissue biopsy may not represent the status of remodeling elsewhere in the uterus; possibly leading to a form of sampling bias. Likewise, roughly 50-60% of biopsy samples are taken accurately from the placental bed.[31] By including a large enough cohort, we were able to counter these effect. Continued research with larger study groups is needed to identify possible causal role between spiral artery pathology and adverse neonatal outcome. It may then also be possible to use remodeling as a continuous factor instead of the dichotomized score we used for the current study. A longer follow up is needed to determine to what effect unhealthy spiral artery development may impact long term development of children born after these pregnancies. Also, new research may focus on comparison between a group of babies born prematurely for reasons other than preeclampsia and FGR to elucidate the effect of GA in the causal chain. This comparison will most likely be challenging as previous literature has however shown abnormal remodeling to be present in a high amount of women with spontaneous premature delivery.[32-34] Nonetheless, an evaluation in the light of neonatal outcome has not yet been performed and may be of importance for perinatal and neonatal care.

Conclusion

In pregnancies complicated by preeclampsia and FGR delivery is earlier and birthweight (percentile) smaller when maternal spiral artery remodeling is inadequate or absent. The increase in neonatal morbidity is most likely caused by a decrease in gestational age. Future research is needed to test whether these babies have a higher long-term risk for impaired development and health, and whether these are to be attributed to a shorter life in utero or partially a consequence of a hostile in utero environment. These insights may help to improve perinatal care and the outcomes of babies born after preeclampsia and fetal growth problems.

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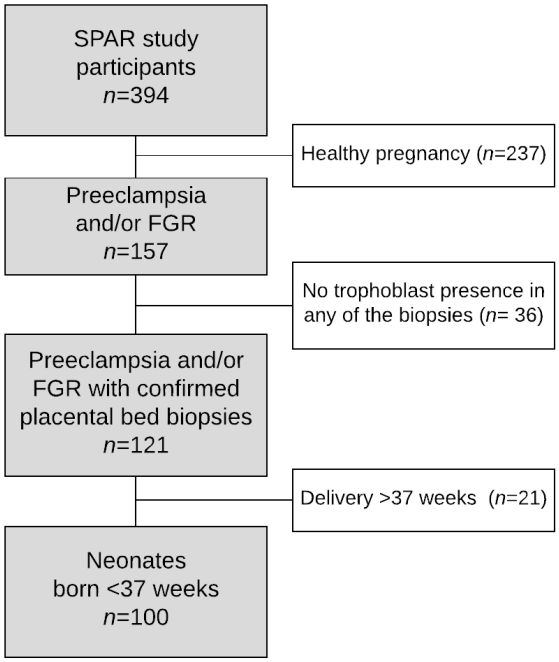
SUPPLEMENTARY MATERIALS

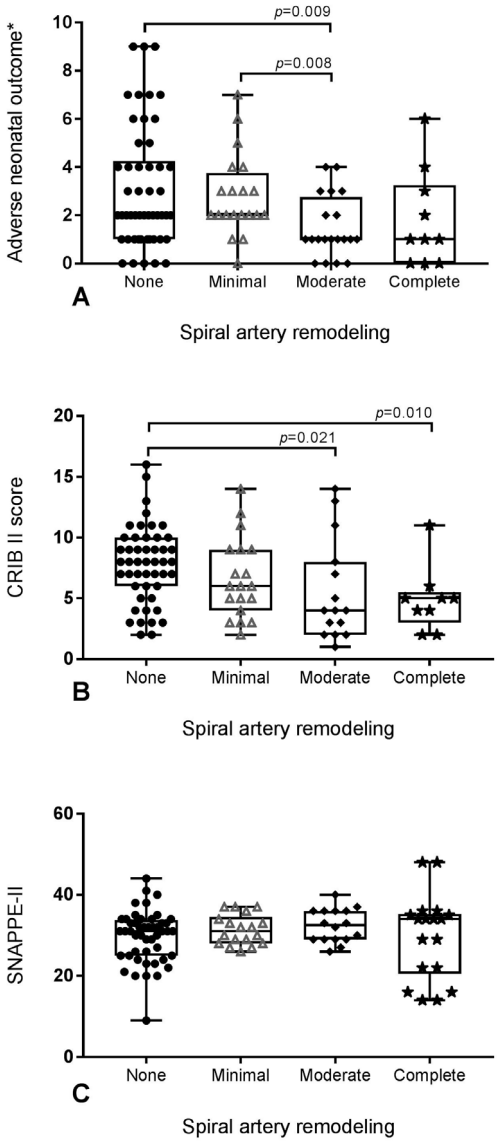
SUPPLEMENTARY TABLE 1. Obstetric and neonatal outcome in association to spiral artery remodeling for neonates born before 32 weeks of gestation.

	Impaired remodeling *n*= 57	**Normal remodeling** *n*=16	***p*-value**
Obstetric outcome			
GA (days)	207 (11)	209 (12)	0.279
BW (grams)	983 (284)	1176 (304)	0.020
BW percentile <p3 (%)	40 (70.2%)	8 (50.0%)	0.133
BW Z-score (%)	-2.4 (1.2)	-1.8 (0.9)	0.041
Head circumference at birth (cm)	25.4 (2.1)	27.5 (3.3)	0.004
Admission NICU (%)	57 (100.0%)	16 (100.0%)	n/a
Apgar score <7 at 5 min (%)	9 (15.8%)	2 (12.5%)	0.745
Adverse neonatal outcome*#			
CRIB II score#	8 (6-10)	5 (4-10)	0.048
SNAPPE-II score#	32 (22-48)	34 (17-59)	0.896
Neonatal outcome score‡	3 (2-5)	3 (1-4)	0.174
Neonatal mortality (%)†	3 (5.3%)	0 (0.0%)	0.349
RDS (%)	36 (63.2%)	10 (62.5%)	0.962
Surfactant (%)	29 (50.9%)	9 (56.3%)	0.704
Mechanical assistance ventilation (%)	30 (52.6%)	9 (56.3%)	0.798
Inotropic support (%)	14 (24.6%)	1 (6.3%)	0.109
PDA with treatment (%)	15 (26.3%)	1 (6.3%)	0.086
IVH ≥ grade II[25] (%)	6 (10.5%)	0 (0.0%)	0.176
Convulsions (%)	1 (1.8%)	0 (0.0%)	0.587
Hyperbilirubinemia (%)	54 (94.7%)	15 (93.8%)	0.878
hypoglycemia (%)	14 (24.6%)	1(6.3%)	0.109
Blood transfusion (%)	30 (52.6%)	7 (43.8%)	0.530
NEC, Bell stage ≥ II[26] (%)	2 (3.5%)	0 (0.0%)	0.447
Post-partum CCS (%)	9 (15.8%)	2 (12.5%)	0.745
Culture proven sepsis (%)	13 (22.8%)	4 (25.0%)	0.854
Additional neonatal outcome			
Total admission (days)	32.3 (30.4)	31.5 (34.4)	0.928
Parenteral feeding (%)	57 (100.0%)	16 (100.0%)	n/a
Hematological measurements◊			
Hemoglobin <8 mmol/L (%)	9 (15.8%)	1 (6.3%)	0.327
Thrombocyte count <150x10^9/L (%)	29 (50.9%)	5 (31.3%)	0.164
NRBC >2x10^9/L (%)	32 (57.1%)	8 (50.0%)	0.612

Obstetric and neonatal outcome in association to the extent of spiral artery remodeling in infants born before 32 completed weeks of gestation. Values are presented as means (standard deviations) or as numbers (%). *P*-values were calculated by the Fisher's exact test for continuous variables and $\chi 2$ test for categorical variables. #Values represented as medians with interquartile ranges and *p*-values calculated by Mann-Whitney U test. *Within the first 60 days of life. †In hospital mortality. ‡Sum score with point awarded for: mortality (2 pts), RDS (1 pt), IVH (1 pt), PDA (1 pt), IVH (1 pt), sepsis (1 pt), NEC (1 pt), inotropic support (1 pt), convulsions (1 pt), hyperbilirubinemia (1 pt), blood transfusion (1 pt), hypoglycemia (1 pt), CCS (1 pt). ◊ within 48 hrs after birth. *Abbreviations*: GA, gestational age; BW, birthweight; NICU, level III neonatal intensive care unit; CRIB II, Clinical Risk Index for Babies II; SNAPPE-II, Score for Neonatal Acute Physiology Perinatal Extension II; RDS, respiratory distress syndrome; PDA, patent ductus arteriosus; IVH, Intraventricular hemorrhage; NEC, necrotizing enterocolitis; CCS, corticosteroids, NRBC, nucleated red blood cell count.



SUPPLEMENTARY FIGURE 1. Inclusion strategy of neonates in this analysis from the Spiral artery remodeling (SPAR) study. *Abbreviations:* SPAR, Spiral artery remodeling; PEFGR, preeclampsia and/or fetal growth restriction; GA, gestational age.



SUPPLEMENTARY FIGURE 2. Original spiral artery remodeling degree (none, minimal, moderate and complete) in association to adverse neonatal outcome score (A), CRIB II (B), and SNAPPE-II (C) scores in neonates born before 37 weeks of gestation. This needs to be further investigated. Boxes indicate medians with interquartile ranges and lines depict the full range, *p*-values are calculate by Mann-Whitney U tests.

5

Human placental bed endothelial cell transcriptomics and systemic biomarker profiling reveals inflammatory endothelial activation in early onset preeclampsia

In preparation

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ABSTRACT

Background

Preeclampsia is a multi-system disorder of pregnancy clinically defined as de novo hypertension and proteinuria, with generalized endothelial dysfunction as a key mechanism leading to these symptoms. Impaired spiral artery remodeling in the tissue underlying the placenta (i.e. the placental bed) is thought to precede systemic symptoms. It is unknown to what extent changes to maternal vascular endothelial cells (ECs) play a role in this complex remodeling process and whether the placental bed EC phenotype is different in pregnancies complicated by preeclampsia and fetal growth restriction (FGR) compared to healthy pregnancies.

Objectives

To determine (1) the transcriptional profile of local placental bed ECs from women with early onset preeclampsia and FGR, compared with healthy pregnancy and (2) a systemic biomarker profile of inflammation, endothelial cell dysfunction and activation.

Methods

Biopsy samples were obtained from the placental bed during primary Caesarean section from five women with early onset preeclampsia with FGR and four healthy controls. The tissue was enzymatically digested and CD31+CD146+ ECs were isolated through flow cytometry-assisted cell sorting. RNA was subsequently isolated for sequencing by CEL-Seq2 protocol. RNA expression patterns were analyzed by unsupervised clustering methods including principal component analysis and hierarchical clustering, and subjected to differential gene expression analysis and pathway analysis. For biomarker profiling, 67 analytes were measured in sera from 20 women with early onset preeclampsia and FGR and 20 women with healthy pregnancy by multiplex immunoassay.

Results

Transcriptional profiling of maternal placental bed ECs showed overlapping gene expression signatures for most patients with preeclampsia compared with healthy pregnant controls, with the exception of a small number of differentially expressed genes. Pathway analysis of upregulated genes in preeclampsia identified 6 significantly enriched pathways related to vasoconstriction, activation of platelets and the innate immune system. Individual upregulated genes in preeclampsia included prostaglandin D2 synthase, olfactomedin 1 and interleukin-3 receptor subunit alpha, while serine peptidase inhibitor kazal type 5 and sestrin 3 were significantly downregulated. Unbiased analysis of endothelium-related serum biomarkers identified separate profiles for healthy pregnancy and preeclampsia. Analytes most contributing to this separation were identified as sFLT-1, endoglin, PlGF, leptin, SAA-1 and sICAM-1. In addition, unsupervised biomarker profiling within women with preeclampsia was able to separate women with and without the complication of hemolysis-, elevated liver enzymes, low platelets (HELLP)-syndrome.

Conclusion

To our knowledge, this is the first study using transcriptomics to investigate human ECs from the placental bed in pregnancy complicated by severe preeclampsia. Both transcriptional profiling of placental bed ECs and systemic profiling of serum biomarkers revealed involvement of innate immune activation, endothelial activation and endothelial dysfunction in preeclampsia compared to controls. These data suggest that preeclampsia is associated with altered local interactions between the maternal immune system and placental bed ECs, as a part of the process of spiral artery remodeling necessary for normal pregnancy outcome.

INTRODUCTION

Preeclampsia is a multi-system syndrome defined by new onset hypertension and proteinuria. Both symptoms implicate generalized endothelial dysfunction as a key pathophysiologic feature.[1] Severe, or early onset, preeclampsia occurs in 1-2% of first pregnancies and often concurs with fetal growth restriction (FGR).[2,3] Besides risk of severe consequences for the fetus, the mother is at increased risk of complications such as pulmonary edema, eclamptic seizures and hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome. Although the pathophysiology of these closely linked pregnancy complications is yet to be fully elucidated and heterogeneity in the disease is wide, several stages can be identified.

Impaired physiological transformation of existent vasculature in uterine tissue underlying the placenta (i.e. the placental bed), known as spiral artery remodeling, is thought to be the essential first stage which precedes the symptomatic phase in many cases.[4] Spiral artery remodeling is necessary for adequate blood flow to the developing placenta and fetus, and is histologically characterized by reorganization of vascular wall components such as ECs, smooth muscle cells and the elastic lamina and new formation of a fibrinous layer within the vessel wall. It has been suggested that intramural invasion and EC replacement in response to migration of extravillous trophoblasts into the placental bed is a key mechanism for successful remodeling of spiral arteries.[5] In preeclampsia and fetal growth restriction, this remodeling response is often impaired or absent, leading to altered blood flow to the placenta. Next to defective remodeling, signs of loss of EC function are often seen in the placental bed, i.e. infiltration of the intimal and medial layer of the spiral arteries with foam cells and lipid deposits, termed 'acute atherosis', and thrombosis.[6-9] Eventually these placental bed characteristics are associated with presentation of a variable sequence of key symptoms. Maternal endothelial dysfunction and subsequent hypertension are thought to be the result of release of several inflammatory and antiangiogenic factors into the maternal circulation.[10]

The systemic inflammatory response has been well documented in studies consistently showing elevation of C-reactive protein (CRP), interleukin (IL)-6 and IL-8.[11] Pro-inflammatory endothelial activation is suggested by elevated plasma levels of the soluble forms of endothelial and vascular cell activation molecules (i.e. E-selection and sVCAM-1).[12] High levels of the anti-angiogenic factors sFLT-1 and endoglin and low levels of pro-angiogenic PlGF have been reported in preeclampsia and are believed to be indicators of an anti-angiogenic environment.[13,14] Additionally, the complex homeostatic balance of vascular tone, which is normally maintained by the endothelium, is disturbed, leading to dysregulated and elevated blood pressure.[15,16] Similarly, ECs exposed to serum from preeclamptic women show signs of dysfunction in vitro, indicating the importance for identifying circulating factors causing systemic endothelial dysfunction.[17,18] Furthermore, preeclampsia has been associated with future cardiovascular disease, implying that a vulnerable maternal vascular constitution may be responsible for vascular pathology at both time points.[9,19,20]

It is unknown to what extent the vascular endothelial cells (ECs) play a role in this complex process and whether EC phenotype is altered in preeclampsia. Here, we hypothesize that endothelial dysfunction within the blood vessels of the placental bed, in particular spiral arteries, may play a role in the ineffective vascular remodeling response associated with poor placentation. In this study, we investigate the transcriptional profile of ECs isolated from placental bed biopsies (representing spiral artery ECs) to identify functional changes and specific pathways at play in severe preeclampsia (with FGR). Furthermore, we investigate systemic biomarker profiles by multiplex immunoassay in order to asses if inflammation and endothelial activation or dysfunction are involved in the pathogenesis these complications in pregnancy.

METHODS

Patient selection and definitions

This study is part of the Spiral Artery Remodeling (SPAR) cohort study. SPAR is a prospective multicenter study investigating spiral artery remodeling and pathology in women with and without preeclampsia and/or FGR. Detailed description of the study set-up and protocol was previously published.[9] In short, women who had a clinical indication for a Caesarean section due to preeclampsia and/or FGR were recruited as the case group of the study. Women who delivered by primary elective Caesarean section after an uneventful pregnancy and without any major underlying pathology were defined as controls. We defined preeclampsia, according to the latest Society for the Study of Hypertension in Pregnancy guideline, as new-onset hypertension (≥140/90mmHg) after 20 weeks of gestation in combination with significant proteinuria (≥300mg/24h or protein/creatinine ratio ≥0.3 mg/mmol), maternal organ dysfunction (i.e. renal insufficiency, liver involvement, neurological or hematological complications) or FGR.[21] HELLP syndrome was defined according to the following criteria: hemolysis (defined as serum lactate dehydrogenase (LDH) >600 U/L and/or haptoglobin 0.3 g/L), elevated liver enzymes (serum aspartate aminotransferase (AST) >50 U/L and/or serum alanine aminotransferase (ALT) >50 U/L), and a low platelet count (<100x10^9/L).[22] FGR was defined according to the Dutch national guidelines, as an ultrasonographically estimated fetal weight or abdominal circumference below the tenth percentile or a reduction in the standardized growth curves of ≥20 percentiles.[23] Placental insufficiency had to be the suspected cause of preeclampsia and FGR and cases with known chromosomal and/or congenital abnormalities were excluded. Other definitions can be found in previous publication.[9]

For this study we included women with early-onset (i.e. delivery before 34 completed weeks of gestation) preeclampsia concurring with FGR. For EC transcriptomics we included only primiparous women, of which five with severe preeclampsia and FGR and four women with healthy pregnancies for comparison. For multiplex immunoassay, 20 patients with severe preeclampsia and FGR with histologically confirmed spiral artery pathology were included and 20 healthy women with uneventful pregnancies with normal spiral artery remodeling served as controls. All patients had primary elective Caesarean indications, i.e. no contractions or rupture of membranes. All patients received study information and signed informed consent prior to participation. This study was reviewed and approved by the local Institutional Ethical Review Board of the University Medical Center Utrecht, protocol reference number: 16-198.

Placental bed biopsies

After delivery of the neonate and placenta the placental bed was manually located and two biopsies of the central placental bed from the inner uterine myometrial wall were obtained as previously described.[9] Additionally, biopsies were taken from the incision site when the placenta was not situated on this part of the uterine wall.

Isolation of placental bed ECs

The biopsy samples were collected in medium consisting of RPMI 1640 (Gibco) supplemented with Penicillin/Streptomycin (Gibco), L-glutamine (Gibco) and 10% fetal calf serum (FCS, Biowest) and minced into pieces of 1 mm^3 in PBS (Gibco). The biopsies were enzymatically digested with 1 mg/mL collagenase IV (Sigma) in medium for 60 minutes at 37^{0}C in a tube shaker under constant agitation at 120 rpm. To dissolve the remaining biopsy pieces after digestion and remove any remaining lumps, the biopsies were pipetted up and down multiple times and poured over a 100 µm Cell Strainer (BD Falcon). Cells were subsequently washed in staining buffer consisting of cold PBS supplemented with 2% FCS and 0.1% sodium-azide (Severn Biotech Ltd.) and filtered through a 70 µm cell strainer. For FACS sorting, the cells were incubated with surface antibodies against CD45, CD31 (PECAM-1), CD146 (MCAM), CD54 (ICAM-1), CD144 (VE-cadherin), CD105 (Endoglin), and CD309 (VEGFR2) (Supplementary Table 1) for 20 minutes in staining buffer at 4^{0}C, washed in the same buffer and filtered through a 50 µm cell strainer (Filcon, BD). 2000 cells of the CD45-CD31+CD146+ cell population were sorted into eppendorfs containing 125 µL PBS on one of the two available FACSAria™ II or III machines (BD). After sorting, 375 µL Trizol LS (Thermo Fisher Scientific) was added to each vial and vials were stored at -80^{0}C until RNA isolation.

RNA isolation

For RNA isolation, the vials were thawed a room temperature and 100 µL chloroform was added to each vial. The vials were shaken well and spun down at 12000xg for 15 minutes at 4^{0}C. The aqueous phase was transferred into a new tube and RNA was mixed with 1ul of GlycoBlue (Invitrogen) and precipitated with 250 µL isopropanolol. Cells were incubated at -20^{0}C for one hour and subsequently spun down at 12000xg for 10 minutes. The supernatant was carefully discarded and the RNA pellet was washed twice with 375 µL 75% ethanol. Vials were stored at -80^{0}C until library preparation.

Whole transcriptome sequencing and data analysis

Low input RNA sequencing libraries from biological sorted cell population replicates were prepared using the Cel-Seq2 Sample Preparation Protocol[24] and sequenced as 2 x 75bp paired-end on a NextSeq 500 (Utrecht Sequencing Facility). The reads were demultiplexed and aligned to human cDNA reference using the BWA (0.7.13).[25] Multiple reads mapping to the same gene with the same unique molecular identifier (UMI, 6bp long) were counted as a single read. RNA sequencing data were normalized per million reads and differentially expressed genes were identified using the DESeq2 package in R 3.4.3 (CRAN). Genes with *p*adj<0.05 were considered differentially expressed. For principal component analysis (PCA), the 1000 most variable genes were used and data were mean-centered per gene. For pathway analysis with Toppgene Suite, genes with nominal *p*-value <0.05 were used.[26]

Multiplex immunoassay

Blood was collected in serum tubes within 4 hours before Caesarean section and spun down at 4000xg at 4°C. Serum was stored at -80°C until analysis. The multiplex immunoassay for 67 analytes was performed as described previously, measuring all analytes simultaneously in 50 µL of serum (xMAP; Luminex).[27] Heterophilic immunoglobulins were pre-absorbed from all samples with HeteroBlock (Omega Biologicals). Acquisition was performed with a Bio-Rad FlexMAP3D in combination with xPONENT software version 4.2 (Luminex). Data analysis was performed with Bioplex Manager 6.1.1 (Bio-Rad).

Data analysis multiplex immunoassay

Multiplex data were analyzed using GraphPad Prism 7.0, SPSS Statistics 24 (IBM) and R 3.4.3 (CRAN). Out of ranges values on the lower end were imputed as 0.5x lowest measured value; out of ranges values on the upper end were imputed as 2x highest measured value. Analytes with more than 35% of measured values below the lower or above the upper limit of detection were excluded from the analyses (Granzyme B, Galectin-7, TRANCE-sRANKL, MIF, TNFa, IL-4, IL-1RA). For comparisons between two groups, the Mann-Whitney U test was used, with correction for multiple testing of all 60 analytes by Bonferroni or FDR as indicated where applicable. For principal component analysis (PCA) and heatmap analysis, data were mean-centered per analyte. Unsupervised hierarchical clustering was performed by Ward's method with Euclidian distance. Random forest analysis was performed via http://www.metaboanalyst.ca/ with standard settings. Correlations were assessed by spearman rank correlation. Adjusted p-values <0.05 were considered significant.

RESULTS

Baseline characteristics

Maternal and pregnancy baseline characteristics for both the EC transcriptomics and the multiplex immunoassay are presented in Supplementary Table 2 and 3. Two patients with preeclampsia were excluded from the biomarker analyses due to cross-reactivity with the multiplex beads, which may yield false-positive results. One additional preeclampsia case was excluded when histopathology showed normal spiral artery remodeling. Baseline characteristics for both analyses were very similar, in short; women with preeclampsia were less often of white European descent and were more often obese. Preeclampsia was early in onset (i.e. before 34 weeks of gestation) with FGR in all cases, and women often received antihypertensive medication, magnesium sulphate or antepartum corticosteroids. As expected, gestational age and birthweight were lower in the women with preeclampsia. For EC transcriptomics only nulliparous women were included; within the biomarker profiling group, controls were less often nulliparous compared to women with preeclampsia. Additionally, in the latter almost half of patients presented with HELLP syndrome. Eight patients were included in both the biomarker and the transcriptional profiling experiments.

Placental bed endothelial cell transcriptomics

In short, biopsy samples were obtained from the placental bed during primary Caesarean section from five women with preeclampsia and four healthy controls. The tissue was enzymatically digested and CD31+CD146+ ECs were isolated through flow cytometry assisted cell sorting. RNA was subsequently isolated for sequencing by Cel-Seq2 protocol.

High expression of key endothelial genes such as Van Willebrand Factor, PECAM1 (CD31), MCAM (CD146) endoglin, claudin-5, CCL14, Tie1, CD34, and CTGF in addition to expression of 65 out of 72 endothelial-specific genes identified by Chi et al. confirmed endothelial cell identity (Supplementary Figure 1A).[28] In addition, the maternal origin of EC was confirmed by high expression of the female-specific XIST gene and absent expression of the male-specific SRY gene in all samples. Although largely overlapping gene expression signatures were found between patients with preeclampsia and healthy pregnancies, we identified a small set of differentially expressed genes (Figure 1A). Differential gene expression analysis produced three significantly up- and two significantly downregulated genes in preeclampsia compared to healthy pregnancy ($p$adj<0.05; Figure 1B). Upregulated genes were prostaglandin D2 synthase (PTGDS), olfactomedin 1 (OFLM1) and IL-3 receptor subunit alpha (IL3RA). Downregulated genes were serine peptidase inhibitor Kazal type 5 (SPINK5) and sestrin 3 (SESN3). Three additional up- and three downregulated genes were identified with $p$adj<0.10 (Figure 1B), including nestin. A heatmap comparing expression of the differentially expressed genes in preeclampsia and healthy pregnancy is shown in Figure 1C. In order to perform pathway analysis of upregulated genes in preeclampsia we lowered the significance threshold (to a nominal p-value <0.05) as even though these genes

are not significantly upregulated after correction, they are still enriched in preeclampsia and may have a role in pathogenesis. We identified 6 significantly enriched pathways (Figure 1D) related to innate immune activation and platelet activation. Comparison of EC transcriptional profiles at two sites within the uterus, i.e. the incision and the placental bed site, between preeclampsia and healthy pregnancy, also revealed partially overlapping gene signatures of up- and downregulated genes, including PTGDS and SPINK5 (Supplementary Figure 1B and 1C).

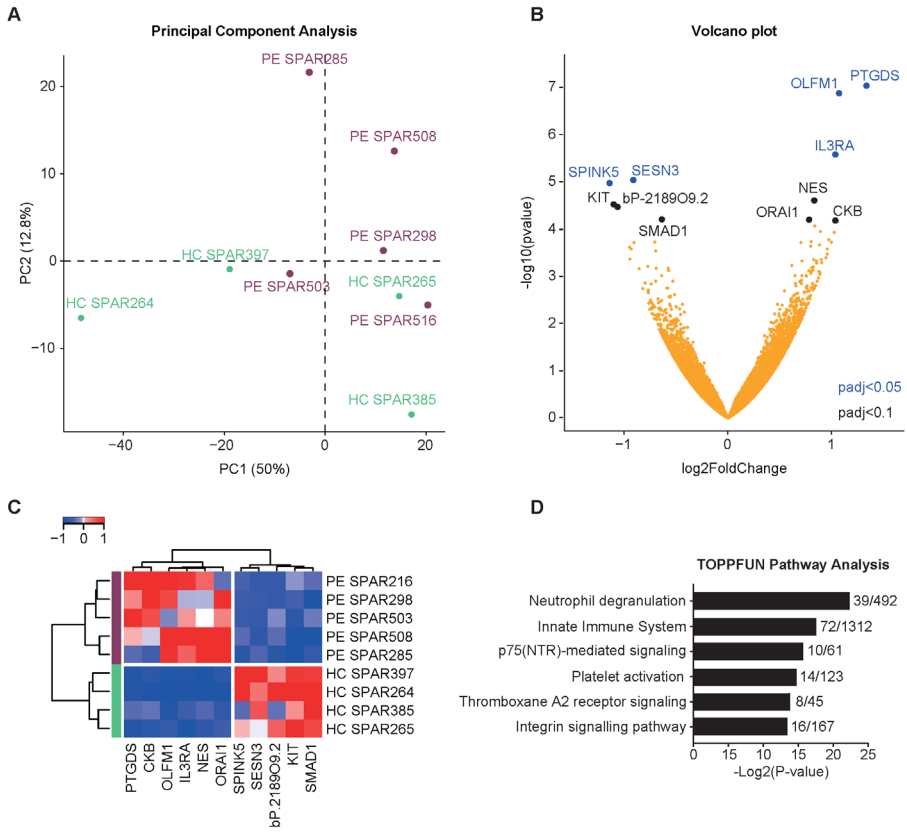

FIGURE 1. Transcriptomic profiling of spiral artery endothelial cells from the placental bed, comparing preeclampsia with FGR and healthy pregnancy. 2000 CD45-CD31+CD146+ endothelial cells were isolated from each biopsy by flow cytometry assisted cell sorting and RNA was sequenced by CEL-seq2 protocol. (**A**) Principal component analysis of preeclampsia cases and healthy controls using the 1000 genes with the highest variance (purple = preeclampsia, green = healthy controls). Genes were mean-centered. (**B**) Volcano plot showing differentially expressed genes with a *p*adj<0.05 (blue) and *p*adj<0.1 (black). (**C**) Heatmap of differentially expressed genes with a *p*adj<0.1. Genes were mean-centered and hierarchically clustered by Ward's method and Euclidian distance. (**D**) Pathway analysis in ToppGene Suite on the 617 upregulated genes in preeclampsia compared to healthy pregnancy with a nominal *p*- value<0.05. Numbers indicate the number of overlapping upregulated genes in endothelial cells from preeclampsia samples, compared to the total known genes in the indicated pathway. *Abbreviations*: PE, preeclampsia (in this study early onset, in combination with fetal growth restriction); HC, healthy pregnancy; SPINK5, serine peptidase inhibitor Kazal type 5; SESN3,sestrin 3, KIT, KIT proto-oncogene receptor tyrosine kinase; SMAD1, Mothers against decapentaplegic homolog 1; PTGDS, prostaglandin D2 synthase; OLFM1, olfactomedin 1; IL3RA, interleukin 3 receptor subunit alpha; NES, nestin; ORAI1, Calcium release-activated calcium channel protein 1; CKB, creatine kinase B.

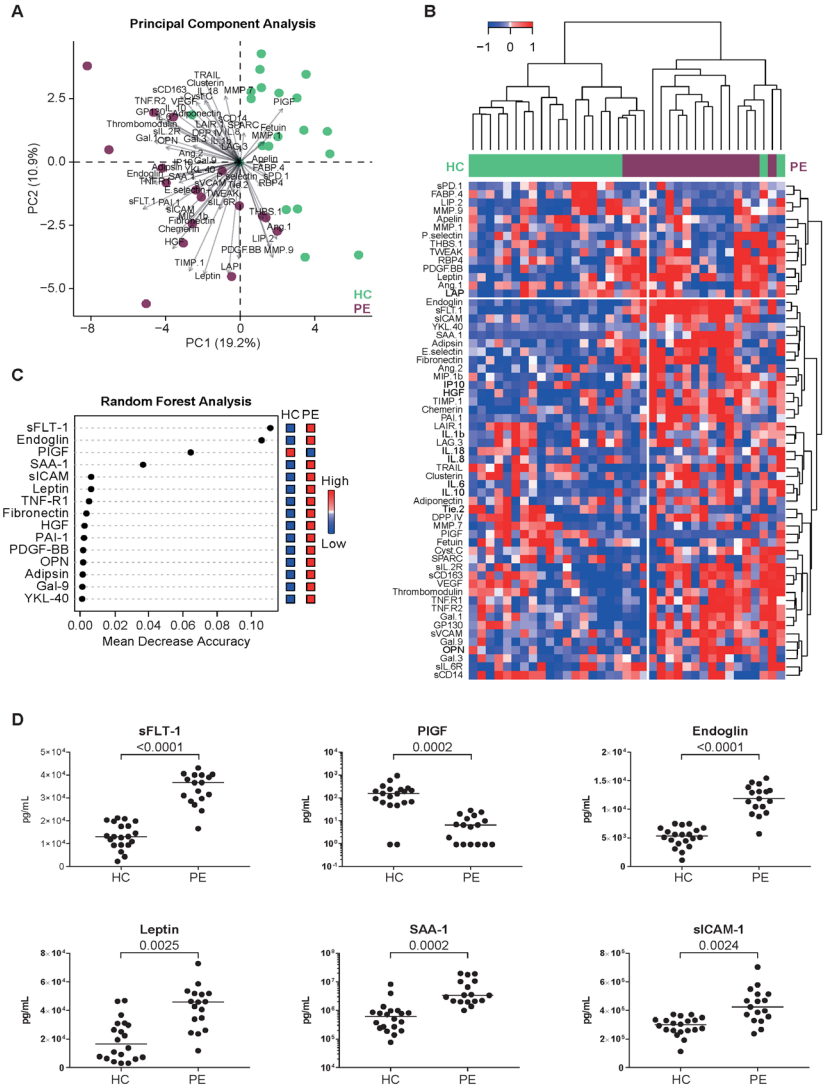

FIGURE 2. Systemic biomarker profiling of markers related to inflammation, endothelial activation and endothelial dysfunction, comparing preeclampsia with FGR and healthy pregnancy. Biomarkers were analyzed in serum by multiplex immunoassay. (**A**) Principal component analysis of preeclampsia cases and healthy controls using all 60 markers (purple = preeclampsia, green = healthy controls). Analytes were mean-centered. (**B**) Heatmap with hierarchical clustering of all 60 markers. Markers were mean-centered and patients were clustered by Ward's method with Euclidian distance. (**C**) Random Forest analysis with 1000 trees yielding an out-of-bag error of 0.00 and showing the analytes most important for separation of preeclampsia and healthy groups. Analytes were mean-centered. (**D**) Scatter dot plots of sFLT-1, PlGF, Endoglin, Leptin, SAA-1, and sICAM-1. Line represents median; FDR with correction for multiple testing of 60 analytes is indicated. Mann-Whitney U test. *Abbreviations*: PE, preeclampsia (in this study early onset, in combination with fetal growth restriction); HC, healthy pregnancy; Multiplex Immunoassay abbreviations may be found in the Supplementary Tables.

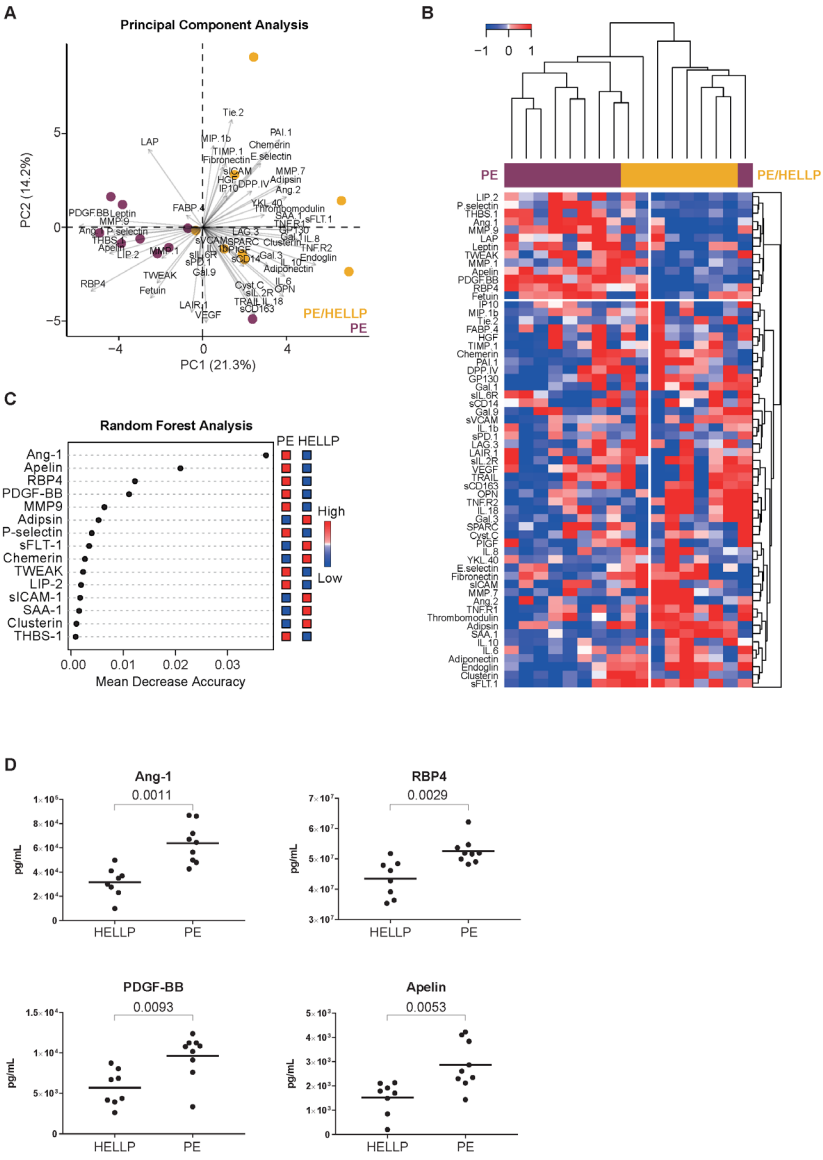

FIGURE 3. Systemic biomarker profiling of markers related to inflammation, endothelial activation and endothelial dysfunction within preeclampsia and a subgroup with HELLP syndrome. Biomarkers were analyzed in serum by multiplex immunoassay. (**A**) Principal component analysis of preeclampsia cases with and without HELLP using all 60 markers (purple = preeclampsia without HELLP, yellow = preeclampsia with HELLP). Analytes were mean-centered. (**B**) Heatmap with hierarchical clustering of all 60 markers. Markers were mean-centered and patients were clustered by Ward's method with Euclidian distance. (**C**) Random Forest analysis with 1000 trees yielding an out-of-bag error of 0.294 and showing the analytes most important for separation of preeclampsia and healthy groups. Analytes were mean-centered. (**D**) Scatter dot plots of Ang-1, RBP4, PDGF-BB, and apelin. Line represents median; nominal p value without correction for multiple testing is indicated. Mann-Whitney U test. *Abbreviations*: PE, preeclampsia (in this study early onset, in combination with fetal growth restriction, without HELLP); HELLP, hemolysis, elevated liver enzymes, low platelets syndrome; Multiplex Immunoassay abbreviations may be found in the Supplementary Tables.

Multiplex immunoassay

To determine systemic endothelial-related effects in preeclampsia we measured markers associated with inflammation, endothelial activation and endothelial dysfunction in a cohort of preeclampsia patients and healthy pregnancies. Baseline characteristics can be found in Supplementary Table 3. Systemic biomarker profiling yielded clear distinct biomarker signatures in preeclampsia and healthy pregnancy by PCA (Figure 2A). Unsupervised hierarchical clustering also separated preeclampsia from healthy pregnancy, with the exception of five individuals (Figure 2B). No clinical, histological or laboratory parameters could be identified to explain these exceptions. Analytes most contributing to separation of the two groups were identified as sFLT-1, endoglin and PlGF by random forest analysis, yielding an out-of-bag (OOB) error of 0.0 (Figure 2C). Accordingly, the sFLT-1/PlGF ratio was significantly increased in preeclampsia (Supplementary Figure 2). In addition to these already established markers in preeclampsia and the recently identified preeclampsia marker leptin, the acute phase reactant SAA-1 was highly discriminative, supporting the inflammatory phenotype in preeclampsia (Figure 2D). This was also confirmed by consistently increased levels of other pro-inflammatory proteins in preeclampsia patients (i.e. IL-6, TNF-R1, MIP-1β, Supplementary Table 4). Markers of endothelial activation sICAM-1 and E-selectin were also significantly higher in preeclampsia compared to healthy pregnancy, suggesting systemic endothelial activation (Figure 2D, Supplementary Table 4).

To further explore heterogeneity within preeclampsia, unbiased analysis within the case group was performed. PCA and hierarchical clustering separated the case group into two clusters (Figure 3A and B). Review of clinical and biochemical lab parameters revealed a diagnosis of HELLP syndrome in 6 out of 7 cases clustering separately, in addition to 2 other cases with only preeclampsia. Analytes most contributing to separation of preeclampsia with and without HELLP were identified as Ang-1, PDGF-BB, RPB4 and Apelin by random forest analysis with an OOB error of 0.294 (Figure 3C). All of these analytes were lower in patients with HELLP syndrome, and correlated with decreased thrombocyte counts (*data not shown*). sFLT-1, SAA-1, adipsin, chemerin, and clusterin were higher in women with preeclampsia complicated by HELLP syndrome. After correction for multiple testing, comparison of individual markers left no significant differences between the groups (Supplementary Table 5).

DISCUSSION

In this study we used state of the art techniques to isolate ECs from the placental bed for transcriptomic profiling, comparing pregnancies complicated by preeclampsia to healthy pregnancy. We identified 5 differentially expressed genes (PTGDS, OLFM1, IL3RA, SPINK5 and SESN3) and pathways related to innate immune activation and platelet activation to be upregulated in preeclampsia. Differential expression of PTGDS and SPINK5 was confirmed at the incision site, suggesting that some of the transcriptional changes observed in ECs from preeclampsia are not confined to the placental bed but are present in the entire uterus. This may indicate a more generalized uterine EC involvement in these patients. Besides the few differentially expressed genes, transcriptomic profiling showed large overlap between the two groups, which could be expected due to the specific nature and location of the isolated cells. Systemic profiling of biomarkers related to inflammation, endothelial activation and dysfunction yielded distinct signatures for preeclampsia and healthy pregnancy, which were driven mostly by the established 'preeclampsia markers' sFLT1, endoglin and PlGF, but also inflammation marker SAA-1, the adipokine leptin and markers of endothelial activation. Biomarker profiling within the preeclampsia group revealed two distinct subgroups, which clinically could be identified as preeclampsia with or without HELLP syndrome. The impact of the differentially expressed genes and biomarkers and their association with pregnancy and preeclampsia remain to be further investigated, but we can cautiously speculate on the importance of some of them here.

Comparison with other studies

PTGDS, upregulated in preeclampsia, catalyzes the conversion of prostaglandin H2 to prostaglandin D2 (PGD_2) and is important for inhibition of platelet aggregation, relaxation and contraction of smooth muscle and reduction of vascular permeability.[29,30] ECs are known to produce PTGDS under shear stress, which is likely present in the case of insufficient remodeling and high blood pressure in preeclampsia.[31-33] PGD_2 may be involved in inflammation through recruitment of T helper (Th) type 2 cells.[34] Although many studies show increased Th1 type and decreased Th2 type immunity in preeclampsia, at least as many reports demonstrate the opposite.[11] PTGDS upregulation has been associated with uterine contraction and spontaneous preterm birth, which may be linked to preeclampsia due to belief of some that spontaneous preterm birth serves as an internal rescue mechanism aiming to protect both mother and baby from damaging effects of prolonged preeclamptic and growth restricted pregnancy.[35-40] However, we cannot exclude the term difference between both groups to influence the regulation of PTGDS, although none of the women included in the experiments had been in labor before Caesarean section and logic would dictate PTGDS to be upregulated in the women closer to term (healthy controls) in preparation for labor.

Studies on olfactomedin 1 (Olfm-1) in the context of reproduction are limited. Human recombinant Olfm-1 suppresses the attachment of spheroids onto endometrial cells and downregulation of Olfm-1 during the receptive period may favor embryo attachment for successful implantation. Yet the decrease in Olfm-1 expression in the endometrium of patients treated with ovarian stimulation was not associated with an increase in endometrial receptivity in vivo.[41]

IL-3RA is a receptor for IL-3. IL-3 was shown to mediate positive signals for embryo implantation and to promote placental development and fetal growth.[42] IL-3RA receptor expression on EC increases migration of dendritic cells into tissues, which may regulate the Th1/Th2 balance within the decidua to maintain a Th2-dominant state, which is essential for maintenance of pregnancy.[43-45] A pathologic implication of high IL3RA expression in reproduction has not been reported.

Nestin (NES), a type VI intermediate filament protein known to participate in remodeling of the cell, was borderline upregulated in preeclampsia. In animal models, NES upregulation characterizes vascular remodeling secondary to hypertension. In comparison to multiple other reproductive tissues, NES is most strongly expressed by ECs of newly vascularized tissues.[46,47] In humans, urinary NES levels are significantly increased in preeclampsia patients and positively correlate with proteinuria.[47]

The SPINK5 gene, downregulated in ECs from preeclamptic patients, codes for the protein LEKT1, a serine protease inhibitor. Polymorphisms in SPINK5 have been associated with hypersensitivity of the immune system, especially in skin (atopy).[48] Other members of the SPINK family (i.e. SPINK1) have been shown to be highly up-regulated in decidua of recurrent pregnancy loss and to be predictive of preeclampsia, but no evidence is available for similar functions of SPINK5.[49]

Sestrin 3, also downregulated in preeclampsia reduces the levels of intracellular reactive oxygen species and is stress-induced.[50] It is required for normal regulation of blood glucose, insulin resistance, plays a role in lipid storage in obesity and is associated with increasing severity of coronary artery disease.[51,52] SESN3 has not previously been investigated in reproduction.

Pathway analysis of the genes enriched in ECs from preeclampsia identified 6 significant pathways involving the innate immune system and platelet activation. The biomarker profiling by multiplex immunoassay confirmed upregulation of factors related to immune activation and platelet activation in preeclampsia. We found a significant raise in soluble fms-like tyrosine kinase-1 (sFlt-1) and endoglin, which are known to cause a decrease in vascular endothelial growth factor (VEGF) and placental growth factor (PlFG), leading to increased vascular damage and endothelial dysfunction. We confirmed low PlGF levels in women with preeclampsia.[14] Lower PlGF concentrations in early pregnancy are found in women who subsequently develop preeclampsia, but sFLT-1 levels are similar, suggesting that PlGF production within the placenta may be decreased before sFLT-1 rises, subsequently binding PlGF and lowering serum levels even more.[53] Amongst the many inflammatory markers we

found increased in preeclampsia, we found TNF receptor 1 to be interesting as it mediates through TNF-α, inducing hypertension either directly or through endothelin (ET) production. ET is thought to be the most powerful vasoconstrictor in humans and perhaps to be of great importance in ultimately causing hypertension in pregnancy related disorders.[54] Similar to other reports, we found increased levels of the pro-inflammatory IL-6 and Macrophage inflammatory protein (MIP-1β).[11] The acute-phase protein SAA-1, which is released in response to tissue injury and infection, was significantly increased.

Comparing biomarker profiles between women with and without HELLP syndrome, platelet derived angiopoietin 1 (Ang-1) and PDFG-BB, both of which have important roles in vascular development and angiogenesis, were borderline decreased in HELLP.[54] The low levels of these markers may however be explained by low platelet counts, as this is a key diagnostic feature in HELLP syndrome. Other markers discriminating between the two phenotypes were apelin, sFLT-1, sICAM-1, RBP4 and several adipokines. Apelin has been known to have a hypotensive effect and promotes migration and proliferation of ECs, inducing angiogenesis.[55-57] With both sFLT-1 and sICAM-1 being higher in HELLP, we speculate that endothelial dysfunction is more pronounced when HELLP syndrome is present, possibly explaining liver and hematological implications.

In conclusion, placental bed EC transcriptomics and circulating profiles both point to a role for anti-angiogenic factors and endothelial-, inflammatory- and platelet activation in the pathogenesis of preeclampsia.

Strengths and limitations

To our knowledge this is the first study using transcriptomics to investigate human ECs from the placental bed in patients with pregnancy complicated by severe preeclampsia and controls. State of the art techniques were used to isolate very pure ECs from placental bed spiral arteries in which failed remodeling was histologically confirmed, and describe their functional alterations. We performed extensive biomarker profiling by multiplex immunoassay to identify systemic markers for inflammation and endothelial dysfunction, taking in regard the evidence that most cytokines act in concert with each other, analyzing combined rather than separate effects by unbiased statistical methods.

One limitation of this study is the term difference at which pregnancies are ended and tissue and serum samples are taken, which should be taken under consideration when interpreting the results. This limitation is inseparably connected to the study set-up and pathophysiology of disease, and the alternative choice of a control group with (spontaneous) premature parturition was not preferred due to the knowledge that spiral artery remodeling is comparably defective in spontaneous premature birth. Secondly, we point out that corticosteroid use close to delivery of all women with preeclampsia may have had an effect on systemic markers and local gene profiles, although one would mostly expect an attenuation of the contrast between cases and controls. Lastly, the low-input sequencing technique used in this study is a strength, as it made the study feasible with

small tissue samples. A strength we need to add is that isolating ECs by flow cytometry assisted cell sorting, is a sensitive and highly selective method, ensuring certainty and purity of the cell type analyzed in this study. For future studies, a larger number of samples will increase the statistical power and will possibly reveal more differentially expressed genes.

Conclusion and perspectives for future research

In this paper, we identified several candidate genes which were differentially expressed in endothelial cells from the placental bed that have a role in inflammatory response, vascular function and endothelial dysfunction. Systemic markers of these pathways can be found in women who suffer from severe and early onset preeclampsia with FGR.

Further steps in our ongoing research will focus on confirming these findings with larger series and different phenotypes of preeclampsia and/or FGR. The specific biological functions of the indicated pathways and individual candidate genes within the placental bed and their systemic consequences need to be further evaluated. Additional experiments are needed to test whether these endothelial targets are involved in orchestrating the remodeling process of spiral arteries. Alternatively, it is important to verify if these gene expressions are involved only in the uterus or whether they are involved with generalized endothelial dysfunction elsewhere in the body in preeclampsia. Last, but not least, it would be interesting to assess if different expression patterns of placental bed ECs are in any way representative of the underlying impaired pre-existent (or chronic) vascular-endothelial health known to be associated with preeclampsia.[13,58]

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE 1. Antibodies used for flow cytometry assisted cell sorting.

CD marker	Name	Fluorochrome	Company	Catalog no.	Clone
CD31	PECAM-1	FITC	BD	555445	WM59
CD144	VE-Cadherin	PE	BD	561714	55-7H1
CD146	MCAM	PerCP-Cy5.5	Biolegend	342014	SHM-57
CD309	VEGFR2	Pe-Cy7	Biologend	359912	7D4-6
CD54	ICAM-1	APC	BD	559771	HA58
CD105	Endoglin	eFluor450	eBioscience	48-1057-42	SN6
CD45	Leukocyte common antigen	Pacific Orange	Life technologies	MHCD4530	HI30

Abbreviations: CD, cluster of differentiation; PECAM-1, Platelet-endothelial cell adhesion molecule-1; VE-Cadherin, vascular endothelial cadherin; MCAM, melanoma cell adhesion molecule; VEGFR2, Vascular endothelial growth factor receptor 2; ICAM-1, Intercellular Adhesion Molecule 1; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; PerCP-Cy5.5, Peridinin-chlorophyll proteins- Cyanine5.5; Pe-Cy7, Phycoerythrin-cyanine 7; APC, Allophycocyanin.

SUPPLEMENTARY TABLE 2. Baseline characteristics for nulliparous women with preeclampsia and healthy pregnancy included for endothelial cell transcriptional profile comparison.

	Healthy control *n*=4	**Preeclampsia** *n*=5	***p*-value**
General characteristics			
Age (years)	35.3 (5.3)	28.2 (7.1)	0.145
White European (%)	4 (100%)	2 (40.0%)	0.058
BMI (kg/m^2)	26.6 (5.5)	28.1 (5.6)	0.699
Obesity (%)	2 (50.0%)	2 (40.0%)	0.764
Smoking (%)	0 (0.0%)	0 (0.0%)	n/a
General and obstetric history			
Pre-existent hypertension (%)	0 (0.0%)	0 (0.0%)	n/a
Pre-existent kidney disease (%)	0 (0.0%)	0 (0.0%)	n/a
Pre-existent Diabetes (%)	0 (0.0%)	0 (0.0%)	n/a
Nulliparity (%)	4 (100.0%)	5 (100.0%)	n/a
Pregnancy characteristics			
Early onset (%)	n/a	5 (100%)	n/a
FGR (%)	n/a	5 (100%)	n/a
HELLP (%)	n/a	2 (40%)	n/a
GDM with insulin use (%)	0 (0.0%)	0 (0.0%)	n/a
HPP (%)	1 (25%)	1 (20%)	0.858
Maternal characteristics			
Systolic blood pressure (mmHg)	122 (10)	162 (22)	0.010
Diastolic blood pressure (mmHg)	76 (8)	101 (9)	0.003
Antihypertensive treatment oral (%)	0 (0.0%)	4 (80.0%)	0.016
Antihypertensive treatment iv (%)	0 (0.0%)	1 (20.0%)	0.343
Antepartum MgSO4 iv (%)	0 (0.0%)	4 (80.0%)	0.016
Antepartum CCS (%)	0 (0.0%)	5 (100%)	0.003
Neonatal characteristics			
GA at delivery (days)	277 (7)	216 (16)	<0.001
Birthweight (grams)	3529 (525)	1135 (338)	<0.001
Birthweight <3rd percentile (%)	0 (0.0%)	4 (80.0%)	<0.001
Fetal sex, male (%)	2 (50%)	3 (60%)	0.764

Values are presented as means (standard deviations) or as numbers (%). *P*-values were calculated by the χ^2 test for continuous variables and Fisher's exact test for categorical variables. Abbreviations: BMI, Body Mass Index; Obesity was defined as an BMI >30kg/m2; FGR, fetal growth restriction; HELLP, Hemolysis Elevated Liver enzymes Low Platelets syndrome; GDM, gestational diabetes mellitus, HPP, hemorrhage post-partum, defined as blood loss >1000mL; iv, intravenous; MgSO4, magnesiumsulphate; CCS, corticosteroid treatment; GA, gestational age (days); n/a; not applicable.

SUPPLEMENTARY TABLE 3. Maternal and pregnancy baseline characteristics in women with severe preeclampsia (with and without HELLP syndrome) and healthy pregnancy for the multiplex immunoassay comparison of systemic biomarkers for inflammation, endothelial activation and dysfunction.

	Healthy control *n*=20	**Preeclampsia** *n*=17	***p*-value†**	**Preeclampsia** *n*=9	**Preeclampsia with HELLP syndrome** *n*=8	***p*-value‡**
General characteristics						
Age (years)	33.6 (3.9)	31.0 (5.6)	0.110	30.0 (6.5)	33.8 (5.7)	0.228
White European (%)	20 (100.0%)	10 (58.8%)	0.001	4 (44.4%)	6 (75%)	0.201
BMI (kg/m^2)	23.2 (2.2)	25.3 (4.9)	0.094	28.0	22.3	0.016
Obesity (%)	0 (0.0%)	3 (20.0%)	0.036	3 (37.5%)	0 (0.0%)	0.070
Smoking (%)	0 (0.0%)	1 (5.9%)	0.272	1 (11.1%)	0 (0.0%)	0.331
General and obstetric history						
Pre-existent hypertension (%)	0 (0.0%)	2 (11.8%)	0.115	1 (11.1%)	1 (12.5%)	0.929
Pre-existent kidney disease (%)	1 (5.0%)	0 (0.0%)	0.350	0 (0%)	0 (0%)	n/a
Pre-existent Diabetes (%)	0 (0.0%)	0 (0.0%)	n/a	0 (0%)	0 (0%)	n/a
Nulliparity (%)	5 (25.0%)	12 (70.6%)	0.006	6 (66.7%)	6 (75.0%)	0.707
CS in history (%)*	13 (86.7%)	2 (40.0%)	0.037	2 (66.7%)	0 (0.0%)	0.136
HDP in history (%)*	0 (0.0%)	2 (40.0%)	0.010	2 (66.7%)	0 (0.0%)	0.136
FGR in history (%)*	0 (0.0%)	2 (40.0%)	0.010	2 (66.7%)	0 (0.0%)	0.136
Pregnancy characteristics						
Early onset (%)	n/a	17 (100%)	n/a	9 (100%)	8 (100%)	n/a
FGR (%)	n/a	17 (100%)	n/a	9 (100%)	8 (100%)	n/a
HELLP (%)	n/a	8 (47.1%)	n/a	n/a	8 (100%)	n/a
GDM with insulin use (%)	0 (0.0%)	0 (0.0%)	n/a	0 (0%)	0 (0%)	n/a
HPP (%)	1 (5.0%)	0 (0.0%)	0.350	0 (0%)	0 (0%)	n/a
Maternal characteristics						
Systolic blood pressure (mmHg)	120 (8)	169 (23)	<0.001	164 (21)	171 (26)	0.567
Diastolic blood pressure (mmHg)	75 (7)	108 (10)	<0.001	105 (10)	108 (12)	0.591
Antihypertensive treatment oral (%)	0 (0.0%)	14 (82.4%)	<0.001	8 (88.9%)	6 (75.0%)	0.453
Antihypertensive treatment iv (%)	0 (0.0%)	7 (41.2%)	0.001	2 (22.2%)	5 (62.5%)	0.092
Antepartum MgSO4 iv (%)	0 (0.0%)	13 (76.5%)	<0.001	7 (77.8%)	6 (75.0%)	0.893
Antepartum CCS (%)	0 (0.0%)	17(100.0%)	<0.001	9 (100%)	8 (100%)	n/a
Neonatal characteristics						
GA at delivery (days)	276 (4)	214 (14)	<0.001	215.1 (12.1)	211.9 (16.0)	0.642
Birthweight (grams)	3528 (400)	1048 (278)	<0.001	1015.8 (277.6)	1095.9 (274.2)	0.554
Birthweight <3rd percentile (%)	0 (0.0%)	14 (82.4%)	<0.001	9 (100%)	5 (62.5%)	0.043
Fetal sex, male (%)	8 (40.0%)	8 (47.1%)	0.666	4 (44.4%)	4 (50.0%)	0.819

Values are presented as means (standard deviations) or as numbers (%). P values were calculated by the χ^2 test for continuous variables and Fisher's exact test for categorical variables. *Obstetric history is calculated amongst multiparous patients only. †*p*-value between healthy pregnancy and all cases of preeclampsia with FGR. ‡ *p*-value between cases of preeclampsia with and without HELLP syndrome. Abbreviations: BMI, Body Mass Index; Obesity was defined as an BMI >30kg/m2; CS, Caesarean section; HDP, hypertensive disease of pregnancy; FGR, fetal growth restriction; HELLP, Hemolysis Elevated Liver enzymes Low Platelets syndrome; GDM, gestational diabetes mellitus, HPP, hemorrhage post-partum, defined as blood loss >1000mL; iv, intravenous; MgSO4, magnesiumsulphate; CCS, corticosteroid treatment; GA, gestational age (days); n/a; not applicable.

SUPPLEMENTARY TABLE 4. Individual marker comparison by multiplex immunoassay between pre-eclampsia and healthy pregnancy.

Marker	Healthy controls *n*=20	Preeclampsia *n*=17	Nominal *p**	Corrected *p*-value†	FDR‡
sFLT-1	13021 (9818)	36593 (10929)	<0.001	<0.001	0.000
Endoglin	5322 (2499)	11854 (3664)	<0.001	<0.001	0.000
SAA-1	611653 (698678)	3370800 (8368350)	<0.001	0.001	0.000
PlGF	154 (175)	6 (15)	<0.001	0.001	0.000
sICAM	298010 (74524)	424138 (170489)	<0.001	0.012	0.002
Leptin	16590 (24017)	45833 (21860)	<0.001	0.015	0.003
Fibronectin	3940350 (85956650)	264720000 (252699500)	<0.001	0.026	0.004
YKL-40	49269 (31248)	80929 (103123)	0.001	0.043	0.005
TNF-R1	5112 (1609)	6921 (2129)	0.001	0.082	0.009
HGF	713 (527)	1122 (713)	0.002	0.092	0.009
PAI-1	260281 (169489)	445399 (774301)	0.002	0.096	0.009
Chemerin	21215 (9372)	29814 (14405)	0.002	0.125	0.010
sIL-2R	215 (421)	645 (583)	0.002	0.148	0.011
MIP-1b	78 (32)	112 (39)	0.004	0.227	0.016
TIMP-1	221695 (50772)	277035 (103473)	0.006	0.365	0.024
IL-6	6 (7)	13 (11)	0.007	0.401	0.025
E-selectin	37510 (22478)	63530 (38702)	0.010	0.575	0.032
OPN	31031 (9983)	46889 (31280)	0.010	0.575	0.032
IP10	316 (196)	439 (180)	0.019	1.137	0.060
PDGF-BB	5304 (2909)	8074 (6557)	0.021	1.232	0.062
Gal-9	22030 (6233)	26245 (15174)	0.022	1.336	0.064
Adipsin	536 (359)	853 (680)	0.026	1.562	0.071
IL-10	3 (6)	6 (9)	0.037	2.209	0.096
GP130	42194 (6669)	44972 (7236)	0.046	2.753	0.114
FABP-4	25018 (12684)	20040 (9522)	0.048	2.856	0.114
sIL-6R	25297 (11062)	32534 (11260)	0.051	3.066	0.118
sPD-1	441 (299)	316 (129)	0.059	3.529	0.131
Gal-1	22035 (5283)	25366 (9627)	0.100	5.989	0.214
sVCAM	2641450 (593475)	3175200 (1198850)	0.106	6.376	0.220
LAP	4290 (2123)	5201 (1938)	0.116	6.986	0.232
TNF-R2	1371 (422)	1546 (860)	0.120	7.207	0.232
Ang-2	2494 (2018)	4489 (6135)	0.170	10.215	0.319
VEGF	7 (6)	8 (5)	0.185	11.091	0.335
RBP4	47079500 (6995250)	49048000 (7432500)	0.190	11.402	0.335
Gal-3	15908 (10207)	20115 (14824)	0.235	14.077	0.402
Thrombomodulin	2575 (1384)	2951 (2346)	0.247	14.808	0.411
IL-8	21 (37)	28 (16)	0.259	15.569	0.421
TWEAK	4314 (1083)	4715 (2219)	0.273	16.355	0.429
SPARC	1804650 (3669059)	681058 (2634289)	0.286	17.166	0.429

SUPPLEMENTARY TABLE 4 CONTINUED.

Marker	Healthy controls *n*=20	Preeclampsia *n*=17	Nominal *p**	Corrected *p*-value†	FDR‡
Fetuin	255200000 (108872500)	250070000 (73740000)	0.286	17.167	0.429
Apelin	1824 (1317)	2122 (1143)	0.322	19.315	0.471
Adiponectin	90217000 (45889250)	110140000 (61881500)	0.345	20.687	0.493
MMP-1	92106 (85164)	75686 (42535)	0.361	21.634	0.503
LAG-3	854 (482)	756 (434)	0.411	24.632	0.547
Clusterin	197982 (41166)	180935 (60490)	0.411	24.635	0.547
sCD163	8483 (6436)	10977 (5644)	0.428	25.688	0.558
MMP-7	2865 (4093)	2013 (3626)	0.465	27.871	0.581
THBS-1	126100000 (125142500)	77871000 (154575000)	0.465	27.871	0.581
Ang-1	52821 (16822)	47937 (33180)	0.522	31.330	0.639
LIP-2	230467 (449878)	260186 (133947)	0.626	37.549	0.747
DPP-IV	1065650 (579388)	1139300(648400)	0.648	38.854	0.747
MMP-9	31766000 (31350250)	33745000 (27716000)	0.648	38.854	0.747
Tie-2	2026 (1417)	1701 (618)	0.703	42.193	0.796
LAIR-1	1331 (517)	1512 (594)	0.784	47.032	0.871
Cyst C	646181 (434890)	724342 (596285)	0.807	48.443	0.881
IL-1b	5 (5)	5 (3)	0.855	51.293	0.900
P-selectin	304331 (269833)	287851 (240170)	0.855	51.295	0.900
IL-18	162 (203)	168 (279)	0.903	54.178	0.934
TRAIL	84 (127)	88 (122)	0.951	57.045	0.964
sCD14	2418500 (1386475)	2267000 (2090233)	0.964	57.812	0.964

Values are median concentrations (pg/ml) with interquartile ranges, *p*-value was calculated with Mann-Whitney-U tests. *Analytes with more than 35% of measured values below the lower or above the upper limit of detection. †*P*-value adjusted for multiple testing by Bonferroni. ‡*P*-value adjusted for multiple testing by False Discovery Rate (FDR). *Abbreviations*: FDR, false discovery rate; sFLT-1, soluble fms-like tyrosine kinase-1; SAA-1, serum amyloid A1; PlGF, placental growth factor; sICAM, soluble Intercellular Adhesion Molecule; YKL-40, human cartilage glycoprotein 39; TNR-R1, tumor necrosis factor-receptor 1; HGF, hepatocyte growth factor; PAI-1, plasminogen activator inhibitor-1; sIL-2R, soluble interleukin-2 receptor; MIP-1b, macrophage inflammatory protein-1 beta; TIMP-1, Tissue Inhibitor of Metalloproteinase 1; IL-6, interleukin 6; OPN, osteopontin; IP10, Interferon gamma-induced protein 10; PDGF-BB, Platelet-derived growth factor subunit B; Gal-9, galectin 9; IL-10, interleukin 10; GP130, glycoprotein 130; FABP-4, Fatty acid-binding protein 4; sIL-6R, soluble interleukin 6 receptor; sPD-1, soluble programmed cell death protein 1; Gal-1, galectin 1; sVCAM, soluble Vascular cell adhesion protein; LAP, leucine-amino-peptidase; TNF-R2, tumor necrosis factor receptor 2; Ang-2, Angiopoietin 2; VEGF, vascular endothelial cell growth factor; RBP4, Retinol binding protein 4; Gal-3, galectin 3; IL-8, interleukin 8; TWEAK, TNF (tumor necrosis factor)-related weak inducer of apoptosis; SPARC, secreted protein acidic and rich in cysteine; MMP-1, matrix metalloprotease 1; LAG-3, Lymphocyte-activation gene 3; sCD-163, soluble cluster of differentiation 163; MMP-7, matrix metalloprotease 7; THBS-1, Thrombospondin 1; Ang-1, Angiopoietin 1; LIP-2, Lipase 2; DPP-IV, Dipeptidyl peptidase-4; MMP-9, matrix metalloprotease 9; Tie-2, Tyrosine kinase with immunoglobulin-like and EGF-like domains 2; LAIR-1, Leukocyte-associated immunoglobulin-like receptor 1; Cyst C, Cystatin C; IL-1b, interleukin 1 beta; IL-18, interleukin 18; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; sCD14, soluble cluster of differentiation 14.

SUPPLEMENTARY TABLE 5. Individual marker comparison by multiplex immunoassay between women with preeclampsia with or without HELLP syndrome.

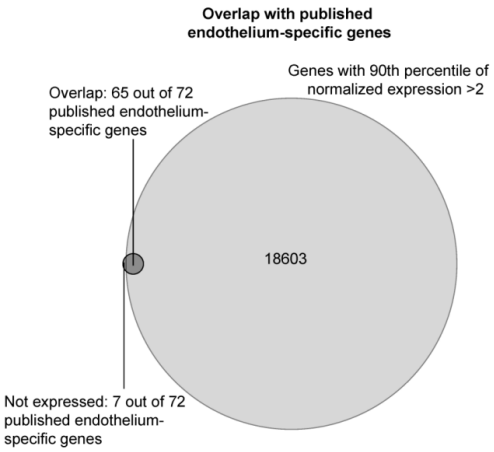
Marker	Preeclampsia *n*=9	Preeclampsia with HELLP syndrome *n*=8	Nominal *p**	Corrected *p*-value†	FDR‡
Ang-1	64475 (30089)	32536 (15733)	0.001	0.064	0.064
RBP4	51902000 (4672000)	44490500 (11239000)	0.003	0.171	0.086
Apelin	2613 (1764)	1747 (1054)	0.005	0.316	0.105
PDGF-BB	10789 (2871)	5552 (3771)	0.009	0.560	0.124
sFLT-1	29677 (11717)	40018 (6668)	0.012	0.741	0.124
P-selectin	361210 (678401)	199624 (212380)	0.012	0.741	0.124
LIP-2	308570 (235993)	216820 (68172)	0.016	0.969	0.138
SAA-1	2152800 (1799600)	10419500 (16176975)	0.021	1.255	0.144
Adipsin	609 (652)	992 (148)	0.024	1.419	0.144
Clusterin	173716 (31083)	205257 (83254)	0.027	1.613	0.144
MMP-9	38172000 (18494500)	15480500 (27122425)	0.027	1.613	0.144
PAI-1	329237 (602442)	702866 (873613)	0.034	2.056	0.144
IL-10	5 (7)	10 (19)	0.043	2.598	0.144
LAG-3	693 (284)	955 (343)	0.043	2.598	0.144
IL-8	26 (9)	36 (22)	0.043	2.598	0.144
Endoglin	10456 (3512)	13188 (2584)	0.043	2.598	0.144
Chemerin	24906 (14321)	35253 (8072)	0.043	2.598	0.144
THBS-1	181170000 (222636500)	61244500 (92863250)	0.043	2.598	0.144
E-selectin	41700(35183)	74198 (42960)	0.054	3.258	0.171
GP130	43643(14851)	48886 (7553)	0.068	4.050	0.193
sICAM	357172 (195616)	466600 (181756)	0.068	4.050	0.193
Gal-1	22128 (8862)	29001 (10229)	0.083	4.996	0.217
Ang-2	2365 (4026)	5696 (7261)	0.083	4.996	0.217
TNF-R1	5749 (1907)	7199 (2544)	0.102	6.113	0.235
TNF-R2	1412 (650)	1910 (923)	0.102	6.113	0.235
LAP	5420 (2165)	4558 (1746)	0.102	6.113	0.235
Leptin	51168 (18453)	39824 (22068)	0.112	6.728	0.249
Fibronectin	210150000 (218089825)	329035000 (329784250)	0.123	7.364	0.263
TWEAK	5407 (2120)	4317 (735)	0.149	8.935	0.298
Tie-2	1699 (697)	2150 (1201)	0.149	8.935	0.298
Thrombomodulin	2526 (2500)	3394 (2003)	0.178	10.676	0.334
DPP-IV	1073200 (626920)	1258550 (512500)	0.178	10.676	0.334
MMP-1	76927 (45814)	67427 (64107)	0.211	12.658	0.384
Fetuin	251270000 (57415000)	213730000 (79657500)	0.290	17.390	0.497
TIMP-1	251428 (88968)	281139 (75567)	0.290	17.390	0.497
FABP-4	22066 (11863)	19132 (6272)	0.336	20.155	0.530
sVCAM	3028100 (939800)	3379500 (1542825)	0.336	20.155	0.530
Adiponectin	91688000 (57801500)	121705000 (66442500)	0.336	20.155	0.530

SUPPLEMENTARY TABLE 5 CONTINUED.

Marker	Preeclampsia *n*=9	Preeclampsia with HELLP syndrome *n*=8	Nominal *p**	Corrected *p*-value†	FDR‡
OPN	44073 (18272)	52769 (47517)	0.386	23.189	0.580
MMP-7	1999 (2219)	2894 (5488)	0.386	23.189	0.580
SPARC	1433700 (2914207)	622578 (1582056)	0.441	26.485	0.646
IL-6	13 (10)	15 (11)	0.501	30.035	0.698
HGF	944 (854)	1189 (612)	0.501	30.035	0.698
Gal-3	20115 (13396)	23106 (23100)	0.630	37.826	0.824
sCD14	2543500 (2219787)	2167300 (2068225)	0.630	37.826	0.824
IL-1b	5 (4)	5 (5)	0.665	39.889	0.824
VEGF	8 (4)	6 (6)	0.700	41.998	0.824
IL-18	168 (250)	185 (327)	0.700	42.019	0.824
sIL-2R	645 (642)	717 (574)	0.700	42.019	0.824
Cyst C	724342 (916234)	727035 (514915)	0.700	42.019	0.824
Gal-9	23763 (16392)	27282 (14181)	0.700	42.019	0.824
MIP-1b	105 (39)	114 (72)	0.773	46.370	0.875
sPD-1	326 (176)	302 (54)	0.773	46.370	0.875
IP10	418 (164)	440 (410)	0.847	50.843	0.924
sCD163	8599 (4445)	11659 (8178)	0.847	50.843	0.924
PlGF	6(14)	4 (16)	0.883	52.963	0.946
sIL-6R	32534(13135)	32003 (10516)	0.923	55.398	0.955
LAIR-1	1512 (557)	1482 (693)	0.923	55.401	0.955
TRAIL	88 (101)	65 (154)	0.961	57.683	0.978
YKL-40	80929 (74124)	105167 (126816)	1.000	60.000	1.000

Values are median concentrations (pg/ml) with interquartile ranges, *P*-value was calculated with Mann-Whitney-U tests. †*P*-value adjusted for multiple testing by Bonferroni. ‡*P*-value adjusted for multiple testing by False Discovery Rate (FDR). *Abbreviations*: HELLP, hemolysis, elevated liverenzymen, low platelets; FDR, false discovery rate; Ang-1, Angiopoietin 1; RBP4, Retinol binding protein 4; PDGF-BB, Platelet-derived growth factor subunit B; sFLT-1, soluble fms-like tyrosine kinase-1; LIP-2, Lipase 2; SAA-1, serum amyloid A1; MMP-9, matrix metalloprotease 9; PAI-1, plasminogen activator inhibitor-1; IL-10, interleukin 10; LAG-3, Lymphocyte-activation gene 3; IL-8, interleukin 8; THBS-1, Thrombospondin 1; GP130, glycoprotein 130; sICAM, soluble Intercellular Adhesion Molecule; Gal-1, galectin 1; Ang-2, Angiopoietin 2; TNR-R1, tumor necrosis factor-receptor 1; TNF-R2, tumor necrosis factor receptor 2; LAP, leucine-amino-peptidase; TWEAK, TNF (tumor necrosis factor)-related weak inducer of apoptosis; Tie-2, Tyrosine kinase with immunoglobulin-like and EGF-like domains 2; DPP-IV, Dipeptidyl peptidase-4; TIMP-1, MMP-1, matrix metalloprotease 1; Tissue Inhibitor of Metalloproteinase 1; FABP-4, Fatty acid-binding protein 4; sVCAM, soluble Vascular cell adhesion protein; OPN, osteopontin; MMP-7, matrix metalloprotease 7; SPARC, secreted protein acidic and rich in cysteine; IL-6, interleukin 6; HGF, hepatocyte growth factor; Gal-3, galectin 3; sCD14, soluble cluster of differentiation 14, IL-1b, interleukin 1 beta; VEGF, vascular endothelial cell growth factor; IL-18, interleukin 18; sIL-2R, soluble interleukin-2 receptor; Cyst C, Cystatin C; Gal-9, galectin 9; MIP-1b, macrophage inflammatory protein-1 beta; sPD-1, soluble programmed cell death protein 1; IP10, Interferon gamma-induced protein 10; sCD-163, soluble cluster of differentiation 163; PlGF, placental growth factor; sIL-6R, soluble interleukin 6 receptor; LAIR-1, Leukocyte-associated immunoglobulin-like receptor 1; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; YKL-40, human cartilage glycoprotein 39.

A



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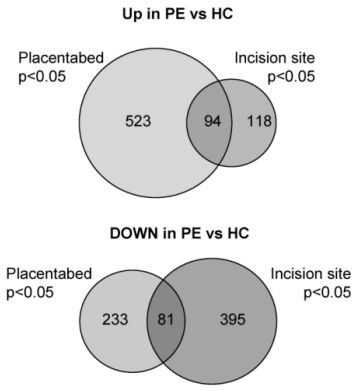
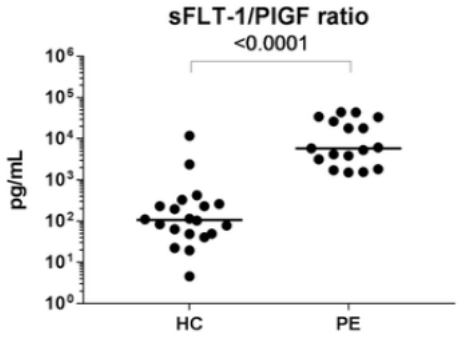


C

Gene	p value	padj
Up in PE		
PTGDS	<0.0001	0.0031
OLFM1	0.0004	0.1369
IL3RA	0.0072	0.4639
Down in PE		
SPINK5	<0.0001	0.0340
SESN3	0.0028	0.3265

SUPPLEMENTARY FIGURE 1. Endothelial identity and overlap of upregulated and downregulated genes in placental bed with incision site. (A) Overlap between 72 endothelium-specific genes published by Chi *et al*. and all genes with 90th percentile of normalized expression >2 in endothelial cells from the placental bed (irrespective of preeclampsia or healthy controls).[27] (B) Overlap between upregulated and downregulated genes in preeclampsia compared to healthy pregnancies in placental bed and incision site, with a nominal *p*-value <0.05. (C) *P*-value and *p*adj of differential gene expression between preeclampsia and healthy controls at the incision site, for significantly differentially expressed genes with a *p*adj<0.05 in placental bed. *Abbreviations*: PE, preeclampsia (in this study early onset, in combination with fetal growth restriction); HC, healthy pregnancy; PTGDS, prostaglandin D2 synthase; OLFM1, olfactomedin 1; IL3RA, interleukin 3 receptor subunit alpha; SPINK5, serine peptidase inhibitor Kazal type 5; SESN3,sestrin 3.



SUPPLEMENTARY FIGURE 2. Ratio between serum sFLT-1 and PlGF in preeclampsia and healthy pregnancy. Scatter dot plots of sFLT-1/PlGF ratio. Line represents median; nominal *p*-value without correction for multiple testing is indicated and calculated by Mann-Whitney U test. *Abbreviations*: PE, preeclampsia (in this study early onset, in combination with fetal growth restriction); HC, healthy pregnancy; sFLT-1, soluble fms-like tyrosine kinase-1; sICAM, soluble Intercellular Adhesion Molecule; PlGF, Placental growth factor.

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Association of maternal prepregnancy body mass index with placental histopathological characteristics in uncomplicated term pregnancies

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ABSTRACT

Introduction

Prepregnancy obesity is a growing global health problem and has several risks for mother and child. The aim of this study was to systematically examine the effect of increased maternal body mass index (BMI) on placental pathology in otherwise uneventful term pregnancies.

Methods

In this analysis, we studied data of the Netherlands Amniotic Fluid study, a prospective study of women delivering in Utrecht, the Netherlands, between 2006 and 2007. We included women with uncomplicated pregnancies, vaginal delivery, and data on prepregnancy weight and height (*n*=382). Placental histopathology was compared between women of normal BMI (≤24.9 kg/m2), overweight (25–29.9 kg/m2), and obese (≥30 kg/m2).

Results

Increasing prepregnancy BMI was associated with heavier placentas and higher mean infant's birth weight. In addition, obesity was positively associated with high-grade chronic villitis (odds ratio [OR]: 18.1, 95% confidence interval [CI]: 1.6–205.2), accelerated villous maturation (OR: 1.1, 95% CI: 1.0–1.2), and lower incidence of placental weight below the 10th percentile for gestational age (OR: 0.5, 95% CI: 0.3–1.0). There was a substantial effect of parity on maternal, placental, and neonatal weights.

Conclusions

Even in uncomplicated pregnancies, maternal obesity is associated with characteristic changes in placental pathology. Further research is needed to evaluate these changes in view of later-life health of infants born to obese mothers.

INTRODUCTION

Obesity is one of the fastest growing global health problems among women of reproductive age.[1] In the United States, almost two-thirds of women of child-bearing age are overweight and one-third is obese.[2] In most European countries, including the Netherlands, obesity prevalence is lower (approximately 15%), but trends over time project similar rates within the next decade.[1-4] Women with a higher prepregnancy body mass index (BMI) are at risk of a several obstetric complications (ie, gestational diabetes mellitus [GDM], preeclampsia, large-for-gestational-age [LGA] birth weight babies) associated with higher morbidity for both mother and child.[2,5-9]

A long-term higher risk of obesity and metabolic syndrome is seen in offspring of these obese mothers. Most of these complications are likely mediated by the effect of obesity on placental function, including placental production of growth factors, hormones, and the transport of nutrients to the fetus, causing pregnancy complications and long-term fetal programming concerns.[10] It is unknown to what extent high BMI affects characteristics of the placenta and infants in healthy uncomplicated pregnancy.

Animal studies have shown that maternal obesity is independently associated with increased placental messenger RNA levels of toll-like receptor (TLR)-2 and TLR-4 and show higher levels of pro-inflammatory cytokines.[11] In humans, studies have suggested an increased maternal inflammatory response (MIR) in obese pregnancy, as shown by elevated levels of circulating IL-6 and higher levels of placental pro-inflammatory cytokines.[12] Only few studies have evaluated the relationship between obesity and placental pathology, reporting an effect of increased MIR in placentas of obese mothers (ie, chorioamnionitis), a higher prevalence of chronic villitis, and a higher prevalence of vascular lesions (ie, acute atherosis and villous infarctions) related to maternal vascular under perfusion.[13-15] These reports, however, were based on both complicated and uncomplicated pregnancy.[13-15]

The aim of this study was to systematically examine the association between maternal BMI and placental pathology, using a well-defined cohort of women with uneventful term pregnancies with unassisted vaginal delivery stratified into different clinically relevant BMI groups.

METHODS

Study Population

This study was part of the Netherlands Amniotic Fluid study, which was set up to investigate the role of intrauterine inflammation in the onset of spontaneous term labor. The full setup of this study was described previously.[16,17] In short, a cross-sectional study was performed in 1 secondary and 1 tertiary hospital in Utrecht, the Netherlands. Women with uncomplicated pregnancies who had a spontaneous vaginal delivery between 2006 and 2007 were included (*n*=578). Explicitly, women in this study were screened for specific pregnancy complications (ie, GDM, preeclampsia, fetal growth restriction [FGR]) when indicated according to Dutch standard prenatal care guidelines and did not get diagnosed with any of these conditions before delivery. The ethical review board approved the study, and all parents provided written informed consent for study participation. In this study, we included the women of whom information was available regarding both placental pathology and self-reported weight and height measurements before pregnancy (*n*=382).

Clinical Characteristics

All necessary clinical maternal (ie, age, parity, gestational age, length, and weight) and fetal characteristics (ie, birth weight, APGAR scores) were collected as previously described.[16,17] BMI was calculated according to the standard formula (kg/m^2), and patients were divided in 3 groups according to World Health Organization classification: normal BMI (≤24.9 kg/m^2), overweight (25.0–29.9 kg/m^2), and obese (≥30.0 kg/m^2).[18]

Placental Pathology

All placentas were stored at 4°C and were processed by 1 experienced pathologist (PGJN) within 72 hours. Because some of the pathological findings depend on gestational age (ie, maturation of placenta parenchyma, weight percentiles), information on gestational age at delivery was disclosed. Other clinical parameters were not disclosed to the pathologist before review. Placental weight was defined without membranes or umbilical cord, and weight for gestational age percentiles were used from Pinar *et al*.[19]

Placental weight below the 10th percentile and above the 90th percentile was classified as abnormal. Fetal/placental weight ratios were calculated. Macroscopic cord coiling index was determined by measuring the number of complete coils (360°) divided by the total length of the attached umbilical cord (cm). Coiling was determined fresh, before formalin fixation. A coiling index below 0.1 was considered as hypocoiling, and hypercoiling was defined as a coiling index above 0.3.

Histology

Two sections of umbilical cord, a membrane roll, and 3 slides of macroscopically normal placental tissue (including both decidua and chorionic plate) were collected and stained

with hematoxylin and eosin. Additional samples were taken from areas that macroscopically appeared abnormal. A full histology examination was performed based on the criteria as previously reported.[16,20,21] Placental lesions are defined in Supplementary Table 1. MIR was defined as an abnormal presence of polymorphonuclear leukocyte cells in the chorionic plate or extraplacental membranes (also described as acute chorioamnionitis). Fetal inflammatory response (FIR) was defined as aggregates of neutrophils in umbilical vascular wall with or without expansion in Wharton's jelly (also known as acute funisitis). Chronic villitis was defined as infiltration of lymphocytes and macrophages in the placental villi.

Statistical Analysis

Statistical analyses were performed using SPSS version 21.0 (IBM Corp). $\chi 2$ test was used to examine differences between placental pathological lesions among the groups in case of categorical variables. For continuous variables, 1-way analysis of variance testing was conducted. Linear regression was used to determine correlation coefficients between continuous variables. We considered differences to be statistically significant at $p<0.05$.

RESULTS

Baseline Characteristics

We included 382 women with 273 women with a normal BMI, 77 patients were overweight, and 32 were obese. Table 1 presents the baseline characteristics of the groups. Compared with patients who had a normal BMI, overweight and obese women were significantly less often nulliparous (43.6% vs 24.7% and 21.9%, respectively, *p*=0.001) but had similar neonatal characteristics among the BMI categories. Of note, within this group of term uneventful pregnancies, no differences were observed in gestational age at delivery, birth weight and birth weight percentile, small-for-gestational age (SGA; birth weight <10th percentile) or LGA babies (birthweight >90th percentile) associated with BMI category.

Placental Pathology

The placental pathological lesions (means and percentages) by maternal prepregnancy BMI are shown in Table 2. The risk of placental pathological lesions by prepregnancy maternal BMI category is presented in Table 3.

TABLE 1. Maternal and neonatal characteristics per BMI category

	Normal BMI <24.9 *n*=273	**Overweight BMI 25-29.9** *n*=77	**Obese BMI > 30** *n*=32	***p*-value**
Maternal characteristics				
Age (years)	30.8 (5.1)	30.6 (5.2)	31.0 (3.4)	0.909
Nulliparity (%)	119 (43.6%)	19 (24.7%)	7 (21.9%)	<0.001
Height (cm)	168.5 (7.4)	166.6 (6.6)	165.3 (7.8)	0.015
Weight (kg)	61.2 (6.7)	75.7 (6.4)	90.8 (12.7)	<0.001
BMI (kg/m^2)	21.5 (1.8)	27.3 (1.4)	33.1 (3.0)	<0.001
Neonatal characteristics				
Male gender (%)	143 (52.4%)	37 (48.1%)	13 (40.6%)	0.413
GA at delivery (days)	279.8 (7.2)	281.0 (7.4)	278.7 (8.1)	0.217
Birth weight (grams)	3531 (437)	3629 (453)	3621 (391)	0.239
Birth weight percentile	51.5 (27.5)	54.8 (28.3)	58.8 (23.9)	0.331
SGA (%)	19 (6.9%)	6 (7.8%)	0 (0.0%)	0.227
LGA (%)	23 (8.4%)	7 (9.0%)	2 (6.3%)	0.887
APGAR score (at 5 min)	9.8 (0.6)	9.7 (0.6)	9.8 (0.5)	0.463

Values are presented as means (standard deviation) or as numbers (%). *P*-values were calculated by one way anova for continuous variables and chi square test for categorical variables. *Abbreviations:* BMI, Body Mass Index (kg/m^2); normal BMI (<24.9 kg/m^2), overweight (25.0-29.9 kg/m^2) and obese (>30.0 kg/m^2); GA, gestational age (days); SGA, small for gestational age (birthweight < 10th percentile); LGA: large for gestational age (birthweight > 90th percentile). APGAR scores were determined 5 min post-partum.

TABLE 2. Placental pathology characteristics per BMI category.

	Normal BMI <24.9 *n*=273	**Overweight BMI 25-29.9** *n*=77	**Obese BMI > 30** *n*=32	***p*-value**
Placenta weight (grams)	490.9 (87.1)	513.5 (97.1)	511.8 (77.5)	0.120
SGA placenta (%)	76 (27.8%)	12 (15.6%)	5 (15.6%)	0.067
LGA placenta (%)	23 (8.4%)	9 (11.7%)	4 (12.5%)	0.567
Fetal/placental weight (ratio)	7.3 (1.1)	7.2 (1.1)	7.2 (1.0)	0.595
Coiling index	0.141 (0.069)	0.149 (0.078)	0.136 (0.077)	0.629
Hypocoiling (%)	82 (30.0%)	18 (23.4%)	13 (40.6%)	0.190
Hypercoiling (%)	4 (1.5%)	3 (3.9%)	1 (3.1%)	0.384
Meconium stained amniotic fluid (%)	54 (19.8%)	18 (23.4%)	5 (15.6%)	0.418
MIR ≥ stage 2 (%)	17 (6.2%)	8 (10.4%)	3 (9.4%)	0.417
FIR ≥ stage 2 (%)	8 (2.9%)	2 (2.6%)	2 (6.3%)	0.568
High grade chronic villitis (%)	1 (0.4%)	1 (1.3%)	2 (6.3%)	0.008
Fetal Thrombosis (%)	13 (4.8%)	6 (7.8%)	3 (9.4%)	0.395
Delayed villous maturation (%)	64 (23.4%)	25 (32.5%)	9 (28.1%)	0.181
Accelerated villous maturation (%)	0 (0%)	0 (0%)	2 (6.3%)	<0.001
Elevated NRBC (%)	3 (1.1%)	1 (1.3%)	0 (0%)	0.822
Placental infarction (%)	76 (27.8%)	16 (20.8%)	4 (12.5%)	0.128
Chorangiosis (%)	4 (1.5%)	1 (1.3%)	2 (6.3%)	0.150

Values are presented as means (standard deviation) or as numbers (%). *P*-values were calculated by one way Anova for continuous variables and chi square test for categorical variables. *Abbreviations:* BMI, Body Mass Index; normal BMI (<24.9 kg/m^2), overweight (25.0-29.9 kg/m^2) and obese (>30.0 kg/m^2); SGA placenta: Small for gestational age placenta; placental weight <10th percentile. LGA placenta: large for gestational age placenta; placental weight > 90th percentile. MIR: relevant maternal inflammatory response; chorioamnionitis ≥ stage 2. FIR: relevant Fetal Inflammatory Response; acute funisitis ≥ stage 2. Chronic villitis: relevant when high grade/diffuse. Thrombosis: relevant when medium/high grade. NRBC; nucleated red blood cells. Placental infarction: presence of <5% infarction in the placenta vs. none.

When comparing the occurrence of high-grade chronic villitis (≥grade 2) in the overweight (n=1 [1.3%]) and obese group (*n*=2 [6.3%]) to women with a normal BMI (*n*=1 [0.4%]), there was a significant difference among the groups with higher occurrence in the heavier women (OR: 3.7, 95% CI: 0.3–59.3, *p*=0.338 and OR: 18.1, 95% CI: 1.6–205.2, *p*=0.001, respectively). We did not find a statistical difference when looking at low-grade chronic villitis (occurrence 16.5%–18.7% among the groups, data not shown). Furthermore, we found a difference in maturation of the placental villi, with more accelerated maturation in the obese group *(n*=2 [6.3%] in the obese group vs n=0 (0%) in women with normal BMI; OR: 1.1, 95% CI: 1.0–1.2, *p*<0.001). We found more delayed maturation in the overweight group, although this did not reach statistical significance (OR: 1.6, 95% CI: 0.9–2.8, *p*=0.07) and the data did not show the same trend in the obese group. We found that overweight women had a smaller chance of delivering a placenta <10th percentile *(n*=12 [15.6%] in overweight BMI vs *n*=76

[27.8%] in normal BMI, OR: 0.5, 95% CI: 0.3–1.0, *p*=0.05). The obese group showed a similar trend, although not statistically significant (*n*=5 [15.6%] in obese BMI, OR: 0.5, 95% CI: 0.2–1.3, *p*=0.14). We found no significant differences in the presence of placental thrombosis, infarction (none vs. <5%), chorangiosis, MIR, or FIR. Placental weight, coiling index, and fetoplacental weight ratio did not vary significantly among the groups.

Furthermore, we found a significant correlation between maternal BMI and birth weight (Figure 1A, *r*=.113; *p*=0.037) and weight of the placenta (Figure 1B, *r*=.109; *p*=0.027) but not with birth weight percentile or fetoplacental weight ratios (data not shown). As we found that parity was consistently and significantly different over the BMI groups (Supplementary Figure 1, r=.243; *p*<0.001), we also performed linear regression analysis comparing parity to both birth weight and placental weight and found they were both significantly correlated (*r*=.134; *p*=0.0083 and *r*=.139; *p*=0.0065, respectively, Supplementary Figures 2 and 3).

TABLE 3. Risk of placental pathology characteristics per BMI category.

	Normal BMI <24.9 *n*=273	**Overweight BMI 25-29.9** *n*=77	**Obese BMI > 30** (*n*=32)
SGA placenta	Ref	**0.5 (0.3-1.0)**	0.5 (0.2-1.3)
LGA placenta	Ref	1.5 (0.7-3.3)	1.5 (0.5-4.8)
Hypocoiling	Ref	0.7 (0.4-1.3)	1.6 (0.7-3.4)
Hypercoiling	Ref	2.8 (0.6-12.8)	2.2 (0.2-19.6)
Meconium staining amniotic fluid	Ref	1.3 (0.7-2.4)	0.8 (0.3-2.1)
MIR ≥ stage 2 (%)	Ref	1.8 (0.7-4.3)	1.6 (0.4-5.6)
FIR ≥ stage 2 (%)	Ref	0.9 (0.2-4.4)	2.2 (0.4-10.8)
High grade chronic villitis (%)	Ref	3.7 (0.3-59.3)	**18.1 (1.6-205.2)**
Fetal Thrombosis (%)	Ref	1.7 (0.6-4.7)	2.1 (0.6-7.7)
Delayed villous maturation	Ref	1.6 (0.9-2.8)	1.3 (0.6-2.9)
Accelerated villous maturation	Ref	N/A	**1.1 (1.0-1.2)**
Elevated NRBC	Ref	1.2 (0.1-11.8)	1.0 (1.0-1.0)
Absence of placental infarction	Ref	0.7 (0.4-1.3)	0.4 (0.1-1.1)
Chorangiosis	Ref	0.9 (0.1-8.2)	4.5 (0.5-25.4)

Values are presented as odds ratio's (OR) and 95% confidence intervals. Values and statistical significance were calculated by chi square tests. Highlighted OR's were statistically significant with a *p*-value<0.05. *Abbreviations:* BMI; Body Mass Index, normal BMI (<24.9 kg/m^2), overweight (25.0-29.9 kg/m^2) and obese (>30.0 kg/m^2); SGA placenta; Small for gestational age placenta; placental weight <10th percentile. LGA placenta: large for gestational age placenta; placental weight > 90th percentile. MIR: relevant maternal inflammatory response; chorioamnionitis ≥ stage 2. FIR: relevant Fetal Inflammatory Response; acute funisitis ≥ stage 2. Chronic villitis: relevant when high grade/diffuse. Thrombosis: relevant when medium/high grade. N/A: not applicable. NRBC; nucleated red blood cells.

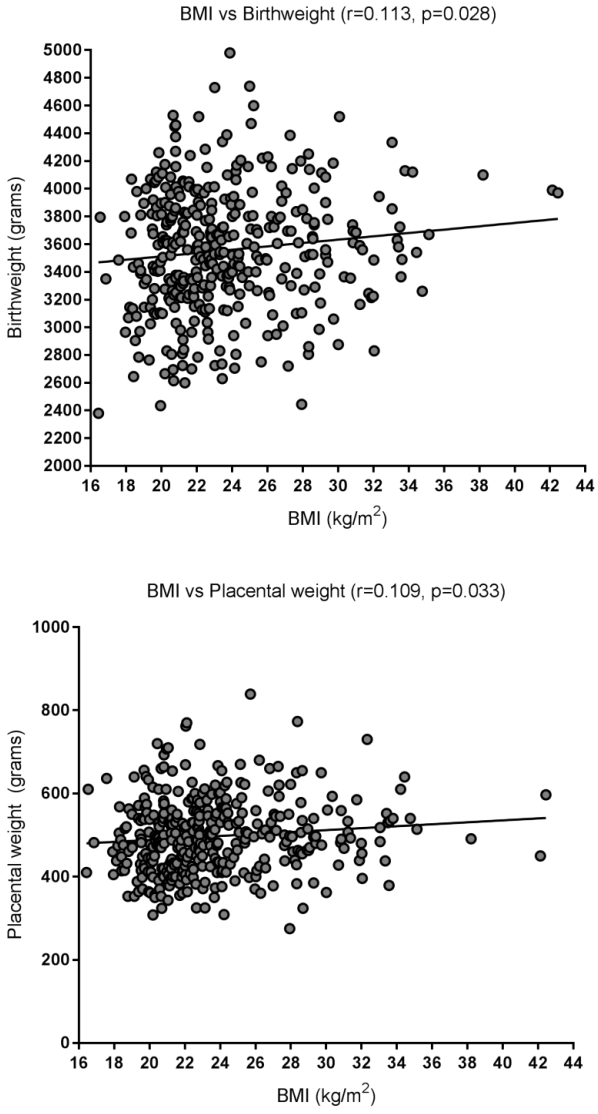

FIGURE 1. Neonatal birth weight (a) and placental weight (b) Pearson correlation with body mass index (BMI; kg/m^2) in singleton pregnancies in women with uncomplicated pregnancy *(n*=382).

DISCUSSION

This study shows the findings of prospective and systematic assessment of placental pathology in uncomplicated term pregnancies in a low-risk obstetric care setting, in association with prepregnancy BMI. We found that women with increasing BMI deliver placentas of a higher weight and babies with a higher birth weight. Despite the fact that these were all uncomplicated pregnancies with spontaneous vaginal delivery, we found that prepregnancy BMI was associated with characteristics of specific placental pathology, that is, more high-grade chronic villitis, more accelerated villous maturation, and less SGA placentas. We also found a substantial effect of parity on maternal, placental, and neonatal weights.

The relationship between higher BMI and adverse perinatal events is established, but the role of the placenta in mediating these complications is not entirely clear.[1] Evidence shows that women with a higher BMI deliver babies with a higher birth weight and heavier placentas.[22-24] Similar to previous reports, high BMI appears to affect placental weight more than the effect on neonatal birthweight.[22] It has been well established that restrictive dietary behavior is correlated to a lower placental weight, without necessarily decreasing neonatal birth weight.[25] How the placenta responds to higher amounts of lipids, glucose, and other metabolic factors is not clear. Placental metabolism is thought to increase and lipid induced inflammatory changes take place. Consequences of these effects are unknown but are thought to affect the future health of the fetus by altering the immune response.[7,26,27] Suboptimal maternal nutrition (either over or under) as well as abnormal placental weight (both high and low) have been shown to increase the risk of health problems in the adult offspring, including adult cardiovascular and metabolic morbidity.[7,28,29] We found a significant correlation between BMI and parity. Parity was also correlated with higher placental and fetal birth weight. Increase in BMI is associated with parity and shows an independently higher risk of pregnancy-related complications irrespective of being overweight or obese. A higher BMI is nonetheless related to higher risk of pregnancy complications irrespective of parity.[30]

Although numbers were relatively low, we found more chronic villitis present with increasing BMI. Previously published studies have shown obese women to present chronic higher levels of pro-inflammatory cytokines.[12,31] This chronic pro-inflammatory state is thought to extend to in utero life and therefore shows signs in the placenta.[7] A recent study by Leon-Garcia *et al.* also reported more chronic villitis within placentas from obese mothers, with specific differences when women delivered a girl or a boy. When they corrected for specific maternal variables (ie, hypertensive disorders and delivery by Caesarean section), the relationship with chronic villitis remained intact showing a robust association between this placenta characteristic and obesity.[15] This leads us to believe that even though our groups may have been smaller, the results remain relevant due to the uncomplicated nature of the group. Although low-grade villitis is not an uncommon finding in normal healthy placentas, high-grade villitis has a significant relation to FGR, neurodevelopmental impairment, fetal demise, and a chance of (increasingly severe) recurrence.[32-35] Interestingly, about half of women who

show recurrent placental chronic villitis are obese.[34] In a study in women who delivered SGA babies, increased BMI was related to increased presence of villitis and its pathological severity.[36] In our group, 3 of 4 (75%) women with high-grade villitis had placental weight below the 10th percentile (1 with normal BMI and 2 in the obese group) with 1 patient delivering a baby with low birth weight (normal BMI). Fetoplacental weight ratios in these pregnancies were high (above the 90th percentile in 3 patients with villitis) and therefore are thought to be highly effective placentas, producing babies with a normal weight despite chronic villitis.[24] Leon-Garcia *et al*. speculate that chronic villitis may play a role in downsizing placentas and babies that were destined to be large in women with obesity as a counter mechanism to obstetrical risks associated with LGA babies (ie, obstructed labor, dystocia).[15] The clinical relevance of chronic villitis and its relation to high BMI and/or placental metabolism, however, remains unclear and deserves further research with attention to its association with adverse pregnancy outcome.[7,35] Even though we did not find a statistical difference, other studies reported conflicting results on inflammatory lesions of both maternal and fetal origin found in the placenta of obese women.[13,14] The different findings among these studies or the lack of significant difference within groups is perhaps clarified by the high background inflammatory lesions in women with uncomplicated pregnancies and the inclusion of both healthy and complicated pregnancy in these cohorts.[13,14]

Besides chronic villitis, we found more accelerated and delayed villous maturation in the obese and overweight group. Accelerated villous maturation is a common pattern that can be seen with maternal vascular under perfusion and it may be present when the baby is growing faster than the placentas capacity.[20] This finding was not present throughout the groups, possibly due to the small number of obese patients and the uncomplicated pregnancy and history of the women in this study. Delayed villous maturation has been previously associated with both GDM and pregestational diabetes mellitus.[37,38] None of the women in this cohort were diagnosed with GDM, but it is possible that these women were underdiagnosed in clinical practice and their placental pathology showed signs of glucose regulatory problems.

Strengths and Limitations

Including only healthy uncomplicated pregnancy with unassisted delivery resulted in a relatively small group of obese women. This may be seen as a limitation to ascertain statistical significance. In other studies on this subject, almost half of the obese patients investigated either had (gestational) hypertension or were delivered by Caesarean section.[13,15] As many obese women are at the risk of pregnancy complications, we did not include these patients to explore the role of obesity on placental pathology without interaction through pregnancy disorders that have an established association with placental pathology. As we were not able to find information on prepregnancy BMI in all patients from the original study, we were only able to study a part of this group. The pregnancy characteristics in the selected study group were similar to the ones we had to exclude due to missing data on maternal BMI and therefore show relevant and unbiased outcomes that can be extrapolated to the general

uncomplicated obstetric population. The self-reported nature of prepregnancy weight and height has been shown not to be an issue when categorizing women into BMI groups.[39]

A strength of this study is that all placental examinations were performed by 1 specialized pathologist and systematically analyzed by the new Amsterdam criteria.[20] Prospective collection of all patients eligible for the study in a low-risk setting is another strong point. We believe that the study therefore gives rise to the idea that obesity may affect placental function not only in high-risk, or complicated, pregnancies but also in low-risk uneventful cases. Other limitations of the study include unanswered questions regarding the role of socioeconomic factors and ethnicity (as this was a relatively homogenous cohort), which may play an additional role in driving maternal BMI, and other potential residual confounders and/or mediators.

Conclusion

Prepregnancy BMI is significantly correlated with higher placental weight and a higher birth weight of the baby. Even though we studied a group of healthy and uncomplicated pregnancies, specific placental pathology (ie, chronic villitis and difference in villous maturation) was associated with a higher BMI. With obesity being a growing health issue among women of childbearing age, the risk of adverse pregnancy-related events for both mother and child increases. Further research is needed to evaluate these changes in view of later-life health of infants born to obese mothers.

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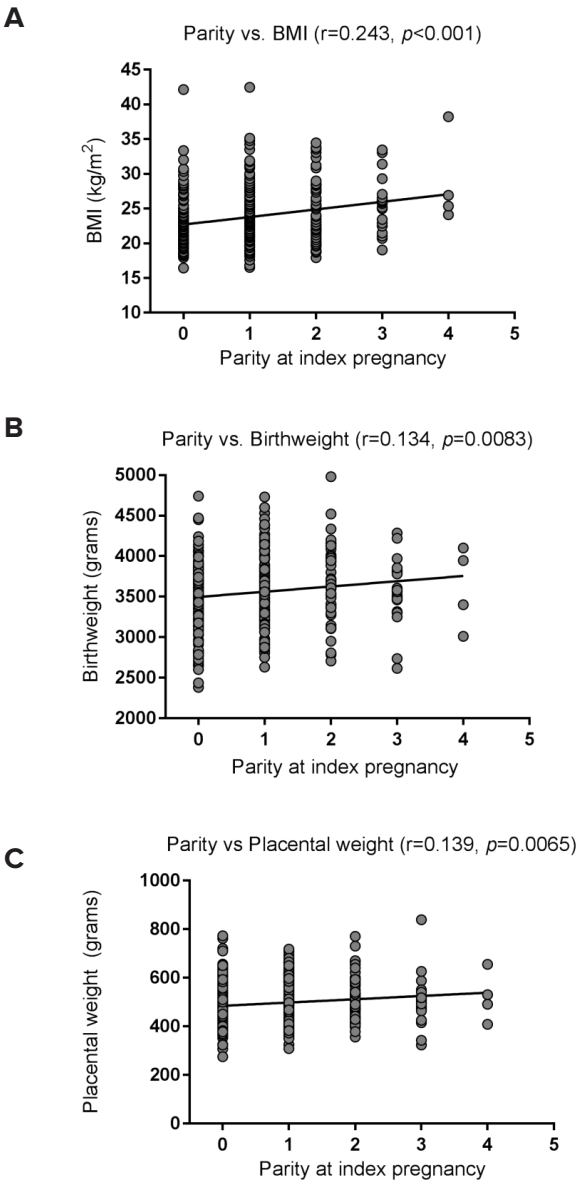
SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE 1. Definitions for placental histopathology.

Placental finding	Definition
Placental weight	Weight (grams), measured without membranes or umbilical cord
Fetoplacental weight ratio (f/p)	Rate of fetal to placental weight
Maternal Inflammatory Response (MIR)	Presence of polymorphonuclear leukocyte cells (PMN) in the chorionic plate or extraplacental membranes (acute chorioamnionitis)
Stage 0:	No inflammation; no presence of PMN
Stage 1:	Acute subchorionitis or chorionitis; frequent presence of PMN
Stage 2:	Acute chorioamnionitis: invasion of PMN in the chorionic plate, fibrous chorion and/or amnion
Stage 3:	Necrotizing chorioamnionitis: karyorrhexis of PMN, amniocyte necrosis, and/or amnion basement membrane hypereosinophilia
Grade 1:	Not severe as defined
Grade 2:	Severe: confluent PMN or with subchorionic microabscesses
Fetal inflammatory Response (FIR)	Aggregates of neutrophils in umbilical vascular wall, with or without expansion in Wharton's jelly (acute funisitis)
Stage 0:	No inflammation
Stage 1:	Chorionic plate vasculitis or umbilical phlebitis
Stage 2:	Involvement of the umbilical vein and one or more umbilical arteries
Stage 3:	Necrotizing funisitis
Grade 1:	Not severe as defined
Grade 2:	Severe: near-confluent intramural PMN with attenuation of vascular smooth muscle
Chronic villitis	Infiltration of lymphocytes and macrophages in the placental villi
Grade 0:	No inflammation
Grade 1 (low):	Inflammation <10 contiguous villi in any one focus; >1 focus
Focal	In one slide, all foci affecting <10 villi, with >1 focus
Multifocal	more than one slide, all foci affecting <10 contiguous villi
Grade 2 (High):	Multiple foci, on more than one section, >1 showing inflammation in >10 contiguous villi
Patchy	Multiple foci; >1 focus with ≥10 contiguous villi, ≥1 slides
Diffuse	>30% of all distal villi are involved
Fetal thrombosis	Diagnosed as at least 5 avascular fibrotic villi without inflammation or mineralization or if adherent thrombi in stem vessels were present
Delayed villous maturation	Monotonous villous population with reduced numbers of vasculosyncytial membranes for the period of gestation, as well as a continuous cytotrophoblast layer and centrally placed capillaries in at least one slide
Accelerated villous maturation	The presence of small or short hypermature villi for gestational period, usually accompanied by an increase in syncytial knots
Elevated NRBC	Circulating nucleated erythrocytes present in at least two capillaries in a random 10x field.
Placental abruption	Based on clinical diagnosis
Choriangiosis	≥ 10 vascular profiles per terminal chorionic villus in 10 villi in 10x field



SUPPLEMENTARY FIGURE 1. (**A**) Pearson correlation parity with body mass index (BMI; kg/m^2) in singleton pregnancies of women with uncomplicated pregnancy (*n*=382). (**B**) Pearson correlation parity with neonatal birthweight (grams) in singleton pregnancies of women with uncomplicated pregnancy (*n*=382). (**C**) Pearson correlation parity with placental weight (grams) in singleton pregnancies of women with uncomplicated pregnancy (*n*=382).

PART II

Maternal cardiovascular health after preeclampsia

7

Recurrence of preeclampsia and the risk of future hypertension and cardiovascular disease: a systematic review and meta-analysis

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ABSTRACT

Background

Women with a history of hypertensive disorders, including preeclampsia, during pregnancy have a two- to-fivefold increased risk of cardiovascular disease (CVD). In 15% of women, preeclampsia recurs in the following pregnancy.

Objectives

To evaluate all evidence on the future risk of developing hypertension and CVD after multiple pregnancies complicated by preeclampsia compared with preeclampsia in a single pregnancy followed by normal subsequent pregnancy.

Search strategy

Embase and Medline were searched until June 2017.

Selection criteria

All relevant studies on the risk of developing hypertension, atherosclerosis, ischaemic heart disease, cerebrovascular accident (CVA), thromboembolism, heart failure or overall hospitalisation and mortality due to CVD after having had recurrent preeclampsia.

Data collection and analysis

Twenty-two studies were included in the review. When possible, we calculated pooled risk ratios (RR) with 95% CI through random-effect analysis.

Main results

Recurrent preeclampsia was consistently associated with an increased pooled risk ratio of hypertension (RR 2.3; 95% CI 1.9–2.9), ischaemic heart disease (RR 2.4; 95% CI 2.2–2.7), heart failure (RR 2.9; 95% CI 2.3–3.7), CVA (RR 1.7; 95% CI 1.2–2.6) and hospitalisation due to CVD (RR 1.6; 95% CI 1.3–1.9) when compared with women with subsequent uncomplicated pregnancies. Other studies on thromboembolism, atherosclerosis and cardiovascular mortality found a positive effect, but data could not be pooled.

Conclusions

This systematic review and meta-analysis support consistent higher risk for future development of hypertension and CVD in women with recurring preeclampsia as opposed to women with a single episode of preeclampsia.

INTRODUCTION

At present, the World Health Organization states that one in five women suffer from hypertension and almost half of mortality in women is caused by cardiovascular disease (CVD).[1,2] In the past decades, large cohort studies have consistently shown an increased association of CVD in women with a history of preeclampsia compared with women with uncomplicated pregnancies.[3,4] This has led to a better understanding of female-specific risk factors for developing CVD.[5,6] Preeclampsia complicates approximately 3–5% of first pregnancies and recurs in approximately 15% of subsequent pregnancies.[3,7,8] Common underlying risk factors such as obesity, dyslipidaemia, inflammatory pathways and endothelial dysfunction are thought to contribute to both CVD and preeclampsia complicated pregnancies.[9-12] As pregnancy requires comprehensive physiological changes in the endocrine, respiratory and circulatory systems, a complicated pregnancy may reveal a predisposition to CVD and act as a 'stress test' identifying women at risk for future disease. Whether metabolic and cardiovascular changes induced by preeclampsia independently create a higher risk of CVD remains unknown.

Some countries have recently started notifying formerly pre-eclamptic women of their increased risks of CVD and advise women to actively test for modifiable risk factors at an early age.[13-16] Pre-emptive screening, early recognition and treatment may prove to be useful in preventing long term morbidity and mortality.[1] It is well known that women with preeclampsia in their first pregnancy tend to have a milder variant or no disease in following pregnancies.[8]B. It is conceivable that women who experience multiple episodes of preeclampsia fail to adjust to the physiological changes and physical stresses more than women with subsequent uncomplicated pregnancies. As not all women with preeclampsia develop CVD later in life, the recurrence of disease may be a helpful indicator for the necessity of screening. Previous studies briefly mention an effect of multiple preeclampsia-affected pregnancies on the risk of future hypertension and CVD. Although several reviews have been conducted on the relationship between pregnancy outcome and CVD, to the best of our knowledge, no systematic review or meta-analysis has been conducted on recurrent pregnancy complications and future lifetime cardiovascular risk. This study aims to evaluate all available evidence on the effect that recurrent preeclampsia has on long-term CVD risk compared with a single episode of preeclampsia with subsequent uncomplicated pregnancy.

METHODS

Study Population

Medline and Embase were searched (until 1 June 2017) using search terms for 'preeclampsia', 'hypertension' and 'cardiovascular disease'. We restricted the search to various synonyms for 'recurrent', 'follow up', 'risk' and 'history', as many articles have been published on CVD after preeclampsia. A detailed description of the search strategy can be found in the Supplementary Appendix 1. Reference lists of original and review articles were reviewed. Articles in languages other than English or Dutch were translated using Google Translate and included when translation quality was sufficient. Unpublished studies were not included. The core outcome set for CVD after pregnancy complications (COMET registration number 701) is currently being developed and could not be used for this systematic review. There was no patient or public involvement in the carrying out of this study.

Inclusion and exclusion criteria

Inclusion criteria were as follows: (1) original articles; (2) studies that compared women with recurrent preeclampsia to women with a single episode of preeclampsia followed by uneventful pregnancies; (3) cerebrovascular accident (CVA), ischaemic heart disease (IHD), thromboembolism, atherosclerosis, heart failure, CVD mortality, hypertension or cardiovascular hospitalisation as outcome; (4) full-length article available; (5) inclusion of more than ten women; and (6) adult population. Studies with a follow-up duration of < 1 year were excluded. We only included studies in which specific data on preeclampsia could be subtracted.

Study selection and data extraction

Two reviewers (LB, and CS or AJM) independently reviewed the title/abstract of all potential studies. As most articles do not show data on recurrence in their respective abstracts, many were reviewed as full text. Disagreement was resolved by discussion and consensus; if needed, the opinion of a third reviewer was decisive. Authors of articles with a promising study set-up but no data in association with recurrence of preeclampsia were contacted through email and allotted 3 months for a response. Data were extracted from each paper independently and included all relevant study specifics (i.e. definition of preeclampsia, follow-up time, outcome measures).

Assessment of study quality and bias

The methodological quality of studies was assessed using the Newcastle–Ottawa quality assessment Scale for cohort and case–control by two researchers (LB/AJM) independently. The Newcastle–Ottawa Scale uses a scoring system of three categories: selection, comparability and outcome (cohort studies) or exposure (case–control studies).[17] When a cohort study was based on one single cohort of women, the scale was adjusted

accordingly. The Newcastle–Ottawa Scale scoring of each included study can be found in Supplementary Table 1.

Statistics

Incidence numbers were extracted from the data reported in each paper. When extracting hazard ratios (HR) and odds ratios (ORs), the most complete multivariate models were used to adjust for potential confounders. Most studies compared single or multiple occurrence preeclampsia affected women to women without any complicated pregnancies, without making a comparison between the two affected groups. All hazard ratios described below are in comparison with women with solely uncomplicated pregnancy. We performed a meta-analysis to give a direct overview of the risk when comparing the groups among themselves. *Review manager* 5.3.5 was used to calculate pooled risk ratios (RR) with 95% CI using a random-effects model. To measure the amount of between-study variation that is due to systematic heterogeneity rather than chance, the I^2 metric was used. We used the MOOSE checklist and PRISMA guidelines for this systematic review.[18,19] This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

TABLE 1. Characteristics and outcomes of studies about recurrent preeclampsia in association with hypertension.

Author, year published	Country, baseline years study	Study design	Exposure	Definition of Preeclampsia		Definition of outcome	No. of participants in study	Follow-up time (median, range) (years)	Age at follow-up (median, range) (years)	Outcome‡
				SBP, DBP (mmHg)	Proteinuria					
Singh, 1974[36]	UK, NS	RCS	Severe PE	NS	NS	Mean BP (mmHg)	147	17.8	40	sPE 137 (1.9)/87 (1.2) rPE 152 (3.9)/92 (2.8)
Sibai, 1986[24]	USA, NS	RCS	Severe PE /E	> 160, >110	> 1g/ 24hrs	Antihypertensive medication	815	6.6	30.9	OR 7.4 (NS)
Sibai, 1991[38]	USA, NS	RCS	Severe PE (2nd trimester)	NS	NS	Antihypertensive medication	125	5.4	NS	OR 10.7 (NS)
Nisell, 1995[30]	Sweden 1986	RCS	PIH / PE	>140, >90	>0.3g/24hrs	Antihypertensive medication OR two measurements of DBP ≥90 mmHg with a 15 min. interval	138	7	NS	Incidence sPE 21% rPE 25%
Gaugler-Senden, 2008[27]	The Netherlands 1993-2003	CCS	Severe PE/E/HELLP (GA<24wks)	NS, >110 >140,>90	2+ [1g/L)	>140 and/or >90 mmHg, and/or use of antihypertensive medication	40	5.5	38.8	NS
Lykke, 2009[21]	Denmark 1978-2007	RCS	PE/E/HELLP	ACOG criteria		ICD-8/ICD-10: DI10-13, DI15, 400-404	536.419	12.9	42.6	sPE HR 2.7 (2.5-2.9)* rPE HR 6.0 (5.4-6.7)*
Magnussen, 2009[22]	Norway 1967-1995	PCS	PE	>140, >90	>0.3g/24hrs	Mean BP (mmHg)	15.065	16.5	40.1	sPE 132.2 (130.7-133.7) rPE 136.7 (133.6-139.9)
						Antihypertensive medication				sPE OR3.1 (2.2-4.3) rPE OR 11.6 (7.1-26.3
Smith, 2009[28]	Canada, NS	PCS	PE	>140 >90	>0.3g/24hrs ≥2 on dipstick	Mean BP (mmHg)	70	1	NS	NS
Spaan, 2012[39]	The Netherlands 1996-2010	RCS	PE	>140, >90	>0.3g/24hrs	Self-reported use of antihypertensive medication	339	6	NS	HR 4.3 (1.6-11.5)
Van Oostwaard, 2012[40]	The Netherlands 2000-2002	RCS	PIH/PE/HELLP preterm	>140, >90	>0.3g/24hrs	Self-reported chronic hypertension	189	8.5	NS	RR 2.4 (1.0-5.4)†
Ghossein-Doha, 2014[32]	The Netherlands, 1996-1999	NCCS	PE	>140, >90	>0.3g/24hrs	BP ≥ 140/90 mmHg	28	14	43	Incidence sPE 22% rPE 86%
van Oostwaard, 2014[41]	The Netherlands 2000-2002	RCS	PIH/PE/HELLP term	>140, >90	>0.3g/24hrs	Self-reported chronic hypertension	120	11	NS	RR 1.8 (1.1-3.0)†
Engeland, 2015[20]	Norway 1967-2012	PCS	PE/E/HELLP	>140, >90	>0.3g/24hrs	Antihypertensive medication	978.493	46	NS	sPE HR 2.0 (2.0-2.0)* rPE HR 2.8 (2.7-3.0)*
Scholten, 2015[35]	The Netherlands 2008-2010	PCS	PE	>140, >90	>0.3g/24hrs	BP ≥ 140/90 mmHg	104	4.6	36	Incidence sPE 15.9% rPE 27.8%
Veerbeek, 2015[37]	The Netherlands 2008-2010	PCS	PE/ PIH	>140 >90	>0.3g/24hrs ≥2 on dipstick	Mean BP (mmHg)	152	2-5	31-34	sPE 124.2 (13.2)/81.9 (9.4)† rPE 123.7 (18.3)/83.8 (12.6)†
Zhang, 2015[34]	China 2009-2013	PCS	PE/E/ superimposed PE	NS	NS	NS	115	5	NS	Incidence sPE 23.3% rPE 47.5%
Auger, 2017[26]	Canada 1989-2013	PCS	PE	ICD-9: 642.3, 642.4, O13, 642.5, 642.6, O14, O15, 642.7, O11		ICD-9: 401-405, 416.8, 437.2, 461.0, ICD-10: I10-I15, I27.0, I67.4	1.108.581	14.5	NS	sPE HR 3.7 (3.5-3.9)* rPE HR7.2 (6.6-7.8)*

NS: data not shown or specified. *Hazard ratio in comparison to women without any complicated pregnancies. ‡ Outcome is specified in either odds ratio, hazard ratio or risk ratio with a 95% confidence interval or mean blood pressure (mmHg) with standard deviation or 95% confidence interval as presented in the corresponding study. † Numbers or data received from the author. Abbreviations; SBP: systolic blood pressure (mmHg), DBP: diastolic blood pressure (mmHg) RCS: retrospective cohort study, CCS: case-control study, PCS: prospective cohort study, NCCS: nested case-control study. PE: preeclampsia, sPE: women with one pregnancy with preeclampsia and normal subsequent pregnancy, rPE: women with recurrent preeclampsia, E: eclampsia, PIH: pregnancy induced hypertension, HELLP: haemolysis elevated liver enzymes low platelets syndrome. GA: gestational age. HT: hypertension, CH: chronic hypertension, BP: blood pressure.

RESULTS

Characteristics of the studies

We identified 22 studies for this review, a detailed description of the selection process can be found in the Supplementary Figure 1. Tables 1 and 2 summarise the characteristics of the studies included for hypertension and cardiovascular morbidity and mortality, respectively. There was a large variance in sample sizes (28–1.108.581) and study populations were selected from all over the world with a majority from northwest Europe, Canada and the USA. Follow up ranged from 1 to 45 years. Most studies used the American Congress of Obstetricians and Gynecologists criteria to identify women with preeclampsia. The definition of hypertension as an outcome varied between measured mean blood pressure, antihypertensive treatment, hospital diagnosis, International Classification of Diseases (ICD) coding and self-reported disease or treatment. When looking at cardiovascular morbidity and mortality, the majority of studies used record linkage through ICD codes and death certificates.

Quality of evidence

The quality score of the included studies can be found in the Supplementary Table 1. Six studies reached the respective maximum of stars among the cohort and case–control studies (maximum nine stars)[20-25]; three studies received eight stars,[26-28] two received seven,[29,30] four received six,[31-34] one received five[35] and one study obtained three stars.[36] For studies comparing a single cohort, one study received the maximum score of seven stars.[37] The remaining four studies received five of seven stars.[38-41]

Hypertension

Overall, 17 papers were found to report on developing hypertension after recurrent preeclampsia, details can be found in Table 1. Four studies reported on mean blood pressure after variable lengths of follow up.[22,28,36,37] Two studies performed their analysis 1–5 years postpartum and did not find any difference between the groups.[28,37] Two studies followed women for almost two decades, finding a significant increase in mean blood pressure in the group with recurrent preeclampsia.[22,36] Five studies found higher risk of antihypertensive medication use when preeclampsia was recurrent compared with a single complicated pregnancy and when compared with women with uncomplicated pregnancy only.[20,22,24,38,39] Two smaller studies looked at the proportion of recurrent preeclampsia in women who were hypertensive at follow up after having had preeclampsia in the index pregnancy and found conflicting results.[32,35] Three studies observed women who went on to have subsequent pregnancies after preeclampsia, in the first two articles a nonsignificant association was mentioned in the text.[27,30,34]

One study found a higher incidence of hypertension among women with recurring preeclampsia, although information on their study set-up was limited.[34] Van Oostwaard *et*

al.[40,41] published on the risk of hypertension in women with recurrent pregnancy induced hypertension or preeclampsia. The author kindly shared data regarding preeclampsia only, resulting in relatively small study groups. For women delivering at term (n = 74) and preterm (n = 59) there was a significantly higher chance of hypertension after recurrence of preeclampsia compared with women with a normal subsequent pregnancy (RR 1.83; 95% CI 1.11–3.02 and RR 2.35; 95% CI 1.02–5.43, respectively). Two groups performed large registry-based cohort studies with ICD codes comparing women with preeclampsia with women with only non-hypertensive pregnancy.[21,26] One study showed a risk gradient, with a higher risk of hypertension after preeclampsia in the first pregnancy (HR 2.70; 95% CI 2.51–2.90) compared with women with two or more normotensive pregnancies. The risk increased (HR 4.34; 95% CI 3.98–4.74) for women with preeclampsia in their second pregnancy only, and increased again for women with preeclampsia in both pregnancies (HR 6.00; 95% CI 5.40–6.67).[21] Auger *et al*.[26] reported increasing hazard ratios of 3.7 (95% CI 3.5–3.9) for a single episode of preeclampsia and 7.2 (95% CI 6.6–7.8) for recurrent preeclampsia when compared with women who only had normotensive pregnancies, 25 years after the index pregnancy.

Meta-analysis

Incidence data on 52.544 women could be extracted from seven studies to perform a meta-analysis.[21,24,26,34,38,40,41] In the pooled analysis the risk ratio for hypertension after follow up was increased in women with recurrent preeclampsia (pooled RR 2.33; 95% CI 1.86–2.92, Figure 1.1.1). Heterogeneity between studies was considerable (I^2 = 82%), so a sensitivity analysis was performed. When excluding the largest study by Auger *et al*.[26] from the pooled analysis, heterogeneity tested was lower (I^2 = 1%), nonetheless the found effect did not change (pooled RR 2.57; 95% CI 2.32–2.85).

Atherosclerosis

Two studies reported on atherosclerosis after recurrent preeclampsia.[26,31] An ICD-code for atherosclerosis was found to be significantly more present in the recurrent preeclampsia group (HR 4.0; 95% CI 3.0–5.3) than in single affected women (HR 2.1; 95% CI 1.8–2.5) when compared with women with solely uncomplicated pregnancies.[26] Akhter *et al*. performed carotid artery intima-media thickness measurements in 42 women with previous preeclampsia. Although they found significantly higher intima-media thickness for women who had preeclampsia, they did not find a higher measurement when preeclampsia had recurred.[31]

Thromboembolism

Two record-linkage studies reported on various types of thrombosis after one or multiple preeclampsia-affected pregnancies compared with women with only uncomplicated pregnancy.[21,26] One study discusses both deep venous thrombosis and pulmonary embolism in one category showing increasing hazard ratios when preeclampsia was recurrent.[21] Auger

et al.[26] discussed results separately, finding higher hazard ratios for both outcomes when comparing single and recurrent preeclampsia with women with uncomplicated pregnancies (Table 2).

Ischaemic heart disease

Three record-linkage studies reported on IHD after one or multiple preeclampsia-affected pregnancies compared with women with only uncomplicated pregnancies. Riise *et al*. reported an increasing hazard ratio after recurrence of preeclampsia (HR 2.20; 95% CI 0.91–5.32 in recurrent preeclampsia and HR 1.95; 95% CI 1.31–2.91 for a single preeclampsia pregnancy), compared with unaffected pregnancies. When preeclampsia was combined with fetal growth restriction or preterm birth the change in hazard ratio was more significant (HR 4.66; 95% CI 2.31–9.37 in recurrent preeclampsia as opposed to one episode of preeclampsia; HR 2.81; 95% CI 1.70–4.61).[23] The other two ICD-coded studies showed a similar increase in hazard ratio when comparing women with single or multiple affected pregnancies with women without pregnancy complications (Table 2).[21,26]

Meta-analysis

With all studies combined, 10 522 women who had recurrent preeclampsia contributed to the meta-analysis on IHD.[21,23,26] In the pooled analysis an increased risk of IHD was observed for recurrent preeclampsia (RR 2.40; 95% CI 2.15–2.68; Figure 1.1.2). Heterogeneity between the studies was low (I^2 = 0%).

Heart failure

Three studies described the development of heart failure. The two record-linkage studies mentioned above indicated higher hazard ratios for recurrent preeclampsia than for single preeclampsia-affected pregnancy compared with solely uncomplicated pregnancies (Table 2).[21,26] Ghossein-Doha *et al*.[29] reported on (non-symptomatic) heart failure type-B diagnosed by cardiac ultrasound 4–10 years postpartum. They did not find recurrence of preeclampsia to be significantly associated with this type of heart failure (OR 2.0; 95% CI 0.7–5.2).

Meta-analysis

Due to the difference in outcome measures, only the data from two studies were comparable (Figure 1.1.3).[21,26] In total, 9585 women had recurrent preeclampsia and showed a pooled risk ratio 2.88 (95% CI 2.23–3.72). Heterogeneity was low (I^2 = 27%).

Cerebrovascular accident

The same two record-linkage studies performed analysis on ischaemic and haemorrhagic CVA, finding higher adjusted hazard ratios for the women with recurrent preeclampsia than women with a single pregnancy with preeclampsia when compared with solely uncomplicated pregnancy (Table 2).[21,26]

Meta-analysis

When results of both studies were combined a risk ratio of 1.69 (95% CI 1.21–2.35) was found with heterogeneity of 75% (Figure 1.1.4).

Cardiovascular events and hospitalisation

Kessous *et al*. performed a retrospective population study reporting on simple and complex cardiovascular events. Simple CVD events (i.e. hyperlipidaemia, hypertension) occurred significantly more in women with two or more pregnancies complicated by preeclampsia (2.2% versus 1.6%; P = 0.001). Complex cardiovascular events (i.e. IHD, heart failure) occurred more frequently in the recurrent preeclampsia group compared with women with one preeclampsia-affected pregnancy (4.6% versus 2.7%; P = 0.001). Patients were also admitted to the hospital more often due to CVD (6.0% versus 4.0%; P = 0.001).[33] This last outcome was also analysed in the record-linkage study by Auger *et al*., finding a similar trend [HR 3.9; 95% CI 3.6–4.2 (recurrent preeclampsia) versus HR 2.3; 95% CI 2.2–2.4 (single preeclampsia), compared with solely uncomplicated pregnancy].[26]

Meta-analysis

When data were pooled for cardiovascular hospitalisations a pooled risk ratio of 1.57 (95% CI 1.31–1.90) was found with some heterogeneity (I^2 = 60%, Figure 1.1.5).

Cardiovascular mortality

Only one study analysed the association between recurrent preeclampsia and cardiovascular mortality. Even though they showed increasing hazard ratios for women with one, two or more preeclampsia-complicated pregnancies, there was no statistical significance when comparing the groups.[25]

TABLE 2. Characteristics and outcomes of studies about recurrent preeclampsia in association with cardiovascular morbidity and mortality.

Author, year published	Country, baseline years study	Study design	Exposure	Definition of Preeclampsia: SBP, DBP (mmHg)	Definition of Preeclampsia: Proteinuria	Outcome	Definition of outcome	No. of participants in study	Follow-up time (median, range) (years)	Age at follow-up (median, range) (years)	Outcome‡
Lykke, 2009[21]	Denmark 1978-2007	RCS	PE/E/ HELLP	ACOG criteria		**IHD**	ICD-9: 410-414, ICD-10: i20-i25	536 419	12.9	NS	sPE: HR 1.3 (1.1-1.5)* rPE: HR 2.8 (2.3-3.4)*
						HF	ICD-8: 42709-42711, 42719, 42799, 42899, 42900, 42908, 42909, ICD-10: i50, i513, i519,				sPE: HR 1.3 (1.0-1.7)* rPE: HR 2.4 (1.6-3.5)*
						TE	ICD-9: 444, 450, 451, ICD-10: i26, i74, i82.				sPE: HR 1.3 (1.1-1.6)* rPE: HR 1.8 (1.3-2.5)*
						CVA	ICD-10: i60-i67, g45. ICD-8: 430-438				sPE: HR 1.2 (1.1-1.4)* rPE: HR 1.5 (1.2-1.9)*
Skjaerven, 2012[25]	Norway 1967-2002	PCS	PE	>140, >90	>0.3g/ 24hrs	**CVD death**	ICD-8/9: 390-459, 410-414, 430-438 ICD-10 i00-i99, i20-i25, i60-69.	700 400	7-42	NS	sPE: HR 1.5 (1.2-1.9)* rPE: HR 2.3 (1.5-3.6)*
Akhter, 2014[31]	Sweden, NS	CCS	PE	>140, >90	>0.3g/ 24hrs ≥2 on dipstick	**Atherosclerosis**	Carotid artery intima-media thickness measurements using ultrasound	42	NS	40-50	NS
Kessous, 2015[33]	Israel 1988-2012	RCS	PE	NS		**Simple cardiovascular events**	ICD-9 4149, 4292, 4438, 4139, 44389, 436, 437, 413, 4371, 4118, 41181, 2722, 2724, 402, 411, 414, 4148, 4299, 440, 4402, 4019, 4439)	1182	0-24	NS	Incidence sPE 1.6% rPE 2.2%
						Complex cardiovascular events	ICD 9 codes 410, 4280, 4281, 4289, 4280, 404, 4049, 4275, 415, 4150				Incidence rPE 2.7% rPE 4.6%
						Cardiovascular hospitalisation	NS				Incidence sPE 4.4% rPE 6.0%
Auger, 2016[26]	Canada 1989-2013	PCS	PE	ICD-9: 642.3, 642.4, O13, 642.5, 642.6, O14, O15, 642.7, O11		**CVD overall**	ICD-9: 401-445, 447-453, ICD-10: i10-i82	606 820	16	NS	sPE: HR 2.3 (2.2-2.4)* rPE: HR 3.9 (3.6-4.2)*
						IHD	ICD-9: 410-414, ICD-10: i20-i25				sPE: HR 1.9 (1.7-2.2)* rPE: HR 3.3 (2.6-4.2)*
						CVA	ICD-9: 433-438 ICD-10: I63-I66.9, I67.2				sPE: HR 1.6 (1.4-1.9)* rPE: HR 3.0 (2.3-4.1)*
						Atherosclerosis	ICD-9:414.0, 414.3, 414.4, 429.2, 440, ICD-10: i25.0, i25.1, i70				sPE: HR 2.1 (1.8-2.5)* rPE: HR 4.0 (3.0-5.3)*
						DVT	ICD-9: 451.1, 451.83, 453.4-453.5, 453.72, 453.82, ICD-10: i80.1-i80.3				sPE: HR 1.7 (1.3-2.1)* rPE: HR 1.3 (0.7-2.3)*
						Pulmonary Embolism	ICD-9: 415.1, ICD-10: I26				sPE: HR 1.2 (1.0-1.5)* rPE: HR 1.4 (0.9-2.1)*
						HF	ICD-9: 428, ICD-10: i50				sPE: HR 2.0 (1.6-2.5)* rPE: HR 4.2 (2.9-6.1)*
Ghossein-Doha, 2017[29]	The Netherlands, NS	CSC	PE	>140 >90	>0.3g/ 24hrs	**HF-B (non-symptomatic)**	Determined by cardiac ultrasound	107	4-10	36-40	OR 2.0 (0.7-5.2)
Riise, 2017[23]	Norway 1980-2002	PCS	PE	>140, >90	>0.3g/ 24hrs	**IHD**	ICD-9: 410-414, ICD-10: i20-i25	281 069	18	NS	sPE: HR 2.8 (1.7-4.6)* rPE: HR 4.7 (2.3-9.4)*

NS: data not shown or specified. *Hazard ratio in comparison to women without any complicated pregnancies. ‡ Outcome is specified in either odds ratio or hazard ratio with 95% confidence interval or incidence (%) as presented in the corresponding study. Abbreviations: SBP: systolic blood pressure (mmHg), DBP: diastolic blood pressure (mmHg), PCS: prospective cohort study, RCS: retrospective cohort study, CCS: case-control study, CSC: cross sectional cohort study. PE: preeclampsia, E: eclampsia, HELLP: haemolysis elevated liver enzymes low platelets syndrome, sPE: women with one pregnancy with preeclampsia and normal subsequent pregnancy, rPE: women with recurrent preeclampsia. CVD: cardiovascular disease, IHD: ischemic heart disease, HF: fear failure, DVT: Deep venous thrombosis, TE: thromboembolism, HF-B: heart failure type B. ACOG: American college obstetrics and gynecology. ICD: international classification of disease. NS: data not shown/specified. HR: hazard ratio, OR: odds ratio. HR: hazard ratio.

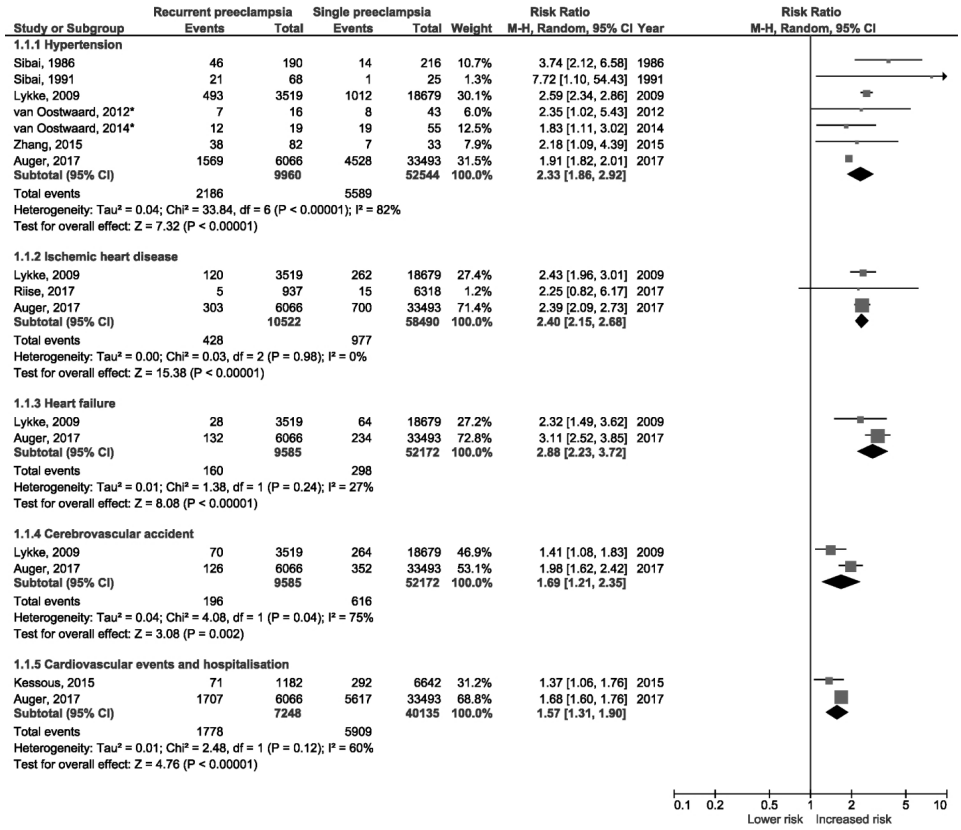

FIGURE 1. Forest plot of studies investigating the risk of hypertension (1.1.1), ischaemic heart disease (1.1.2), heart failure (1.1.3), cerebrovascular accident (1.1.4) and overall hospitalisation due to cardiovascular disease (1.1.5) after recurrent preeclampsia when compared with women with a single pregnancy affected by preeclampsia and subsequent normal pregnancy. Incidence data were extracted from original articles using available figures and tables. *Original data provided by author was used.

DISCUSSION

Main findings

In this systematic review and meta-analysis we aimed to provide a comprehensive overview of available evidence on cardiovascular disease after recurrent preeclampsia. We found that women with recurrent preeclampsia have a threefold increased risk of heart failure, two- to three-fold risk of hypertension and IHD and almost a two-fold risk of CVA and overall CVD, when compared with women with a single event of preeclampsia and subsequent uncomplicated pregnancies. Although the set-up, size and quality of studies were variable, our pooled analysis indicated that the overall association between recurrent preeclampsia and CVD is a robust finding. As women with a history of preeclampsia have been shown to be at increased risk of CVD, this identifies a subgroup of women who are at even greater risk and could benefit from early preventive measures.

Strengths and limitations

This systematic review provides an overview of all available evidence up until June 2017. As most evidence on the risk of CVD after recurrent preeclampsia is based on small groups of women, the only way to obtain reliable results is by performing a meta-analysis. A random-effects model was used to incorporate between-study variation. We only included studies in which preeclampsia was clearly defined, leading to a clear and consistent additional risk of CVD in later life based on recurrence of this disease.

This study also has some limitations which need to be addressed. First, included studies date back to the 1970s and show a wide range of methodological quality. Only seven of 22 studies achieved the maximum score on the Newcastle–Ottawa Scale. Therefore, caution is needed when interpreting the results. Second, comparability between included studies is limited as definitions for exposure, outcome and effect measure differ. Consequently few data could be used for meta-analysis, possibly resulting in an over- or under-estimating of the risk when patient characteristics differ between and within studies. The small number of studies and the large population size of the main studies instantly lead to a higher heterogeneity in the meta-analysis.[42] Also, different measurement of outcome, adjusting for confounders and duration of follow up, can lead to more variation than is to be expected. It could be argued that the addition of meta-analysis from a relatively small number of studies is not likely to improve accuracy in the effect estimates when findings are consistent within the studies, but does illustrate the continuing need for better original data. Third, we identified several studies in which analysis of recurrence of preeclampsia and development of CVD should be possible given the design of the study but was not mentioned in the paper. We experienced a low response rate to multiple emails to authors, hindering our inclusion of more studies. We speculate that it may be possible that some groups looked at this association, but found relatively small groups and minor correlations that were not important enough to mention, possibly leading to a form of publication bias. We believe it

would be beneficial if more studies included recurrence of preeclampsia in their work to allow for improvement of our pooled estimated in the future. Finally, the larger registry-based studies used registered ICD codes upon discharge as outcome for hypertension and CVD.[21-23,26] As the development of CVD and specifically hypertension does not always require hospitalisation, it is possible that only the most severe cases have been included, possibly leading to selection bias.

Interpretation

Several reviews and meta-analyses discuss the risk of CVD after pregnancy complications.[3,4,6,43] Only a few discuss recurrence as a factor, usually stating a higher risk of CVD based on one or two studies. Mechanisms explaining the relation of CVD and preeclampsia are thought to be multifactorial. Several large studies have shown preeclampsia to be an independent risk factor when correcting for several established cardiovascular risk markers, such as hypertension.[3] The significant correlation between preeclampsia, recurrence and the development of hypertension, results in our hypothesis that hypertension does not solely explain the association. We have yet to elucidate whether (recurrent) pre-eclamptic pregnancies induce metabolic and cardiovascular changes or if these women have a stronger predisposition for CVD.[21,22,26,39]

Other mechanisms that potentially plays a role in preeclampsia and CVD are of an inflammatory nature with chronic inflammatory risk markers being significantly higher in former preeclampsia patients.[6,44] Preeclampsia and CVD also share other pathological features indicating similar pathways such as the presence of acute atherosis and endothelial cell dysfunction.[45,46] All of the above strengthens the idea that pregnancy can be seen as a 'stress test' for cardiovascular health, identifying women at risk early in life.

Women with an early onset and/or severe preeclampsia are more likely to experience recurrence of disease compared with those who developed preeclampsia at term.[7,8] Several studies found a consistently higher risk of cardiovascular morbidity and mortality when preeclampsia was early in onset or when combined with (iatrogenic) preterm birth or other complications like fetal growth restriction, irrespective of recurrence of preeclampsia.[21,23,25] In one study from Riise *et al*.,[23] a steadily increasing risk of cardiovascular death after recurrent preeclampsia was found with higher hazard ratios when (recurrent) preeclampsia concurred with preterm delivery or fetal growth restriction. Unfortunately, there were no studies in which data on severity, time of onset and recurrence could be extracted for meta-analysis.[3,33] Therefore, we cannot infer from our review to what extent the association between recurrent preeclampsia and CVD is explained by the onset and/or severity of the first episode.

Several studies within this review discussed timing as a factor in determining cardiovascular risk, morbidity and mortality. When looking at cardiovascular risk markers, studies found significant correlation after many years of follow up, even though these markers may not be apparent soon after pregnancy.[27,28,30-32,35,37,40] A few studies analysed

time between preeclampsia and CVD in their set-up, reporting a significantly shorter time to cardiovascular events in the recurrent group with a significantly accelerated disease progression. The magnitude of this time-specific association appears to decrease over time but remained significant when comparing recurrent and non recurrent preeclampsia.[20,26] Several studies have shown that hypertension is present shortly after preeclampsia.[47,48] After a longer latency period, age-specific risk factors might play a more prominent role and risk for hypertension and CVD in women with normotensive subsequent pregnancies will become more alike. However, studies with a longer follow up still showed significant increased risk in the recurrent group.[21,26,41]

Some studies mention the high risk of having had preeclampsia among women having only one pregnancy. Possibly, women with the most severe form of preeclampsia refrain from subsequent pregnancies because of older age or perceived risk, preventing a dose–response type relationship from becoming apparent.[23,25] There were several studies in our systematic search that did not specify the type of hypertensive pregnancy disorders and were therefore not included in this review. Interestingly, they report that women with recurrent gestational hypertension (including preeclampsia) have a similar increased risk of CVD.[49-54]

Conclusion

Evidence shows a strong relationship between recurrence of preeclampsia and additional risk of developing hypertension and CVD later in life compared with a single pregnancy with preeclampsia. With the increasing burden of CVD on society, this needs to be taken into consideration when establishing prevention programmes. This review shows that multiple complicated pregnancies may need to be weighed more heavily, compared with when subsequent pregnancies were normotensive.

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SUPPLEMENTARY MATERIAL

Supplementary Appendix 1.

Search strings

PubMed search string

#1: "Pre-eclampsia"[MeSH Terms] OR pre-eclampsia [Title/Abstract] OR pre-eclampsia [Title/Abstract]

#2: "Follow-up studies"[MeSH] OR "recurrence"[MeSH] OR "later risk"[Title/Abstract] OR "future risk"[Title/Abstract] OR "follow up"[Title/Abstract] OR "long term"[Title/Abstract] OR subsequent[Title/Abstract] OR "later life"[Title/Abstract] OR "later in life"[Title/Abstract] OR subsequently[Title/Abstract] OR future[Title/Abstract] OR history[Title/Abstract] OR previous[Title/Abstract] OR secondary[Title/Abstract] OR recurrent[Title/Abstract] OR recurrence[Title/Abstract]

#3**:** Hypertension [MeSH Terms] OR hypertension [Title/Abstract]

#4: Cardiovascular disease [MeSH Terms] OR cardiovascular [Title/Abstract]

Hypertension PubMed search: #1 AND #2 AND #3

Cardiovascular disease PubMed search: #1 AND #2 AND #4

Embase search string

#1: 'pre-eclampsia'/exp OR 'pre-eclampsia':ab,ti OR 'pre-eclampsia':ab,ti

#2: 'later risk':ab,ti OR 'future risk':ab,ti OR 'follow up':ab,ti OR 'long term':ab,ti OR 'later life':ab,ti OR 'later in life':ab,ti OR 'subsequently':ab,ti OR 'future':ab,ti OR 'history':ab,ti OR 'previous':ab,ti OR 'secondary':ab,ti OR 'subsequent':ab,ti OR 'recurrence':ab,ti OR 'recurrent':ab,ti OR 'follow up'/exp OR 'recurrent disease'/exp

#3: 'hypertension'/exp OR 'hypertension':ab,ti

#4: 'cardiovascular disease'/exp OR 'cardiovascular':ab,ti

#5: [embase]/lim NOT [medline]/lim

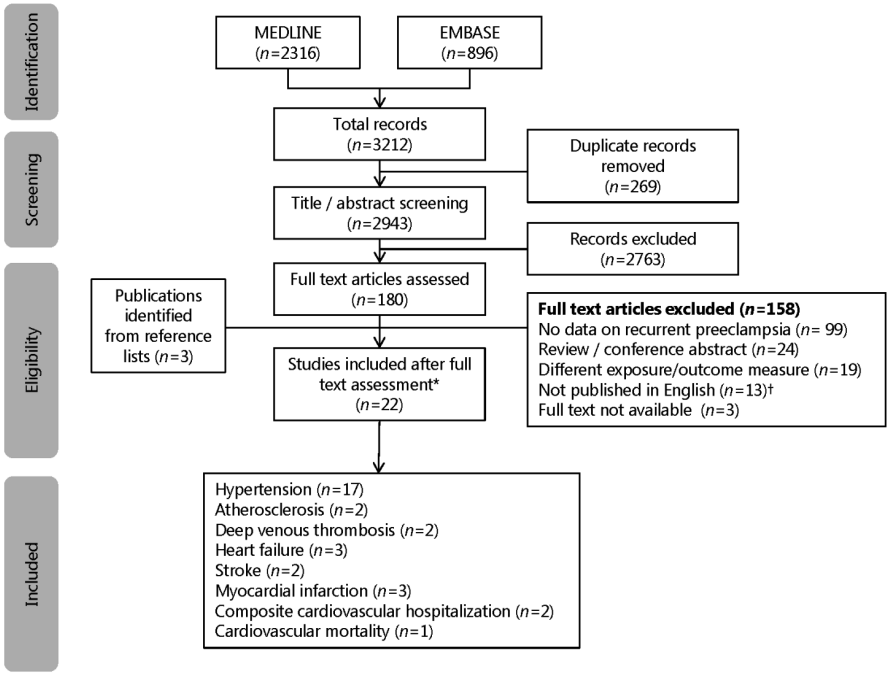
Hypertension Embase search: #1 AND #2 AND #3 AND #5

Cardiovascular disease Embase search: #1 AND #2 AND #4 AND #5

Embase Filters for publication types:

Article/ Article in Press/ Review/ Conference Review

A full list of references and reason of ex- or inclusion is available from the authors.



SUPPLEMENTARY FIGURE 1. Flow chart of selection process.
† Articles in languages other than English or Dutch were translated using Google Translate and included when translation quality was sufficient

SUPPLEMENTARY TABLE 1. Critical appraisal using the Newcastle-Ottawa Quality Assessment Scale for cohort and case-control studies.

Comparative cohort studies	**Selection (max 4 stars)**	**Comparability (max 2 stars)**	**Outcome/Exposure (max 3 stars)**
Singh (1974)[36]	*	*	*
Sibai (1986)[24]	****	**	***
Nisell (1995)[30]	****	*	**
Lykke (2009)[21]	****	**	***
Magnussen (2009)[22]	****	**	***
Smith (2009)[28]	****	**	**
Skjaerven (2012)[25]	****	**	***
Kessous (2015)[33]	****	-	**
Scholten (2015)[35]	***	-	**
Engeland (2015)[20]	****	**	***
Zhang (2015)[34]	****	*	*
Auger (2017)[26]	***	**	***
Riise (2017)[23]	****	**	***
Single cohort studies[†]	**Selection (max 3 stars)**	**Comparability (max 1 stars)**	**Outcome/Exposure (max 3 stars)**
Sibai (1991)[38]	***	-	**
Spaan (2012)[39]	***	*	*
Van Oostwaard (2012)[40]	**	*	**
Van Oostwaard (2014)[41]	**	*	**
Veerbeek (2015)[37]	***	*	***
Case-control studies	**Selection (max 4 stars)**	**Comparability (max 2 stars)**	**Outcome/Exposure (max 3 stars)**
Gaugler-Senden (2008)[27]	****	**	**
Ghossein-Doha (2014)[32]	***	*	**
Akhter (2014)[31]	***	*	**
Ghossein-Doha (2017)[29]	***	*	***

[†]Cohort study based on one single cohort of preeclamptic women: therefore scoring scale was adjusted: selection: max. 3 stars, comparability: max. 1 star based on use of confounders in analysis, outcome; max. 3 stars

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Early onset of coronary artery calcification in women with previous preeclampsia

Submitted

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ABSTRACT

Importance

Preeclampsia is a female-specific risk factor for development of coronary coronary artery calcification (CAC) and atherosclerotic plaque associated with subsequent cardiovascular disease (CVD). At what age CAC develops in these women and which risk factors are involved is unknown.

Objectives

To identify at what age CAC becomes apparent on coronary computer tomography (CCT) in women with a history of preeclampsia and whether modifiable cardiovascular risk factors mediate this risk.

Design

Measurement of modifiable risk factors, CAC by CCT and coronary plaque by CCT angiography (CCTA) in Caucasian women with a history of preeclampsia aged 40-63 in the CREw-IMAGO study (February 2016 until January 2018). Results were compared to age- and ethnicity equivalent women participating in the Framingham heart study (FHS) with normotensive pregnancies.

Setting

Multi-center cross-sectional cohort study.

Participants

258 women (16.3, ±5.9 years postpartum) with a history of preeclampsia and 644 women (20.0 ±8.2 years postpartum) with normotensive pregnancies.

Exposure(s)

Preeclampsia, age and Framingham risk score (FRS).

Main Outcome(s) and Measure(s)

Cardiovascular risk factors including; BMI, blood pressure, smoking, medication use, diabetes, lipid profile and glucose level. CAC score was measured by CCT and coronary plaque prevalence was assessed by CCTA, 10-year CVD risk was estimated by the FRS.

Results

Median age was 46 years in both groups. The prevalence of cardiovascular risk factors and CAC was significantly higher in women with preeclampsia than in those without (CAC: 20.0% vs 13.0%; OR 1.72, 95% CI 1.17-2.52, p=0.003). Women without a history of preeclampsia were 4.9 years (95% CI; 1.8-8.0) older when they had identical CACS as women with a history

of preeclampsia. The latter also had a greater CAC burden with advancing age compared to women without previous preeclampsia (OR 9.36, 95% CI; 2.34-37.45 vs OR 5.81, 95% CI; 2.83-11.82). Mediation analysis showed that the effect was not mediated through systolic blood pressure, total-cholesterol or diabetes. One-third (34.5%) of women with a history of preeclampsia had some form of coronary plaque, but significant stenosis (≥50%) was rare (*n*=2.9%). The 10-year CVD risk was also significantly higher for women with previous preeclampsia (5.25 vs 3.17%, *p*<0.001).

Conclusions and Relevance

Women with a history of preeclampsia have more modifiable cardiovascular risk factors and develop CAC approximately 5 years earlier compared to women with normotensive pregnancies. Women with a history of preeclampsia might benefit from regular cardiovascular screening and follow-up before the age of 45 years.

Trial Registration

Netherlands Trial Register, NTR5531.

INTRODUCTION

Preeclampsia is a hypertensive pregnancy disorder affecting 3-5% of pregnancies in the Western world and up to 9% of pregnancies in sub-Saharan Africa.[1-3] Although clinical symptoms resolve soon after pregnancy it has life-long health implications.[4] Preeclampsia is associated with a two- to seven fold increased risk of developing cardiovascular disease (CVD) compared to having normotensive pregnancies.[5,6] A current hypothesis is that this excessive CVD risk results from a pre-existent predisposition for cardiovascular risk factors, such as hypertension, obesity and dyslipidemia, which also contribute to the development of preeclampsia.[7-11] Although some cardiovascular risk management guidelines acknowledge the increased risk of CVD in former preeclamptic women, suitable recommendations for cardiovascular follow-up are lacking.[12-14] As current cardiovascular risk scores, (such as the Framingham Risk Score [FRS]) used in clinical practice are strongly age-dependent, women with previous preeclampsia are mostly estimated at low risk after pregnancy.[8] As such, cardiovascular risk management is not indicated and these women are often lost to further cardiovascular follow up.

Women with a history of preeclampsia develop modifiable cardiovascular risk factors (i.e. high blood pressure, dyslipidemia and diabetes) five to 10 years earlier than women without such history.[15,16] Our group previously showed that a substantial percentage of these women have coronary artery calcification (CAC), which is a predictor for CVD, assessed by coronary computer tomography (CCT) after the age of 45.[17,18] Nevertheless, it remains unclear at what age the first signs of CAC develop, to what extent modifiable risk factors mediate this effect, and whether this is in line with estimated risk (FRS).

To answer these questions, we investigate the prevalence of cardiovascular risk factors, the FRS and the prevalence of CAC by CCT in asymptomatic women (for several age groups) with a history of preeclampsia compared to women with normotensive pregnancies included in the Framingham Heart Study (FHS). Additionally, we investigate to what extent the association between age and CAC score (CACS) is mediated by modifiable risk factors. Lastly, we determine the prevalence of coronary plaque and significant stenosis by CCT angiography (CCTA) in women with previous preeclampsia.

METHODS

Study population

This study is part of the multicenter, cross-sectional Cardiovascular RiskprofilE: IMaging And Gender-specific disOrders (CREw-IMAGO) cohort, which examines asymptomatic CVD in women with a history of reproductive disorders. A full version of the rationale and design of the CREw-IMAGO cohort has been published previously.[17,19] All Caucasian participants from the previously published CREw-IMAGO study were included in this analysis.[17] For this study, Caucasian women aged over 40 years with a history of preeclampsia were recruited for an outpatient cardiovascular risk assessment and CCT/CCTA scans.[8,20,21] Exclusion criteria were having; a serious illness, previous cardiovascular event (i.e. myocardial infarction, stroke, coronary artery disease) or an increased risk for contrast nephropathy (estimated glomerular filtration rate <60 ml/min/1.73 m^2).

Women underwent cardiovascular risk assessment and both a contrast and non-contract coronary CT scan between February 2016 and January 2018 at either the University Medical Center Utrecht (UMCU) or at the Erasmus Medical Center Rotterdam (EMC). Medical records, with information regarding pregnancy characteristics were available for all participants. Preeclampsia was defined according to international guidelines as new onset hypertension in pregnancy after 20 weeks of gestation in combination with proteinuria, maternal organ dysfunction or uteroplacental dysfunction.[22] The study adheres to the principles of the Declaration of Helsinki and was approved by the medical ethics committee of the University Medical Center Utrecht (reference ID 15-508). All women received study information and provided written informed consent prior to participation.

We compared our results with age- and ethnicity-equivalent women participating in the Third Generation cohort of the FHS. The FHS participants (*n*=2174) were recruited between June 2002 and April 2005, were 20years or older and had at least one parent in the Offspring Cohort. Information on their medical history and medication intake was obtained and they underwent a full cardiovascular physical examination, including blood and urine tests and a non-contrast CCT.[23] The FHS data used for comparison were restricted to women from Caucasian descent aged 40-63 years to match the CREw-IMAGO participants. We excluded women who had never been pregnant and women who had a history of hypertensive disorders in pregnancy. Additionally, women with a previous cardiovascular event were excluded. Details of the in- and exclusion process can be found in Supplementary Figure 1.

Imaging techniques

A full description of the CREw-IMAGO study protocol can be found in previous publications.[17,19] In both research centers, CCT was performed using a 256-slice CT scanner (Philips Healthcare scanner in UMCU or Siemens Drive/Force scanner in EMC) with prospective ECG-triggering. When the heart rate was >65 beats/min, women received an oral or intravenous beta-blocker (Metoprolol) before the start of the CCT scan. First, a non-contrast CCT was performed to

calculate the CACS using the Agatston scoring method.[24] Second, contract-enhanced CCT angiography (CCTA) was performed following sublingual nitroglycerine and injection of non-ionic contrast (Iopromide, Ultravist, Bayer Healthcare, Berlin, Germany). Semi-automated vessel analysis was used to create multiple curved multiplanar reconstructions (MPR) of all coronary arteries. The presence of any coronary plaque (non-calcified, calcified, or mixed) and presence of significant stenosis (≥50%) was assessed in all 17 coronary segments. One of two assigned and experienced cardiovascular radiologists assessed both the scans for each patient.

In the FHS, ECG-triggered CCT scans without contrast were performed with an 8-slice multi-detector row CT scanner (LightSpeed Ultra; General Electric, Milwaukee, WI). CAC scoring was conducted by trained personnel with the Aquarius software package (TeraRecon, San Mateo, CA). Details on CAC scoring performance have been published previously.[25]

The CACS was categorized as no calcifications (CACS=0 AU), minimal (CACS >0 AU and <10 AU); mild (CACS ≥10 AU and <100 AU); moderate (CACS ≥100 AU and <400 AU) and severe (CACS ≥400 AU).

Cardiovascular risk assessment

Traditional cardiovascular risk factors were assessed prior to CCT/CCTA imaging, including age, body mass index (BMI), waist circumference and blood pressure. Additionally, a full history was obtained, including data on smoking, reproductive, cardiovascular and general medical history and the use of medication. Venous blood samples, to assess lipid profile and plasma glucose, were drawn fasting (UMCU women) and non-fasting (EMC women) in the CREw-IMAGO study and fasting in the FHS. LDL-cholesterol was calculated via the Friedewald equation.[26] Ten-year CVD risk was estimated according to the FRS.[27]

Statistical analyses

We estimated that the prevalence of CAC on non-contrast CCT in healthy women without risk factors between the age of 40 and 65 years was 15.6% based on a previous publication by the FHS.[28] For the power calculation we assumed an increased risk of 1.7 for the development of CAC in women with a history of preeclampsia.[29,30] We calculated that a sample size of 231 women in each group would provide 90% power, to detect a difference in CAC, with a two-sided 5% significance level throughout our age range.

Values shown in all tables are not imputed and presented as numbers with percentages for categorical variables, means with standard deviation for variables with a normal distribution and medians with 90% range for variables with a skewed distribution. In total, 0.4% – 17.1% of values were missing in the CREw-IMAGO study and 0.2% - 8.7% in the FHS. Differences between women with and without a history of preeclampsia were tested with Student's t-test for variables with a normal distribution, Kruskal Wallis test for variables with a skewed distribution and Chi square tests for categorical variables.

We examined the distribution of CAC and the FRS per age category (40-45, 45-50, 50-55, and 55-63 years) in relation to a history of preeclampsia. Additionally, for women in the CREw-

IMAGO study the presence of coronary plaque was assessed per age category. We performed a Directed Acyclic Graph (DAG) analysis with a consensus meeting to identify which modifiable cardiovascular risk factors were confounders, mediators or colliders (Supplementary Figure 2).[31] Consensus was achieved by the authors regarding the current structure of our regression models (LBe, LBr, BR, JRvL). The DAG analysis showed three mediating pathways (systolic blood pressure, total-cholesterol and diabetes) for the association between age and CAC. Adjustment for other confounders was not necessary.

We performed ordinal logistic regression analyses to examine the association between age and having a higher CACS for women with a history of preeclampsia and those with no history of preeclampsia. Age was assessed continuously and in categories. Values represented as odds ratios (OR) reflect the odds of being in a higher CAC category when comparing the respective age group to the reference category (40-45 years). We calculated what effect a history of preeclampsia had on the age of CAC onset through: the effect estimate of preeclampsia on CAC / the effect estimate of age on CAC. Lastly, we performed mediation analyses to determine the proportion of the association between age and CAC mediated by systolic blood pressure, total-cholesterol level and diabetes. A two-sided *P*-value < 0.05 was considered statistically significant. All analyses were conducted using Statistical Package for Social Sciences (SPSS) version 24.0 for Windows (SPSS Inc. Chicago, IL, USA).

TABLE 1. Participants' baseline characteristics.

Participant characteristics	Previous preeclampsia (CREw-IMAGO) *n* = 258	No previous preeclampsia (FHS) *n* = 644	***p*-value**
Age (years)	46.0 (41.1, 54.5)	46.0 (40.2, 56.0)	0.41
Time since first pregnancy (years)	16.3 (5.9)	20.0 (8.2)	<0.001
Caucasian ethnicity (n, %)	258 (100%)	644 (100%)	n/a
Education (n, %)			
Elementary school	3 (1.3)	0	<0.001
High school	33 (13.0)	117 (18.2)	
Technical school/community college	97 (38.2)	141 (21.9)	
University	121 (47.6)	385 (59.9)	
Parity (n)	2.0 (1.0, 5.4)	3.0 (1.0, 5.9)	0.01
Ever or current smoker (n, %)	96 (38.2)	339 (52.6)	<0.001
Total-cholesterol (mmol/L)	5.3 (1.0)	5.0 (0.92)	<0.001
LDL-c (mmol/L)	3.2 (0.89)	2.8 (0.81)	<0.001
HDL-c (mmol/L)	1.52 (0.32)	1.62 (0.45)	0.001
Triglycerides (mmol/L)	1.1 (0.60, 2.2)	0.93 (0.51, 2.5)	0.001
Glucose (mmol/L)	5.5 (1.1)	5.2 (1.1)	0.002
Systolic blood pressure (mm Hg)	131.6 (16.6)	114.0 (15.0)	<0.001
Diastolic blood pressure (mm Hg)	82.7 (10.8)	74.9 (9.5)	<0.001
BMI (kg/m2)	26.6 (20.9, 37.6)	25.1 (19.7, 37.8)	0.02
Waist (cm)	87.0 (71.0, 113.4)	88.9 (71.1, 118.1)	0.54
Lipid lowering medication (n, %)	23 (9.0)	37 (5.7)	0.08
Glucose lowering medication (n, %)	8 (3.2)	10 (1.6)	0.12
Anti-hypertensive medication (n, %)	81 (31.6)	64 (10.0)	<0.001
Diabetes diagnosis (n, %)	6 (2.4)	12 (1.9)	0.63
Before menopause (n, %)	101 (39.1)	242 (37.6)	0.66
CAC (AU)	0.0 (0.0, 66.0)	0.0 (0.0, 32.7)	0.001
CACS (n, %)			
No CAC (0 AU)	200 (80.0)	556 (87.0)	0.003
Minimal CAC (1-10 AU)	11 (4.4)	34 (5.3)	
Mild CAC (11-100 AU)	33 (13.2)	37 (5.8)	
Moderate CAC (101-400 AU)	6 (2.4)	12 (1.9)	
Coronary plaque* (n, %)	88 (34.5)	n/a	n/a
Significant stenosis† (n,%)	7 (2.9)	n/a	n/a
Framingham risk score (%)	5.25 (2.06, 15.44)	3.17 (1.09, 10.67)	<0.001

Values are numbers with percentages for categorical variables, means with standard deviation for variables with a normal distribution and medians with 90% range for variables with a skewed distribution. Values represent valid percentages and are not imputed. The *p*-value is the result of student's t-test, Kruskal Wallis and Chi square tests. *Coronary plaque was only assessed in the CREw-IMAGO study and was defined as having either calcified, non-calcified or mixed plaque. †Significant coronary luminal stenosis was only assessed in the CREw-IMAGO study and was defined as having ≥50% stenosis. *Abbreviations*: CREw-IMAGO, Cardiovascular RiskprofilE: IMaging And Gender-specific disOrders; FHS, Framingham Heart Study; AU, Agatston Units; BMI, body mass index; CAC, coronary artery score; HDL, high density lipoprotein; LDL, low density lipoprotein; n, number; n/a, not applicable.

RESULTS

Patient Characteristics

In total, 258 women with a history of preeclampsia were included in the CREw-IMAGO study, and compared with a selection of 644 age- and ethnicity equivalent women from the FHS. Participant's characteristics are presented in Table 1. At the time of screening and CCT and/or CCTA, women were on average 46 years of age in both groups and the majority was higher educated.

Cardiovascular risk factors

Women with a history of preeclampsia were less often smokers but had more cardiovascular metabolic risk factors; they had a more atherogenic lipid profile depicted by higher total-cholesterol, LDL-cholesterol and triglyceride levels and lower HDL-cholesterol compared to women without a history of preeclampsia (Table 1). Moreover, they had a higher BMI and a higher systolic and diastolic blood pressure despite antihypertensive treatment. At comparable age, women with previous preeclampsia had a higher FRS compared to women without such history.

Coronary Artery Calcium Scores

CAC prevalence and distribution is shown in Table 2. In general, the presence of CAC, the CACS and plaque burden increased with age (p<0.001). When comparing these results between women with and those without a history of preeclampsia, we observed no significant difference in CAC distribution under the age of 45 or over 50 years of age (Table 2). CACS > 0 AU was more prevalent amongst former preeclamptic women aged 45-50 years than those without a history of preeclampsia (23.4% vs. 9.7%, p=0.003). Women without previous preeclampsia were on average 4.9 years older (95%CI; 1.8, 8.0) when their prevalence of CAC was similar to that of former preeclamptic women. Also, 34% of former preeclamptic women had visible plaque formation on CCTA, but only 2.9% had a significant stenosis.

Table 3 shows the association between preeclampsia and CAC, and between age (continuously and in groups), the FRS and the CACS for women with and without a history of preeclampsia. Women with a history of preeclampsia had a higher risk of having more severe CAC. We also observed a strong positive and linear association between age and the CACS. Women with a history of preeclampsia consistently showed a higher risk of increased CACS with age compared to women without previous preeclampsia. The FRS was positively associated with the risk of having a higher CACS for women in both groups (Table 3). There was no significant mediation by systolic blood pressure, total-cholesterol level and diabetes on the association of age and CAC in former preeclamptic women. In women with no history of preeclampsia 13% of the association between age and CAC was mediated through systolic blood pressure (*data not shown*).

TABLE 2. Prevalence of CAC per age category in women with a history of preeclampsia (CREw-IMAGO) and with normotensive pregnancies (FHS).

	Age categories			
Previous preeclampsia (CREw-IMAGO)	**40-45 yrs** *n*=85	**45-50 yrs** *n*=115	**50-55 yrs** *n*=45	**55-63 yrs** *n*=12
CAC (AU)	0.0 (0.0, 11.4)	0.0 (0.0, 85.6)*	0.0 (0.0, 113.0)	0.0 (0.0, n.a.)
CACS (n, %)				
No CAC (0 AU)	77 (92.8)	85 (76.6)	31 (70.5)	7 (58.3)
Any CAC (> 0 AU)	6 (7.2)	26 (23.4)*	13 (29.5)	4 (41.7)
Minimal CACS (1-10 AU)	1 (1.2)	6 (5.4)	4 (9.1)	0
Mild CACS (11-100 AU)	5 (6.0)	16 (14.4)	7 (15.9)	5 (41.7)
Moderate CACS (101-400 AU)	0	4 (3.6)	2 (4.5)	0
Coronary plaque[§] (n, %)	11 (13.1)	52 (45.2)	19 (43.2)	6 (50.0)
Framingham risk score (%)	4.10 (1.51, 12.51)*	5.17 (2.32, 12.15)*	7.25 (3.32, 24.44)*	14.00 (3.89,n.a.)*
No previous preeclampsia (FHS)	*n*=254	*n*=206	*n*=123	*n*=56
CAC (AU)	0.0 (0.0, 4.9)	0.0 (0.0, 18.9)*	0.0 (0.0, 87.1)	0.0 (0.0, 106.2)
CACS (n, %)				
No CAC (0 AU)	234 (92.1)	186 (90.3)	98 (79.7)	38 (67.9)
Any CAC (> 0 AU)	20 (7.9)	20 (9.7)*	25 (20.3)	18 (32.1)
Minimal CACS (1-10 AU)	11 (4.3)	9 (4.4)	9 (7.3)	5 (8.9)
Mild CACS (11-100 AU)	7 (2.8)	9 (4.4)	11 (8.9)	10 (17.9)
Moderate CACS (101-400 AU)	2 (0.8)	2 (1.0)	5 (4.1)	3 (5.4)
Coronary plaque[§] (n, %)	n/a	n/a	n/a	n/a
Framingham risk score (%)	2.07 (0.90, 5.61)*	3.43 (1.43, 8.69)*	4.80 (1.83, 15.65)*	7.41 (2.89, 15.27)*

Values are numbers with percentages for categorical variables and medians with 90% range for variables with a skewed distribution. Values represent valid percentages and are not imputed. §Only in women from the CREw-IMAGO study. Coronary plaque was characterized as either calcified, non-calcified or mixed. *Statstically significant difference for the particular outcome between women in this age category from the FHS and those from the CREw-IMAGO study. CACS >4SD were removed from the analyses (n=7). *Abbreviations*: CREw-IMAGO, Cardiovascular RiskprofilE: IMaging And Gender-specific disOrders; FHS, Framingham Heart Study; AU, Agatston Units; CAC, coronary artery calcification; CACS, coronary artery calcification score; n/a, not applicable.

TABLE 3. Ordinal regression for risk of coronary artery calcification in women with a history of preeclampsia (CREw-IMAGO) and without such history (FHS).

	Crude	
Exposures	**CACS** (OR)	***p*-value**
Previous preeclampsia (CREw-IMAGO)		
Preeclampsia	1.72 (1.17, 2.52)	0.006
Age (years)	1.14 (1.06, 1.22)	<0.001
Age categories (years)		
40-45	*reference*	
45-50	3.95 (1.55, 10.08)	0.004
50-55	5.24 (1.83, 14.98)	0.002
55-63	9.36 (2.34, 37.45)	0.002
Framingham risk score	1.16 (1.08, 1.23)	<0.001
No previous preeclampsia (FHS)		
Age (years)	1.14 (1.07, 1.19)	<0.001
Age categories (years)		
40-45	*reference*	
45-50	1.27 (0.66, 2.44)	0.48
50-55	3.06 (1.63, 5.75)	0.001
55-63	5.81 (2.83, 11.82)	<0.001
Framingham risk score	1.21 (1.14, 1.27)	<0.001

Values are OR with 95% confidence interval. Values represent difference in risk of being in a higher CAC score category based on age (both continuous and in categories) or FRS for women with a history of preeclampsia (CREw-IMAGO) and those without (FHS). *Abbreviations*: CREw-IMAGO, Cardiovascular RiskprofilE: IMaging And Gender-specific disOrders; FHS, Framingham Heart Study; CACS, coronary artery score; OR, odds ratio.

DISCUSSION

We showed that women with a history of preeclampsia have a greater risk of CAC and early development of CAC compared to women without such a history. This excessive risk develops around the age of 45 years. Women with a history of preeclampsia developed CAC on average 4.9 years earlier and have more modifiable cardio-metabolic risk factors than women without such history. In our study, this association was not explained through modifiable cardiovascular risk factors (systolic blood pressure, total-cholesterol level and diabetes). Although the FRS was higher in women with a history of preeclampsia, the absolute values did not reach the threshold of >10% for preventive treatment according to current consensus guidelines. Additionally, women with previous preeclampsia often had coronary plaque and prevalence increased with advancing age. Our study provides evidence of an earlier onset of subclinical CVD in women with a history of preeclampsia and justifies management of cardiovascular risk factors before the age of 45 years.

In line with our results, previous studies showed that women with a history of preeclampsia have a 1.6-3.5 fold higher risk of having any CAC than women with normotensive pregnancies.[29,30,32,33] These studies had a smaller sample size and included women who were on average 15-20 years older than those in our study. The prevalence of CAC above the age of 60 years however was found to be persistently higher in these previous preeclamptic women, which is in line with our present findings in women at a younger age.[29,30,32,33] The elevated risk of having any CAC with advancing age associated with preeclampsia is evident when we compare our results to those of other studies.[34] Our findings imply that atherosclerosis, depicted by CAC, develops at a younger age in women with a history of preeclampsia compared to women who had a normotensive pregnancy. We expected that this association would at least partly be explained through the presence of traditional cardiovascular risk factors. However, we did not find a significant mediation effect of the three analyzed risk factors within the current study. These risk factors might still explain the elevated risk of CAC in women with a history of preeclampsia, but not in the association between age and CAC directly. The mediating effect of the traditional cardiovascular risk factors is presumably more important in elderly women. Nonetheless, we cannot exclude that the excess development of CAC in these women might be mediated by other pathways, such as genetics and an elevated pro-inflammatory state.[35-37]

The hypothesis that women with preeclampsia develop cardiovascular risk factors at a younger age, or even have a greater cardiovascular predisposition before pregnancy, was first suggested by Sattar et al.[38] Later, large population-based studies, including the Child Health and Development Studies cohort and the CHAMPS study, showed that these women indeed develop CVD approximately 8 years earlier than women with a history of normotensive pregnancies.[39,40] It has been reported that in general up to 90% of CVD risk can be explained through traditional cardiovascular risk factors (e.g. hypertension, diabetes, obesity, hypercholesterolemia and smoking).[41] Previous studies showed that women with

preeclampsia have more cardiovascular risk factors already shortly after delivery compared to women without such history.[15,16,42,43] In line with these findings, we show that the prevalence of these risk factors in women who experienced preeclampsia is higher in women from the age of 40 years onwards. Although the association between age and CAC could not be explained through these cardiovascular risk factors, timely screening, healthy lifestyle advice and, if necessary, treatment of cardiovascular risk factors is momentarily the most optimal strategy to reduce CVD risk.

Most cardiovascular prevention guidelines do not provide a clear recommendation regarding how and when to perform cardiovascular follow-up of women with a history of preeclampsia.[12-14,44] A long follow up time is required to assess the potential benefit of early screening and treatment of ”hard endpoints”, such as CVD or mortality. This is extremely costly and consequently unlikely to be executed. Up to now it was not possible to compose high-level evidence based recommendations regarding the timing of cardiovascular follow-up of women with a history of preeclampsia. Our results and previous evidence show that women with a history of preeclampsia not only have a more unfavorable cardiovascular risk profile but also more signs of subclinical atherosclerosis by CAC evaluation before the age of 50 years. Therefore, we postulate that cardiovascular follow-up in these women should be initiated before the age of 45 years. Relatively recent, a Dutch obstetric guideline developed by gynecologists, general practitioners, cardiologists and internal medicine specialists regarding the cardiovascular risk management after a pregnancy disorders suggested a similar cardiovascular follow-up protocol.[12] However this guidelines suggests a start of follow-up from the age of 50 years, where as we would like to emphasize to start to monitoring women with a history of preeclampsia at a regular interval (1-2 years) before the age of 45 years and promote a healthy lifestyle. In addition, we believe that early treatment of risk factors may be necessary to prevent irreversible CAC development and to prevent subsequent CVD. We would not recommend the implementation of CTA or CCTA as standard cardiovascular follow-up due to the radiation exposure and high costs.

Strengths and limitations

The strengths of our study are that we prospectively recruited the largest study population up to date of women with previous preeclampsia and performed comprehensive cardiovascular screening including cardiovascular imaging. Moreover, through analysis of the FHS population it was possible to compare women with and without previous preeclampsia and to quantify the additional cardiovascular risk attributed to preeclampsia itself. We included women between 40-63 years and could therefore compare the results between varying ages and study at which age cardiovascular risk diverges from the control population.

Some limitations need to be addressed. All women included in this study were Caucasian and the majority was higher educated. This might have affected the generalizability of our results. Cardiovascular risk factors, including designated mediators, and CAC were measured cross-sectionally. Therefore, mediators may not be true mediators as they were

not all measured in between the exposure and outcome. This might have resulted in a slight overestimation of the effect as these mediators tend to increase the risk of CAC over time. Nevertheless, the total effect estimates remain identical. Due to the age of women included in this study, information on cardiovascular events, and therefore the implication of a positive CAC score, is not available. However, we assume a higher risk of CVD in these women as previous studies have shown a positive association between the presence of CAC and cardiovascular events.[45-47] Lastly, the observational nature of this study does not allow for inference of causality and does not preclude the existence of residual confounding. Additional strengths of this study include that the measurements were taken and graded following standardized protocols. Also, this is the first study measuring coronary plaque burden by CCTA in women with a history of preeclampsia before the age of 45 years. Although we could not compare the results to a control group, presentation of these findings may be important when a control group becomes available in the future.

Conclusion

CAC develops approximately five years earlier, from the age of 45 years, in women with a history of preeclampsia compared to women with normotensive pregnancies. Simultaneously, the prevalence of CAC and cardiovascular risk factors is higher in these women, while 10-year CVD risk estimates remains low. We advise that women with a history of preeclampsia receive regular cardiovascular screening and follow-up before the age of 45 years.

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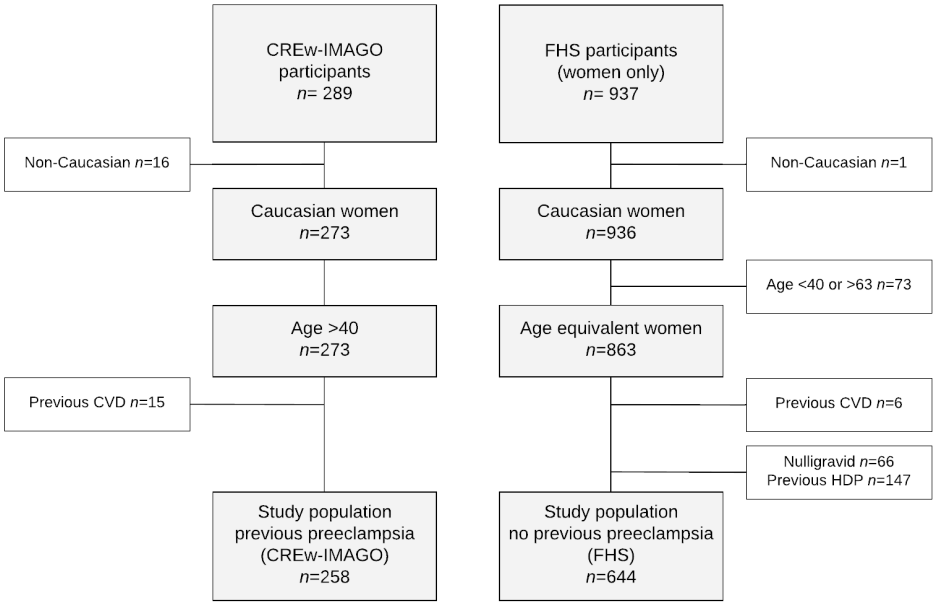
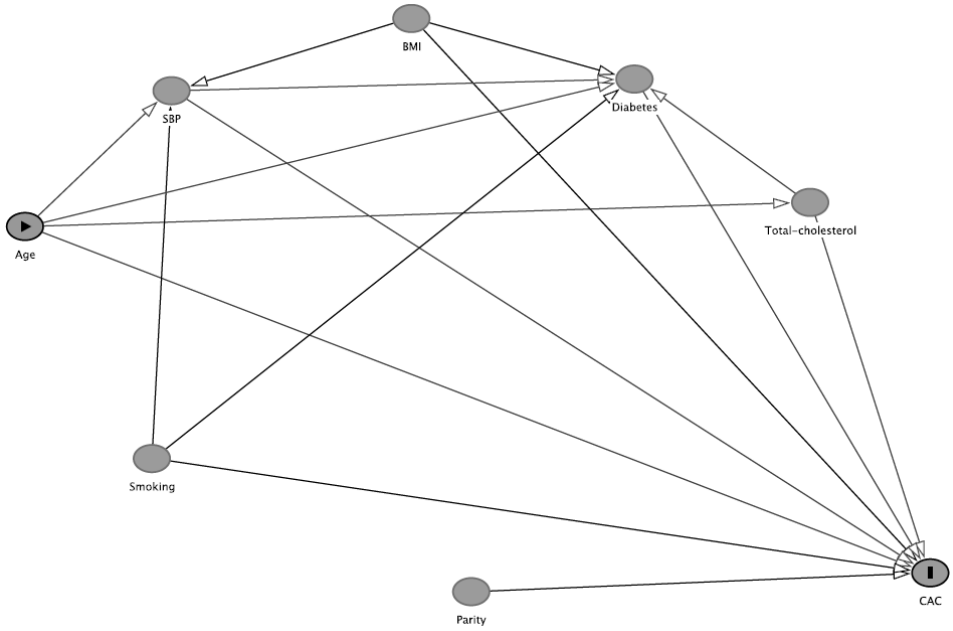
SUPPLEMENTARY MATERIAL



SUPPLEMENTARY FIGURE 1. Flow chart for the inclusion of women with previous preeclampsia (CREw-IMAGO) and without previous preeclampsia (FHS). *Abbreviations*: CREw-IMAGO, Cardiovascular RiskprofilE: IMaging And Gender-specific disOrders; FHS, Framingham heart study; HDP, hypertensive disorders in pregnancy; CVD, cardiovascular disease; CCT, coronary computed tomography; CCTA, coronary computed tomography angiography.



SUPPLEMENTARY FIGURE 2. Directed Acyclic Graph representing the mediating pathways between age and CAC. *Abbreviations*: BMI, body mass index; CAC, coronary artery calcification; SBP, systolic blood pressure.

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9

Impact of preventive screening and lifestyle interventions in women with a history of preeclampsia: model-based micro-simulation study

In preparation

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ABSTRACT

Background

Preeclampsia is a female-specific risk factor for the development of future cardiovascular disease (CVD). Whether early preventive screening and lifestyle interventions in women with previous preeclampsia are beneficial and cost-effective is unknown.

Objectives

To investigate the, both health and economic, impact of preventive screening and lifestyle interventions for CVD in women implemented shortly after pregnancy complicated by preeclampsia.

Methods

Datasets with initial cardiovascular screening at 6 months post-partum and at 10-20 years follow-up were combined to estimate 10-year CVD risks according the Framingham Risk Score (FRS). A micro-simulation model was used to assess the life-long impact of preventive cardiovascular screening initiated directly after women experienced preeclampsia. Screening was started at the age of 30 and was repeated every 5 years. Lifestyle interventions were implemented when women were perceived as high risk (based on FRS with several adjusted thresholds). Comparison was performed in terms of costs and quality-adjusted-life-years (QALYs). Probabilistic sensitivity analysis was performed by Monte-Carlo simulation.

Results

Screening with an absolute FRS threshold of 2% and 5% and implementing lifestyle interventions resulted in an average of 27.47 and 27.46 QALYs, compared to 27.41 QALYs for no screening. Difference in costs between screening were €604 (2% threshold) and €220 (5% threshold) when compared to no screening. Probabilistic sensitivity analysis showed that early screening and lifestyle interventions may be cost effective at a threshold of €20,000/QALY.

Conclusions

Early screening and lifestyle interventions can improve long term health outcomes in women with a history of preeclampsia and may be cost effective. This study underlines that we may need to establish a lifelong cardiovascular prevention program for women starting early after experiencing preeclampsia.

INTRODUCTION

Cardiovascular disease (CVD) is the most prevalent cause of death in women worldwide and is predominantly caused by long term progression of which is associated with a lifelong exposure to risk factors and build-up of atherosclerosis.[1] The global burden of CVD is associated with traditional risk factors, such as hypertension, hypercholesterolemia, obesity, smoking and type II diabetes mellitus and is strongly associated with a prolonged unhealthy lifestyle.[1,2] It has been estimated that, up to 90% of CVD risk can be explained through traditional, and modifiable, risk factors.[3] Over the past decades long-term population studies have identified additional female-specific risk factors. Preeclampsia is one of the strongest female-specific risk factors for CVD, associated with a two- to seven fold increased risk of developing ischemic heart disease and stroke compared to women with normotensive pregnancies.[4-8]

Although several international obstetric guidelines recommend screening of cardiovascular risk profiles for women who have a history of preeclampsia, these are not yet implemented in the leading cardiovascular prevention guidelines.[9-12] These guidelines classically start cardiovascular screening in women at the age of 50 years.[13-15] Additionally, treatment recommendations are based on risk prediction models which calculate 10 years CVD risk and are strongly age-dependent. Women shortly after pregnancy will not usually reach the current risk threshold for preventive measures recommended by these guidelines. Current risk-based selection may therefore not be appropriate for these young women at *relative high* but *absolute low* risk and a lifetime CVD risk-based approach may be preferable.[16]

As the timeline during which benefits from preventive intervention in young women accrue is long, a randomized or cohort setting is not feasible to assess benefits of prevention. A model-based approach is perhaps more realistic although collecting the required evidence is challenging. Two Dutch Markov model-based studies previously showed that early CVD prevention in women with previous preeclampsia is likely to be cost effective.[17,18] However, authentic long-term follow up data from cardiovascular screening, including multiple measures for each participant, were not available at the time these papers were constructed. Furthermore, previous studies used a cohort-model which is not able to include treatment decisions on an individual level, perhaps giving a less realistic representation of clinical practice.

We present a model-based patient-level simulation of early cardiovascular screening and lifestyle interventions to assess both health and economic impact in reducing CVD in women with a history of preeclampsia. We incorporated data from both initial cardiovascular screening six months after delivery in women with preeclampsia and data of screening after 10-20 years follow up to estimate 10-years CVD risks. A life-long horizon was applied to capture all benefits of screening and lifestyle interventions in these women.

METHODS

To assess the impact of early CVD preventive screening strategies, datasets from two studies in the Netherlands were combined. Both studies measured cardiovascular risk parameters at different time intervals after preeclampsia.

The first dataset comprised initial cardiovascular screening performed in 349 women (mean age 30.8, 95% range 22.0-39.6). six months after a first pregnancy was complicated by early-onset preeclampsia. The complete study design has been previously published.[19] In short, this study was performed between 1994 and 2007 and recorded the presence of diabetes and chronic hypertension, calculated BMI, measured blood pressure and fasting blood lipid and glucose levels.

Secondly, we used data from the CREw-IMAGO (Cardiovascular Risk Profile: Imaging and Gender-Specific Disorders) study where women were screened for cardiovascular parameters 10-20 years after pregnancy complicated by (early and late onset) preeclampsia (*n*=291).[20] Women from the first study, and two other cohorts, were invited to participate in the later study. The complete design of the CREw-IMAGO study was published previously.[5,20] In short, asymptomatic women, aged 40 to 63 years (mean age 46.4, 95% range 40.2-57.8), with a history of preeclampsia were assessed for cardiovascular risk factors including BMI, waist circumference, blood pressure, lipid and glucose levels.[19-22]

CVD risk estimates

The Framingham Global Risk Score (FRS) was used to estimate 10-year CVD risk at initial post-partum screening and at follow-up (CREw-IMAGO study).[23] Multiple imputation (with 10 datasets) was performed to substitute missing data using the MICE packages in R.[24] Imputation was based on baseline characteristics, such as age, sex, blood pressure, and cholesterol levels.

Estimated CVD risk estimates and follow-up time were not comparable due to differences in age at screening in both studies. To correct for this, the two CVD risk estimates were recalculated to risk estimates at the same age. The data of 49 women included in both studies (i.e. included in both the initial post-partum screening and the CREw-IMAGO study) was used to calculate relative change in CVD risk, which were then used to re-estimate the CVD risks in both separate datasets for CVD risks at multiple ages (mean age 33.2, 95% range 25.7- 40.3). The re-estimation was done by interpolating the CVD risk estimates to the two fixed points by using the difference in risk estimates. Using the absolute difference, i.e. linear change, was not possible because; a) risk estimates could be become negative, and b) risk estimates were based on a power function rather than a linear function. Therefore, the individual relative change was used to calculating the annual relative change in CVD risk between the two measures.

Based on the annual relative change values of these 49 women, a beta distribution was assessed. This beta distribution was used to draw random values as annual relative change

for CVD risk in the women from the initial post-partum screening and the CREw-IMAGO study who were screened only once. The drawn values were used to calculate CVD risk estimates at the age of 30 and 40 for the post-partum screening, and at the age of 40 and 50 for the CREw-IMAGO study. For an example of this recalculation, see Supplementary Table 1.

Model development and parameters

A discrete time micro-simulation model was developed to assess the impact of early preventive strategies for CVD. The flow-chart of this model is presented in Supplementary Figure 1. The time cycle of the model was one year. Women were followed until death and outcomes were aggregated at population level, i.e. total CVD events, total costs and health outcomes, expressed in Quality-Adjusted-Life-Years (QALYs). Supplementary Table 2 shows an overview of all input parameters that are varied in the modelling. Estimates for model parameters were partially based on expert opinion and consensus. Consequently, relatively wide distributions were used to properly reflect parameter uncertainty.

In total, we simulated risks for 2,000 women, as the incidence of early-onset preeclampsia is currently about 1-2% amongst a total of approximately 171.000 annual pregnancies in the Netherlands.[25,26] Women entered the model at the average age of a first pregnancy in the Netherlands (30 years old).[25] As CVD risk estimates vary with age, we assumed that CVD risk increased over time for each woman. Published long-term data on the development of risk factors, in this specific group, was not available. Therefore, we used 10-years CVD risk estimates from the two cohorts and determined beta distributions accordingly. Additionally, random values (*n*=2,000) were drawn from these beta distributions and used as 10-year CVD risk estimates between the ages of 30 and 50. The 10-year risk estimate at the age of 80 was determined by expert opinion and Dutch prevalence data.[27] The risk estimates between the age of 50 and 80 were then interpolated, after which it was assumed that risks stayed constant. To correlate the risk values for each woman over time, a single correlation estimate was estimated from the two CVD cohorts. The two cohorts, both with two recalculated 10-year CVD risk estimates, were combined and the correlation between the two measures was estimated.

Usual care

Despite a national multidisciplinary guideline recommending that women who experienced preeclampsia should be offered CVD screening by their general practitioner at the age of 50 years, no nationwide primary prevention program is currently offered in the Netherlands.[11] We therefore assumed usual care for these women as follows. We presumed that annually 3% (range 2-4%) of all women above the age of 60 would visit the general practitioner with cardiovascular complaints and could be identified as high risk. Usual care applied a risk threshold of 10% (Framingham Risk Score) to classify women as high risk.[28,29] Lifestyle interventions (including smoking cessation, weight reduction, increasing physical activity) were recommended to high risk women as preventive intervention. We used a risk reduction

(average 0.91, range 0.84-0.96) of the intervention in the model.[30] Because evidence on long-term adherence rates was not available, we assumed that on average 20% of women stayed adherent up to 10 years after initiation of the intervention and derived the annual adherence rate through exponential interpolation.

Preventive strategy for CVD

The CVD prevention strategies for women after preeclampsia was defined as cardiovascular risk screening starting at the age of 30 years, with screening repeated every five years and ending at the age of 55. We used the response rate of the women invited to participate in the CREw-IMAGO study to estimate the proportion that would participate (39%, range 21-60%). As women were young at enrolment in the model, the current age-dependent recommended risk thresholds (FRS>10%) to stratify women as high risk were not suitable. We therefore used lower risk thresholds for the purpose of this study (i.e. FRS>2% and >5%). Women with established CVD were not eligible for preventive screening, but remained in the micro-simulation until death. Women who were assessed as low-risk at the previous screening or if they did not adhere to the lifestyle changes, were invited to the subsequent screening moment(s) after 5 years.

Lifestyle interventions (including smoking cessation, weight reduction, increasing physical activity) were the recommended preventive intervention for women classified as high risk, consistent with usual care. As data on adherence was lacking, we assumed adherence for the younger women to be comparative to the 10-year adherence to lifestyle changes of 20% (range, 18-22%) in older women (see *usual care*).

Model parameters

All model parameters are provided in Supplementary Table 2 and 3. Three CVD event categories are distinguished in this study; coronary artery disease (CAD), cerebrovascular accident (CVA), and other cardiovascular disease (OCVD) events. The CVD events could be either fatal or non-fatal, resulting in incorporation of six total CVD event types. The relative occurrence of the six event types was age-dependent and based on previous literature (Supplementary Table 3).[16,31,32] When a cardiovascular event occurred, the CVD risk estimate was proportionally increased (relative risk ratio 2.1, range 1.7-2.6).

Although we are aware that women experience psychological problems after preeclampsia, little follow-up data is available regarding the long-term clinical effects (and relevance) on quality of life.[33,34] Therefore, we used data available from women with uncomplicated pregnancy, and adjusted for age.[35,36] Quality of life was proportionally reduced after the occurrence of a CVD event.

Utilities after a first CVD event depended on the event type, but remained the same for similar recurrent CVD events. The decrease in utility after a CVD event was lower in the first year, after the first year the utility would slowly increase over time. It was assumed that women with a CVD event would receive medication. We could not take side-effects of medication due

to occurrence of a CVD event into account due to lack of evidence. We therefore assumed that they may be incorporated in the disutility of first and sequential years following a CVD event. We assumed there was no disutility due to screening and preventive lifestyle interventions.

Dutch studies and evidence from NICE were used for the estimation of the costs of CVD events.[35,37-39] Similar to the utilities, costs varied over the six different CVD types. Costs for recurrent events, for all event types, were kept the same. Costs of the first year after a CVD event were set higher than the subsequent years. Costs of the implemented screening included a visit to the general practitioner, including laboratory testing and were applied to all women who participated in screening in the model. Costs of preventive lifestyle interventions were applied to all women who were classified as high risk, i.e. women with CVD risk estimates that exceeded the intervention threshold, regardless of their adherence to these lifestyle interventions.

An overview of all utilities and costs together with the distribution for the sensitivity analyses are presented in Supplementary Table 2 (row 14-44). Following Dutch guidelines, a discount rate of 4% for costs and 1.5% for health outcomes was applied.[40] As preventive screening, CVD events and death due to natural causes can occur at any time during the years (instead of only at the start or end of a year) a half-cycle correction were applied in the model.

Cost-effectiveness analysis

The cost-effectiveness analysis was performed with the incremental cost-effectiveness ratio (ICER) as outcome, according to the Dutch health care perspective. This ratio represents the ratio of the difference in lifetime costs divided by the difference in effectiveness, i.e. health outcomes. The difference in costs and effectiveness is defined as the difference between our modelled early preventive strategy (i.e. screening and lifestyle interventions) and usual care. To determine whether a strategy was cost effective or not, a willingness-to-pay (WTP) threshold of € 20,000 per QALY gained was used. Probabilistic sensitivity analysis was applied to assess how uncertainty in parameter values resulted in uncertainty in the overall cost and effect outcomes. To determine the differences between strategies, we used 1,000 Monte-Carlo simulations applied to a cohort of 2,000 hypothetical, unique women. Furthermore, the probability of a preventive screening to be cost effective compared to usual care was estimated as a function of the WTP and presented in cost-effectiveness-acceptability curves.

RESULTS

Study population

The authentic risk assessment data of women included in both cardiovascular screening studies (i.e. post-partum and at follow up) is presented in Figure 1. The time period between the two real-life screening time points varied significantly within the 49 women who attended both screening moments (average 14.3 years, 95% range 6.6-19.2 years). Similarly, the 10-year CVD risk estimates differ substantially within the group (Figure 1A). The annual relative risk change within the group was quite similar with an average of 1.06 (95% range 1.01- 1.14). There is a substantial variation in CVD risk estimate for similar time periods, indicating substantial heterogeneity in the expected CVD risk in previous preeclamptic women (Figure 1B).

Figure shows the histogram distribution of the re-estimated 10-year CVD risk evaluations for women in all screening cohorts and the projected distributions. Data from screening shortly post-partum is used to estimate 10-year CVD risk at 30 and 40 years, and data from the follow-up study to estimate this risk at 40 and 50 years of age. The red line corresponds with the probability density function of the beta distribution estimated based on the re-estimated 10-year CVD risks. The overall correlation estimate was 0.855 between the risk change between 30-40 years for the post-partum screening and 40-50 years for the CREw-IMAGO study.

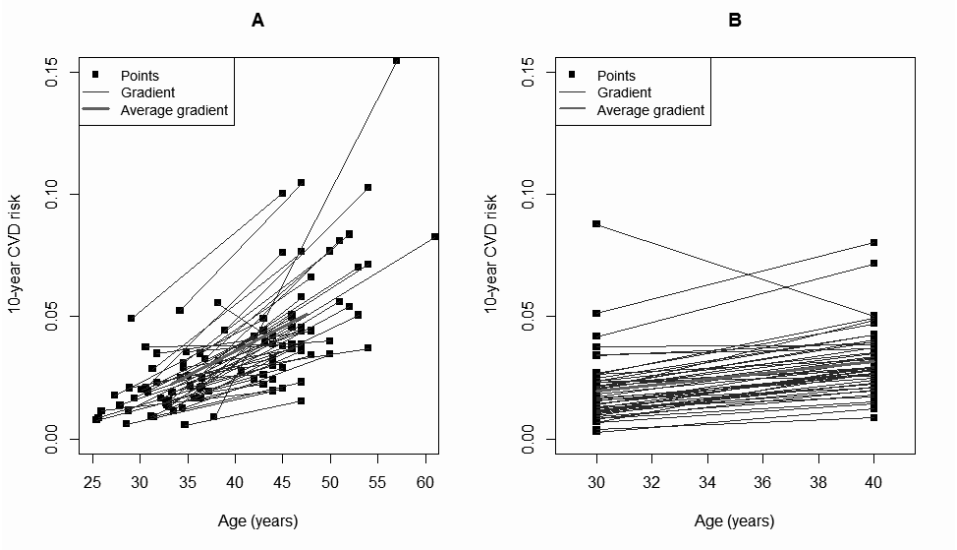

FIGURE 1. Ten-year risk estimates according to the Framingham risk score for 49 women who participated in two cardiovascular screening studies at different time-points after pregnancy complicated by preeclampsia. (**A**) the authentic 10-year CVD risk estimates for 49 women with two CVD screening moments. (**B**) the re-estimated CVD risk assessments to a standardized 10-years' time period. *Abbreviations: CVD, cardiovascular disease.*

Cost-effectiveness analysis

Table 1 presents the results of the cost-effectiveness analysis using the chosen risk thresholds of 2% and 5%. Screening with an absolute risk threshold of 5% was less costly, resulting in incremental costs for the screening and lifestyle intervention program of €220 per woman compared to no screening. However, preventive screening with a 2% threshold, although more expensive at €604 compared to no screening, showed the largest health benefit (0.07 QALYs per woman). Using a 5% threshold was the most cost effective strategy with an incremental cost-effectiveness ratio (ICER) of €3882/QALY gained. Compared to the 5% threshold the model, when a 2% threshold was analyzed, the screening would not be cost effective as the ICER was €40,739/QALY, which is above the WTP threshold of €20,000/QALY.

The incremental cost-effectiveness plane with a WTP threshold of €20,000/QALY is presented in Figure 3. Figure 4 shows the cost-effectiveness acceptability curve. For a WTP threshold of €20,000/QALYs gained, all strategies (including no screening) have a similar likeliness to be cost effective.

TABLE 1. Cost-effectiveness analysis of CVD screening and prevention models.

Preventive screening threshold	**Average costs** (€)	**Average health benefits** (QALYs)	**Incremental cost compared to no screening** (€)	**Incremental health benefits compared to no screening** (QALY)	**NHB**	**ICER** (€/ QALY)
No screening	193 844	27.41	-	-	-	-
5%	194 063	27.46	220	0.057	0.045	3382
2%	194 448	27.47	604	0.067	0.035	40 739

Impact of 5-annual cardiovascular screening in women with previous preeclampsia from the age of 30 years onwards, including implementation of lifestyle interventions when estimated 10-year CVD risk was above the threshold indicated in the table. The analyses was performed with 2,000 women and Monte Carlo 1,000 simulations. A willingness-to-pay threshold of €20,000/QALY was used to estimate the net health benefit (NHB). *Abbreviations*: QALY, quality-adjusted-life-years, CER, cost-effectiveness ratio; NHB, net health benefit; ICER, incremental cost-effectiveness ratio.

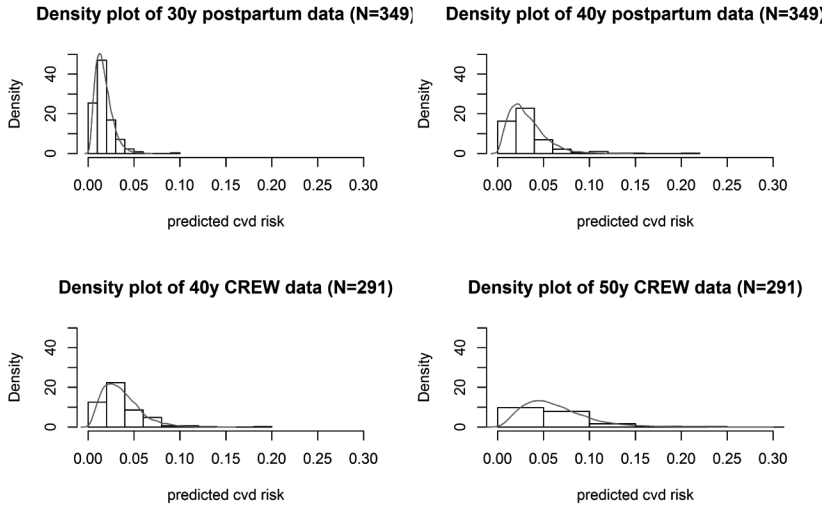

FIGURE 2. Histograms of the re-estimated 10-year CVD risk evaluations for all women included in both authentic cohorts. Ten-year risks were re-estimates for all women included in each screening cohort. Data from the post-partum screening is re-estimated at 30 and 40 years, and data from the follow-up study at 40 and 50 years of age. The red line corresponds with the probability density function of the beta distribution estimated based on the original 10-year CVD risk estimates.

Abbreviations: CVD, cardiovascular disease.

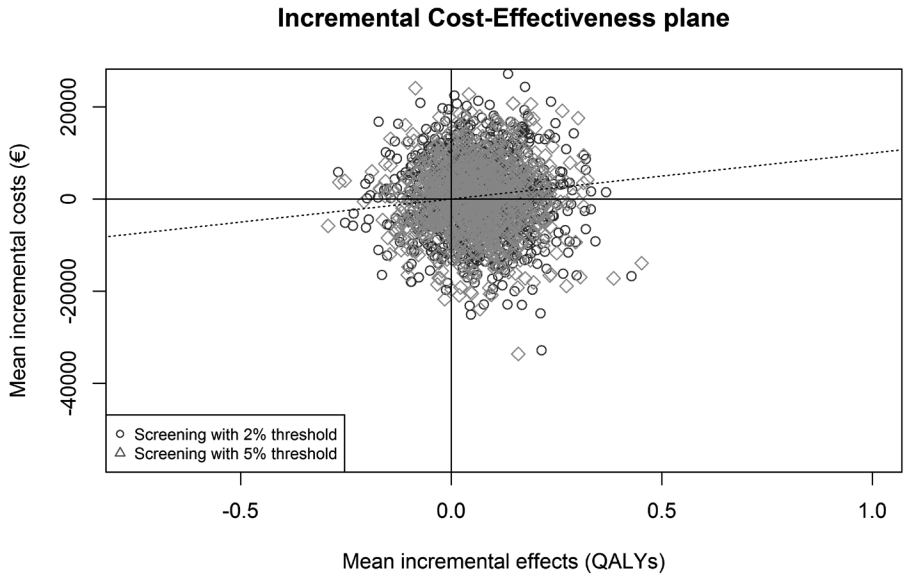

FIGURE 3. incremental cost-effectiveness plane with a WTP threshold of €20,000/QALY.

Abbreviations: WTP, willingness-to-pay; QALY, quality-adjusted-life-years.

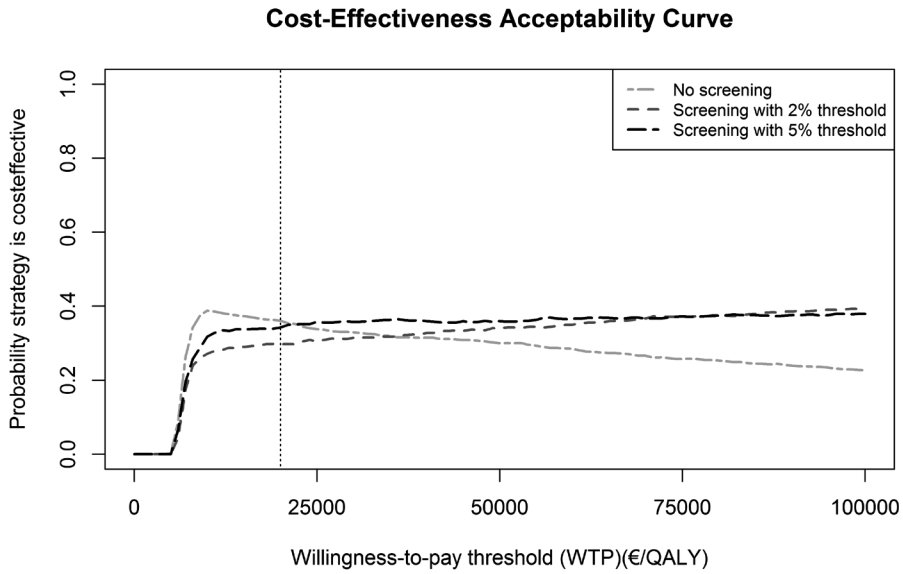

FIGURE 4. Cost-effectiveness acceptability curves for preventive screening and lifestyle interventions in women with a history of preeclampsia. Cost-effectiveness acceptability curve showing preventive screening with a risk threshold of 5% for implementing lifestyle interventions to have the largest probability to be cost-effective for a WTP threshold of 20,000 €/QALY gained.

DISCUSSION

A model-based simulation study offers a feasible way of investigating whether early and long-term preventive screening and lifestyle interventions may reduce CVD burden in women with previous preeclampsia. We found that early (i.e. starting at 30 years old) and repeated (5-annually) screening and lifestyle interventions after preeclampsia can potentially reduce CVD risk and perhaps even increase length of life in good quality. Preventive screening and lifestyle intervention with an absolute risk threshold of two or five percent may be cost effective at a willingness-to-pay threshold of €20,000 per QALY gained. Our analysis suggests that screening and prevention (by life style interventions) shortly after preeclampsia may be cost-effective to improve health and reduce cardiovascular risk.

Strengths and limitations

The strength of this study is based on the incorporation of actual risk factor data from women who underwent extensive cardiovascular screening at several time points after preeclampsia. These data gave insight in the risk distribution among women with preeclampsia for different age categories. Furthermore, these data were used to estimate the correlation between 10-year risk estimates *within* women over time. A micro-simulation model was used to assess the long-term benefits from CVD preventive screening in young women. Using a model with a lifetime horizon is important, as age is a key factor in development of CVD. Moreover, the first manifestation of CVD may take two to four decades following preeclampsia.[41] By including distributions for the parameters of which we were unsure, a realistic estimation of the uncertainty in the expected long-term (health) benefits of preventive intervention is achieved. Additionally, we believe that this model, with some adjustments, can be applied to assess the potential benefits of early CVD prevention in other populations with (female) specific risk factors, such as women with polycystic ovarian syndrome (PCOS) or premature ovarian insufficiency (POI).[42,43] Use of this model may provide information to make evidence-based decisions when establishing cardiovascular prevention programs for women with a complicated obstetric history, while the evidence of intervention studies are still lacking.

Our analysis also has limitations. Because evidence on several parameters within the model was lacking, a degree of assumption and extrapolation was required. To include a degree of uncertainty we allowed a wide distribution for most parameters and incorporated expert opinions on behavior, risks and benefits of interventions in this specific group of women (Supplementary Table 2). We cannot exclude the possibility that lifestyle changes for (older) women may not always be feasible. Also, data on CVD risk after 80 years of age was lacking and was therefore kept constant beyond this age in the model. Furthermore, screening and lifestyle interventions were not combined with any drug therapies, such as statins or antihypertensive medication. However, as women were young during the post-partum risk evaluation, we assumed that some may not be willing to take medication from a young age due to possible side effects. Although the opposite may be true entirely due to the

fact that these are increasingly aware of their CVD risk after experiencing the symptoms of preeclampsia, perhaps making them more likely to be adherent to drug therapy. Additionally, a notable proportion has health complaints due to hypertension shortly after pregnancy, making it more likely they could benefit from early use of antihypertensive medication.[44] Although we did not include this in our model, this needs to be taken in to account for future research. In addition, long term follow-up data regarding impact of such treatment in women with previous preeclampsia are lacking. It must be stated however, that this is likely to be beneficial. Recent CVD preventive guidelines have supported treatment of young individuals even though evidence from randomized or cohort studies for these implementations are not yet available.[45] Taking these possibilities in to consideration, the assumption that women in our 'no screening' scenario are not identified, or treated, before the age of 60 may be an underestimation of usual care in reality. This needs to be evaluated further and may need to be taken in to account when performing similar research in the future.

In this study, we estimated CVD risk with the FRS, which might not be suitable for young women with preeclampsia. Age is a strong contributor in this score and although women with previous preeclampsia develop CVD as soon as 10 years earlier, the FRS is often not raised above the indicative 10% threshold, soon after pregnancy.[13,14] Unfortunately, there is no available risk score for women that includes a (complicated) obstetric history. Additionally, we used data from women with both late and early onset preeclampsia for this study. Although this gives a relevant overview of women with preeclampsia, this may be an underestimation for women with a severe, or early, phenotype. Results from our analysis can therefore also not directly be extrapolated to women with other pregnancy complications (specific including phenotypes of preeclampsia), as the preventive effects are likely to differ in those women. Lastly, we were not able to consider comorbidities or the occurrence of other diseases, like auto-immune disorders or impaired memory, associated with preeclampsia and affecting the outcome and quality of life in these women.[46]

Comparison with other studies

Two Dutch studies showed the potential benefits of early hypertension and metabolic syndrome detection, including medication and/or lifestyle intervention, in women with a history of preeclampsia. [17,18] Whereas the published studies use a Markov model with a number of "health states" with fixed transitions between states, we use an individual-based model. This provides the opportunity to include CVD risk factors, simulated events and outcomes on an individual level which moves closer to individualized care. For the current study, all individual CVD risk factors were combined in one risk estimate and the change in expected risk was modelled over time. A possible benefit of the current model used lies in the possibility of assessing CVD risk factors separately in future investigations, making the model very close to a completely individual assessment. Furthermore, the use of real follow-up data of women at 10-20 years post preeclampsia to estimate the CVD risks and subsequent correlation between the two time points likely has led to more accurate and realistic results.

Clinical implications

Although our model estimates that early screening and lifestyle interventions may be cost effective and increase quality of life, we need to take some aspects into consideration. The first being the fact that women in our proposed model are young and may perhaps be less willing to attend screening and comply with lifestyle interventions. In our experience, offering cardiovascular screening to women after (especially early-onset) preeclampsia results in relatively high percentage of women willing to participate. Unfortunately, many of these mothers with young children eventually do not attend the half day of in-hospital screening that is offered in a study set-up. Although treatment of specific risk factors, such as familiar hypercholesterolemia, should be treated by a vascular specialist, implementation of screening and lifestyle interventions in Dutch primary care would be more efficient. The Dutch GP system is well structured and easily accessible, but it needs to be said that this may not be the case in other countries. Although our model shows a lengthening of individual life in good quality, this effectuates in to approximately 20-25 added days of living in perfect health. When putting the increase in QALYs in population perspective however, it amounts to significant impact on quality adjusted health. In our study we were not able to evaluate willingness to attend screening and follow lifestyle interventions for this relatively modest amount of individual gain.

In addition, our model only included the quality of life gained by reducing cardiovascular outcomes. It is likely that the proposed lifestyle interventions (i.e. weight reduction, smoking cessation) have an additional health benefit in preventing other (non-cardiovascular) health problems, such as preventing joint problems in obese patients and chronic pulmonary problems in smokers. Additionally, the lowering of risk factors will likely reduce risk of other long-term events, such as hypertension and subsequent renal failure, that were currently not incorporated in the model. This may result in further lengthening of life in good health as women age. Taking facts combined, we estimate that, in a real-world setting, more women would, and could, benefit from early cardiovascular screening and intervention when they have experienced preeclampsia.

Conclusion

Our model-based impact assessment demonstrates that screening and lifestyle interventions may beneficial and can be cost effective in order to prevent CVD in women with a history of preeclampsia. This study underlines that we may need to establish a lifelong cardiovascular prevention program for women starting early after experiencing preeclampsia.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE 1. Example of recalculation of 10-year CVD risk estimates.

Box	*Description*	*Value*	*Formula*
1	Age at inclusion initial CVD screening (years)	34.27	
2	Age at inclusion in CREw study (years)	54.00	
3	Difference in time (years)	19.73	box 2 – box 1
4	10-year CVD risk at inclusion in initial CVD screening (%)	1.651	
5	10-year CVD risk at inclusion in CREw-IMAGO (%)	3.701	
6	Absolute difference in 10-year CVD risk	2.050	= box 5 – box 4
7	Relative change in 10-year CVD risk for follow years	2.242	=(box 5)/(box 4)
8	Relative annual change in 10-year CVD risk	1.042	= box 7^(1/box3)
9	New 10-year CVD risk at 30 years (recalculated) (%)	1.386	= box 4*((box8) ^(30-box1))
10	New 10-year CVD risk at 40 years (recalculated) (%)	2.087	= box5*((box8)^(40-box2))

SUPPLEMENTARY TABLE 2. Parameters estimates and values used for the microsimulation.

Uncertain values with unknown range				
Name	*Value*	*95% CI*	*Distribution*	*Source*
Marginal correlation between risk profiles (per 10 years)	0.88	0.79-0.96	uniform	*Data CREw -UMC*
Proportion of women below the age of 60 who participate in early preventive screening	0.39	0.21-0.60	uniform	Data CREw-UMC
Annual proportion of women above the age of 60 who are detected at the GP	0.03	0.02-0.04	uniform	Expert opinion*
Probability of women who are adherent to medication after 10 years	0.20	0.11-0.30	uniform	*Expert opinion**
Relative change of adherence rate after 10 years for women who start with preventive screening, i.e. women below the age of 60	0.99	0.91-1.09	uniform	*Expert opinion**
Modelling choices				
Name	*Value*	*95% CI*	*Distribution*	*Source*
Number of individuals	2000	-	-	[1,2]
Discount rate Cost	4%	-	-	[3]
Discount rate Effect	1.5%	-	-	[3]
Decrease quality of life over age			-	[4,5]
Start age preventive screening – usual Care	60	-	-	-
Follow up screening older age (usual care) (years)	30, 35, 40, 45, 50, and 55 years	-	-	-
Uncertain values but with a certain range/distribution				
Name	*Value*	*95% CI*	*Distribution*	*Source*
Average 10-year CVD risk at age 30	0.02	0.01-0.04	uniform	*Data CREW - UMC*
Average 10-year CVD risk at age 80	0.85	0.80-0.90	uniform	*Expert opinion**
Relative risk ratio after first CVD event (for all years)	2.13	1.70-2.62	gamma	[6]
Relative risk ratio after recurrent CVD event (for all years)	2.16	1.66-2.65	gamma	[6]
Relative risk preventive treatment	0.91	0.84-0.96	beta	[7,8]
Mean Costs†				
Name	*Value*	*95% CI*	*Distribution*	*Source*
Lifestyle intervention	734	693-780	gamma	[9]
Early preventive screening#	143	128-160	gamma	[10]
Event				
Coronary artery disease (CAD)	5036	4989-5087	gamma	[10]
Cerebrovascular accident (CVA)	19475	19337-19606	gamma	[10]
Other CVD (OCVD)	2982	2922-3043	gamma	[11], range assumption

SUPPLEMENTART TABLE 2 CONTINUED.

Mean Costs†				
Name	*Value*	*95% CI*	*Distribution*	*Source*
Recurrent CVD event	1,235	1199-1274	gamma	[11], range assumption
Death due to CVD	2371	2353-2390	gamma	[12]
Post event – annual				
Coronary artery disease (CAD)	762	730-796	gamma	[11], [4]
Cerebrovascular accident (CVA)	10054	9966-10144	gamma	[10]
Other CVD (OCVD)	3370	3304-3434	gamma	[11], range assumption
Recurrent CVD event	687	657-715	gamma	[11], range assumption
Mean Utilities				
Name	*Value*	*95% CI*	*Distribution*	*Source*
Event for whole cycle				
Coronary artery disease (CAD)	0.77	0.59-0.90	beta	[11], range assumption
Cerebrovascular accident (CVA)	0.63	0.46-0.77	beta	[11], range assumption
Other CVD (OCVD)	0.69	0.52-0.83	beta	[11], range assumption
Recurrent CVD event	0.45	0.29-0.61	beta	[11], range assumption
Post-event				
Coronary artery disease (CAD)	0.91	0.64-0.99	beta	[11], range assumption
Cerebrovascular accident (CVA)	0.63	0.42-0.81	beta	[11], range assumption
Other CVD (OCVD)	0.68	0.47-0.85	gamma	[10]
Recurrent CVD event	0.67	0.46-0.84	gamma	[10]

*Expert opinion was formed by 5 of the main authors (GL, LB, AF, AM, BR) † The unit of cost is euro and all costs are updated according to Dutch consumer price indices (2017) and rounded to whole euros. # This includes costs due to a GP visit, pharmacy and laboratory tests. *Abbreviations*: CI, confidence interval; GP, general practitioner; CVD, cardiovascular disease.

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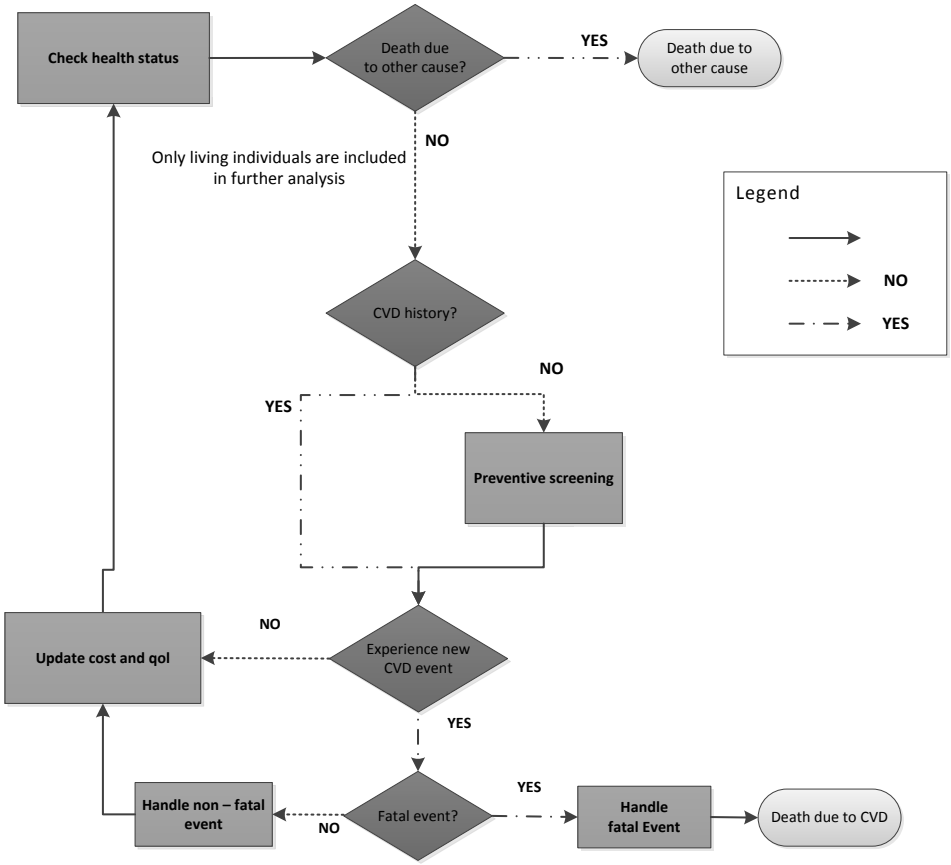
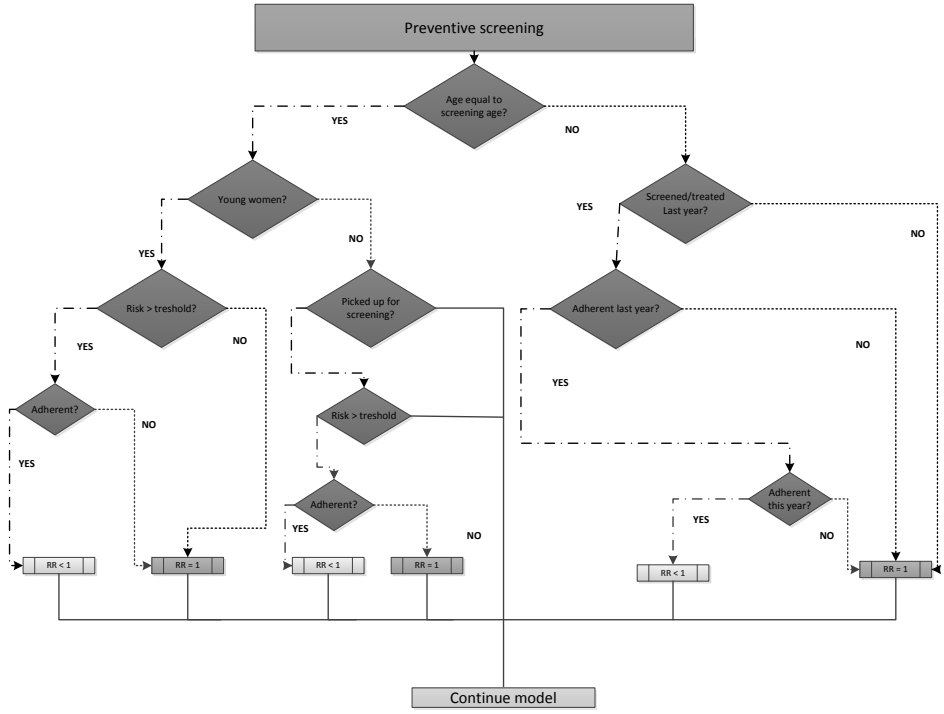
SUPPLEMENTARY TABLE 3. Age dependent event distribution for women

Age	CAD – non fatal	CVA – non fatal	OCVD – non fatal	CAD - fatal	CVA - fatal	OCVD - fatal
20-30	0.158	0.526	0.263	0.000	0.053	0.000
30-40	0.333	0.333	0.238	0.048	0.048	0.000
40-50	0.475	0.263	0.220	0.008	0.008	0.025
50-60	0.536	0.207	0.197	0.032	0.014	0.015
60-70	0.317	0.307	0.010	0.178	0.030	0.158
70-80	0.326	0.326	0.081	0.140	0.012	0.116
80-90	0.317	0.307	0.010	0.178	0.030	0.158
90-100	0.326	0.326	0.081	0.140	0.012	0.116

Abbreviations: CAD, coronary artery disease; CVA, cerebrovascular accident; OCVD, other cardiovascular disease.



SUPPLEMENTARY FIGURE 1. Flowcharts of the simulation model.



SUPPLEMENTARY FIGURE 2. Flowcharts of the simulation model.

Appendix - CREW member list

The CREW consortium consist of (in alphabetical order):
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Summary and general discussion

SUMMARY & MAIN FINDINGS

In **part I** of this thesis, we first aimed to elucidate factors leading to the actual 'failing of the stress test of pregnancy' by studying maternal spiral arteries and the uterine tissue underlying the placenta (i.e. the placental bed) in pregnancy complications.

We produced comprehensive data indicating that impaired spiral artery remodeling is present in almost two thirds of women with fetal growth restriction (FGR) and/or preeclampsia **(chapter 3)**. Impaired remodeling was often combined with absence of intramural trophoblast cells, although presence of interstitial trophoblast was abundant. This challenges the classic hypothesis that impaired spiral artery remodeling is the result of problems with invasiveness of the trophoblast cells. Next to remodeling problems, acute atherosis can be found in almost a third of women presenting with these pregnancy complications **(chapter 3)**. These lesions appear to be associated with modifiable cardiovascular risk factors in the mother underlining the role of pre-existent maternal constitution **(chapter 2)**. This is consistent with the concept of complicated pregnancy as a 'stress test' for future cardiovascular disease (CVD). Furthermore, we found that the baby is born earlier and relatively smaller when preeclampsia and/or FGR is associated with underlying impaired spiral artery remodeling **(chapter 4)**. Studying vascular endothelial cells (ECs) from the placental bed at the transcriptional level, we found evidence of altered inflammation and endothelial dysfunction suggesting that maternal inflammatory predisposition is also at play in women who 'fail the stress test' **(chapter 5)**. Moreover, placental function seems to be directly affected by the mothers health and constitution as we found evidence of histopathological lesions in mothers who were obese before getting pregnant **(chapter 6)**.

In **part II**, we aimed to elucidate some of the missing information regarding the development of cardiovascular disease (CVD) after have 'failed the stress test' previously (i.e. women with a history of preeclampsia). In other words, we investigated factors that may help to identify *who* is at increased risk, *when* cardiovascular damage develops and *if* timely screening and lifestyle interventions may be cost- beneficial and effective.

To further specify *who* would have added benefit from screening and prevention, we performed a systematic review and meta-analysis and found that women who experienced preeclampsia in more than one pregnancy have an increased risk of CVD compared to women having preeclampsia once **(chapter 7).** To pinpoint *when* visible cardiovascular damage develops, we performed coronary computed tomography (CCT) in women with a history of preeclampsia. On average women with previous preeclampsia develop coronary artery calcification (CAC) 5 years earlier than women with uncomplicated obstetric history, around the age of 45 **(chapter 8)**. Although research on effective prevention programs in these women is lacking, one may conclude that the recommended screening after the age of 50 is too late for women who experienced preeclampsia. Lastly, to identify *when* to screen and *if* we may be able to prevent it, we performed a model-based micro simulation study entailing data from the CCT study. We found that early (i.e. at 30 years) and regular screening in combination with lifestyle interventions reduce the risk of CVD in these women, are likely to improve quality of life and to be cost-effective **(chapter 9)**.

IMMUNOVASCULAR PATHOLOGY UNDERLYING PREECLAMPSIA, FETAL GROWTH RESTRICTION AND HEALTHY PREGNANCY (PART I)

Spiral artery remodeling and vascular pathology

As previously stated, evidence regarding defective spiral artery remodeling in pregnancy complications was scarce and underpowered. Although several small series of Caesarean hysterectomies and placental bed biopsies have shown "physiological" spiral artery remodeling to be incomplete in preeclampsia and FGR, prevalence data had not been established.[1,2] As some studies additionally found trophoblast cells to be less present in the placental bed and the vascular wall, it was hypothesized that the remodeling process may be impaired by the reduced 'invasiveness' of these cells.[2,3] In **chapter 3** we investigated spiral artery remodeling in a comprehensive cohort collecting placental bed biopsies from women with preeclampsia and/or FGR compared to uncomplicated pregnancy. In our cohort, impaired remodeling of spiral arteries was observed in over 60% of cases, and was found only in a very small proportion of uneventful pregnancies. In addition, we observed that in almost half of pregnancies with preeclampsia/FGR the spiral arteries showed no intramural trophoblast cells, although trophoblast presence in surrounding tissue was abundant. Previous studies have shown initial stages of adequate spiral artery transformation to rely on vascular receptivity as a result of a strictly maternal response to pregnancy, even in absence of trophoblast cells.[4-6] In our study, we were able to quantify these observations and provide prevalence data regarding the large proportion of pregnancies affected by spiral artery pathology among women with preeclampsia and FGR. These finding makes a strong case for the belief that maternal spiral arterial incompetence precedes pregnancy specific vascular adaptations in the development of preeclampsia and FGR.

Next to defective outward remodeling, we found vascular lesions of the vascular wall of the spiral artery, including 'acute atherosis' and thrombosis, to be common in preeclampsia and FGR **(chapter 3)**. Previous studies mainly showed evidence of these lesions downstream to affected, unremodeled, spiral arteries (i.e. the superficial decidual segments).[7,8] From the observations in our cohort we conclude that women with preeclampsia and/or FGR have spiral artery acute atherosis in almost 30% of cases, and are equally prevalent in the myometrial and decidual segments.

Spiral artery acute atherosis resembles the histopathological features of early-stage atherosclerosis (i.e. leukocyte infiltration, lipid accumulation and fibrin deposition).[9] While atherosclerosis develops slowly throughout life, vascular pathology in the uteroplacental circulation occurs relatively instantly during preeclampsia. This may emphasize the mothers inability for vascular adaptations, when under, acute or chronic, stress. Additionally, **chapter 2** showed that women with acute atherosis had unfavorable lipoprotein profiles directly postpartum. Although our sample size was limited, this may present important information regarding maternal cardiovascular and metabolic constitution and risk.[10] A clear limitation is that these measurements were performed the day after Caesarean section, while

many women were still enduring severe preeclampsia symptoms and receiving several medications. If these CVD risk markers (i.e. higher LDL-cholesterol and triglycerides) remain after pregnancy, needs to be further evaluated. One other group has investigated this relationship and found decidual vasculopathy (in the placenta) to be associated with increased CVD risk parameters 6-12 months post-partum.[11,12] Due to the sizeable loss to follow up in this group of mothers with young children, we have not yet been able to complete inclusion of sufficient additional cardiovascular work-ups at 6-12 months after pregnancy. Future perspectives of this study are aimed at elucidating if placental bed pathology can be used to identify women who are at increased cardiovascular risk and who would surely benefit from early screening and prevention.

Although we now have shown that a causal relationship between spiral artery remodeling problems and preeclampsia/FGR is likely, this does not clarify (patho)physiologic remodeling itself. The mother may not be capable of functional remodeling. Our study was not set up to specify why this may be or what vascular (or inflammatory) component could be key. Several studies revealed that components of the vascular wall (i.e. endothelial cells, extracellular matrix, vascular smooth muscle cells) and an angiogenic imbalance are perhaps both indicators and causal pathways resulting in preeclampsia.[13,14] If these factors lead to impaired remodeling (i.e. abnormal placentation), to a maternal systemic (hypertensive) response, or both, is not known. Different, perhaps separate, pathways may explain the heterogeneous presentation of preeclampsia and FGR. Additionally, the chronic inflammatory state shown in former preeclampsia patients may lead to development of both initial vascular pathology and (accelerated) formation of long-term atherosclerosis and CVD.[13,15]

Potential immunovascular mechanisms

In **chapter 5** we aimed to identify whether ECs derived from the placental bed of patients with early onset preeclampsia with FGR have different transcriptional profiles indicating specific altered pathways. We identified differentially expressed genes in this highly specific cell population which indicated innate immunity, platelet activation and endothelial cell activation to be different when pregnancy is complicated by preeclampsia. Multiplex immunoassay in women with similar preeclampsia/FGR phenotype confirmed a significant role of maternal inflammatory and endothelial cell activation in the symptomatic phase of disease. This study is, to the best of our knowledge, one of the first studies using these techniques to identify candidate genes in this cell type and merits further investigations to identify exact relevance and future clinical possibilities.

We find altered presence of several immune cell populations at the maternal-fetal interface in **chapter 2 and 3**. This finding reconfirms a key role for the maternal inflammatory system in either causing functional (or dysfunctional) spiral artery remodeling or in response to vascular damage.[16-18] Our, and previous, studies were however set up to identify large differences in perivascular and local inflammation and included immunohistochemical markers for general cell populations that do not give us specific information regarding

subsets, phenotype and function, and warrants future research. The wide range of gestational ages at delivery, the only opportunity to obtain placental bed biopsies, needs to be taken in to consideration when interpreting data and constructing possible explanations. As spiral artery remodeling itself occurs much earlier in pregnancy and studies have shown immune and vascular composition to change as pregnancy progresses, these results are even harder to interpret correctly.[19-21] With these considerations in mind, we can only speculate on the significance of altered presence of these cells in the placental bed when pregnancy is complicated.

We found increased perivascular macrophage infiltration **(chapter 2 and 3)**, which has previously been associated with poor intravascular trophoblast invasion and abnormal spiral artery adaptation.[16,17] Uterine macrophages are important for normal placentation by induction of apoptosis and extracellular matrix degradation in the first trimester. Studies show placentas from preeclamptic pregnancies to contain more macrophages and more pro-inflammatory M1 polarization, similar to the phenotype in atherosclerosis.[1,17,22-26] M1 polarization has been associated with impaired trophoblast infiltration and the M2 type seems to play a beneficial role in vascular remodeling.[23,25,27-29] Uterine macrophage function, phenotype, and especially polarization, in regard to spiral artery remodeling needs to be investigated further. A role for macrophages in development of both pregnancy complications and future CVD seems apparent. As this is currently being trialed in CVD research, this may even give relevant opportunities for (immune) therapy once we identify specific mechanisms.[13,28,30,31]

Although the function of NK cells is thought to be important in the first and second trimester, we found diminished uNK cell in spiral arteries clearly apparent in preeclampsia and FGR in **chapter 3**. A higher presence of uterine natural killer (uNK) early in pregnancy has been associated with more advanced spiral artery remodeling.[20,32,33] Investigating presence of uNK cells in the placental bed in preeclampsia yields conflicting results, possibly due to timing and technique of tissue sampling and selection of groups.[16,33-36] This makes a definitive conclusion regarding our findings more complicated, but again shows an important influence of the maternal immune system in regulating pregnancy specific (vascular) adaptations.

Although other studies demonstrated diverse results, we found preeclampsia to be associated with lower perivascular presence of T-cells (CD3+) cells in the placental bed in **chapter 2 and 3**.[33,35,36] Current literature points out a significant higher presence of pro-inflammatory Th17 and lower amount of anti-inflammatory regulatory T cells in both preeclampsia and CVD.[1,37] As CD3+ is a co-receptor shared by many T cells subsets, our results cannot be used to assess the phenotype and function of these cells. Regulatory T-cells acquire an increasingly important role in the development of pregnancy complications and future CVD due to their anti-inflammatory nature, and need to be further evaluated in these high risk women.[38]

Ultimately, we need to know how the maternal immune and cardiovascular system interact at maternal-fetal interface (effectively the black box of pregnancy) and identify at what

level interplay of fetal and placental factors influence pregnancy-specific adaptations.[39,40] In addition, much of the above described research is solely based on research of the placenta. As this thesis provides evidence that maternal uterine environment is extremely important for successful pregnancy, the causative interacting mechanisms may lie 'deeper'. Our group aims to use the study set up show cased in **chapter 5,** using flow assisted isolation of specific (immune) cell types from placental bed biopsies, to further clarify other inflammatory and vascular pathways in preeclampsia and FGR.

Neonatal implications of impaired spiral artery remodeling

In **chapter 4** we found that pregnancies complicated by preeclampsia and FGR ended almost two gestational weeks earlier when spiral artery remodeling is inadequate or absent. Additionally, babies were born with lower birthweight (percentiles). Although the increase of morbidity and mortality in the perinatal period was likely due to a lower gestational age, we hypothesize that babies who are born following ineffectively remodeled spiral arteries may additionally be more deprived in-utero. It is suspected that a hostile in-utero environment (i.e. chronic hypoxia) may 'program' mechanisms in the fetus, having impact on health and disease later in life.[41,42] They may therefore be more susceptible to adverse long-term outcome. Alternatively, the pregnancies with absent remodeling may represent the 'worst' phenotype, which are associated with abnormal prenatal ultrasound findings (i.e. umbilical artery flow) that often leading to a swift decision to deliver the baby in clinical practice.[43] The long term health of these children should be followed to determine the association between pathophysiological mechanisms (childhood and adolescent) health, and the ability to develop normal motor-, neuro- and cognitive function.[44-46] Alternatively, accelerated post-natal growth catch up and perhaps even the carry-over of genetic predisposition may play a role in causing an ultimately worse outcome for these babies.[47,48] Follow up should not only focus on making these distinct associations apparent, but also if the unhealthy environment in utero may be doing more harm than an earlier premature birth would do.

Implications of maternal health for placental function and pregnancy outcome

In **chapter 6** we investigated whether prepregnancy obesity, a pre-existent risk marker for both preeclampsia and CVD, affected placental characteristics in uncomplicated pregnancy. We found that women with increasing BMI deliver heavier babies and heavier placentas and was associated with more high-grade chronic villitis and accelerated villous maturation.

As many traditional risk factors for CVD can be identified in the development of pregnancy complications the growing amount of women with an unhealthy lifestyle and high BMI is worrisome. Additionally, we found a substantial effect of parity on maternal, placental, and neonatal weights. This indicates that having (more) children, even when healthy and uncomplicated, has implications for future disease susceptibility and strengthens our resolve that women should enter pregnancy as healthy as possible. This calls for methods to increase awareness of the population and generalize preconception care. Irrespective

of previous pregnancy outcome, this leads us to hypothesize that regaining fitness and health before entering a second pregnancy may be beneficial in order to improve obstetric outcome and perhaps even the long-term health of mother and child.[49]

MATERNAL CARDIOVASCULAR HEALTH AFTER PREECLAMPSIA (PART II)

Long-term epidemiologic studies have shown pregnancy complications, such as preeclampsia, to be associated with an increased lifelong risk of CVD.[50-53] Although we have been aware of this association for over ten years, preventive healthcare is still poorly implemented in many countries. Despite publication of a national multidisciplinary guideline for cardiovascular risk management in women with pregnancy and reproductive complications (2016), this includes the Netherlands.[54] This guideline recommends that women with a history of preeclampsia should be offered screening for cardiovascular risk factors by their general practitioner after the age of 50 years. Most other cardiovascular risk management guidelines are even more conservative and recommend that screening and treatment should only be advised when 10-year CVD risk (i.e. Framingham risk score) is more than ten percent. As these risk scores are strongly age-dependent, preventive measures are almost never indicated in the first 10 to 20 year after preeclampsia. In other words, current cardiovascular screening and prevention is aimed at middle-aged women and may not be appropriate for many of them. With questions still remaining regarding the timeline of CVD development after pregnancy complications, we aimed to further specify women *who* would benefit the most from screening and preventive measures. Additionally, we aimed to identify *when* (or at what age) screening would be most useful. Lastly, we aimed to investigate *if* screening and lifestyle interventions in women shortly after preeclampsia may be beneficial and cost-effective in order to prevent CVD and raise quality of life.

Recurrence of preeclampsia and cardiovascular risk

Although associated risk is higher, not all women with preeclampsia develop CVD. It may therefore be important, and cost-effective, to narrow down subsets of women *who* would benefit most from early prevention. Previous studies have shown that association of preeclampsia and CVD may be attenuated by time of onset, severity and combination with other pregnancy complications such as preterm delivery or placental abruption.[55-57] Little evidence was available however for estimation of cardiovascular risk in women who experienced preeclampsia in more than one pregnancy. As reviewed in **chapter 7** we found that women with recurrent preeclampsia consistently have a higher risk of CVD compared to women with preeclampsia once and subsequent normal pregnancies (Figure 1).

This supports the concept of pregnancy as a ‘stress test’ for cardiovascular health. It is likely that women who have a worse vascular constitution, and thus a higher risk for CVD, fail to make pregnancy specific adaptations in the second pregnancy (resulting in preeclampsia) when most other women have subsequent uncomplicated pregnancies (approx. 85%).[58,59] Additionally, we postulate that women with a vulnerable cardiovascular system, more often have an early onset or severe phenotype of preeclampsia and are more likely to experience recurrence of it in a next pregnancy compared to those who developed

preeclampsia later or milder.[58,59] A causal relationship cannot completely be dismissed. If preeclampsia does cause lasting damage to the maternal cardiovascular system, recurrence in a next pregnancy will further increase cardiovascular risk. Irrespective of the mechanism, recurrence of preeclampsia is a clear indicator that cardiovascular screening and preventive measures are indicated and need to be initiated.

Coronary artery calcifications and atherosclerosis

Our group previously showed that women with previous preeclampsia over the age of 45, had a comprehensive percentage of coronary artery calcification (CAC), a subclinical form of CVD.[60,61] Additionally almost half of women had coronary atherosclerotic plaques on coronary computed tomography (CCT) Angiography.[60] Several studies have shown women with a history of preeclampsia to develop traditional risk factors, such as hypertension, 5-10 year earlier compared to women with uncomplicated obstetric history.[62,63] The previous study was not set up to identify *when* women develop CAC and consequently *when* measures need to be implemented to prevent coronary artery disease. We therefore conducted the CCT study in younger women with previous preeclampsia to find out at what point in life women develop these irreversible cardiac abnormalities.

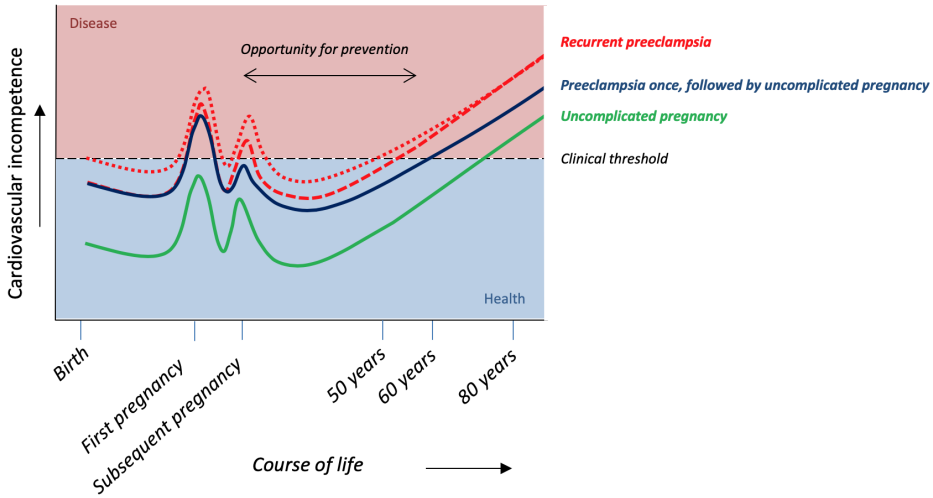

FIGURE 1. Concept of pregnancy as a "stress test", identifying women with recurrent preeclampsia at increased risk for cardiovascular disease in comparison with preeclampsia once and subsequent uncomplicated pregnancy. *Adapted from Sattar & Greer, BMJ 2002*

In **chapter 8** we found that women with previous preeclampsia aged from an age of 45 have similar prevalence of CAC compared to women who were 5 years older with normotensive obstetric history. In each age category the 10-year Framingham risk score was higher amongst women with a history of preeclampsia, but seldom crossed the indicative treatment threshold of 10%. The fact that cardiovascular risk and CAC score are age-dependent is not a surprise.[64] Similarly it is known that older women with a history of preeclampsia have CAC and atherosclerotic plaques more often than women with normal pregnancy.[53,65,66] There was however no data on at what age coronary calcifications develop in women post preeclampsia.

Furthermore, we performed mediation analysis to identify if total cholesterol level, systolic blood pressure or diabetes influenced the association of aging and CAC formation in women with previous preeclampsia. We did not find a significant mediation effect of these parameters in our study. This does however not mean that there isn't an increasing effect, separate from the association with age, on the development on CVD in these women. We found most traditionally measured, modifiable risk factors to be more prevalent in women with previous preeclampsia. This still holds strong indication that current cardiovascular screening and preventive measures need to be adequately implemented.

Even though advised in the previously mentioned Dutch guidelines, general practitioners fail to invite former preeclamptic patients for regular cardiovascular work-ups.[54,67] When they are invited, they will only be asked for screening appointments to indicate preventive measures from the age of 50. The data presented in **chapter 8** strongly suggest that preventive interventions for women with previous preeclampsia should commence before the age of 45. Currently, we would not recommend the implementation of CTA or CCTA as standard cardiovascular follow-up after a preeclamptic pregnancy due to the radiation exposure and high costs that accompany the execution of these procedures. CVD risk may however potentially be lowered by earlier detection and treatment of modifiable risk factors.

Cardiovascular screening & lifestyle interventions

To elaborate on possibilities regarding *when* to start preventive screening and *if* lifestyle interventions may be able to improve cardiovascular outcome, we used a model based micro simulation study (and cost effectiveness analysis) to quantify long-term, life time, benefits from preventive intervening in young women after preeclampsia (**chapter 9**). As the timeline over which these benefits accrue is long, a randomized or cohort setting is not realistic. Intervention studies aimed at women at a young age require different strategies than those in higher age groups, taking in to account different adherence, -endpoints, -effectiveness, and contribution of (other) risk factors. In our model, we incorporated actual data from women with previous preeclampsia who underwent extensive cardiovascular screening both soon after pregnancy (approximately 6 months) and at 10-20 years follow up **(chapter 8)**. This gives us real-life cardiovascular risk data to use in our simulation model.

The model implemented cardiovascular screening in 2000 women just after pregnancy,

at the early age of 30, with repeated screening every 5 years and received lifestyle interventions when perceived CVD risk was above a certain threshold. We found that this strategy was beneficial, raised quality of life in a small amount and was likely to be cost-effective. Using a 5% threshold was the most likely to be cost-effective strategy with an incremental cost-effectiveness ratio of €3882/QALY, resulting in 0.06 QALYs per woman.

When interpreting this paper it needs to be considered that a simulation model does not accurately quantify the benefits of the proposed prevention strategy, but does give an adequate illustration of the potential. Although our model shows increase in individual quality of life, 0.06 QALY effectuates in to approximately 20-25 added days of living in perfect health. Young women may not be willing to attend screening and follow lifestyle advice for this small amount of profit. However, our model only included the quality of life gained by reducing cardiovascular outcomes. It is likely that the proposed lifestyle interventions have an additional health benefit in preventing other diseases, such as type II diabetes and chronic obstructive pulmonary disorders, resulting in further lengthening of life in perfect health as women age. Although we are still working on fine-tuning this model, the combination of these preliminary facts, leads us to believe that many women could benefit from cardiovascular screening and intervention soon after they have experienced preeclampsia during pregnancy.

FUTURE PERSPECTIVES

There is an ongoing debate regarding the pathophysiological mechanisms linking pregnancy complications to CVD. In this thesis, we propose that a compromised maternal cardiovascular constitution is a key etiological component for the development of both. Although the data presented in this thesis support this hypothesis, it cannot be ruled out that pregnancy complications as preeclampsia induce lasting vascular damage or persistence of detrimental pathways as well. Keeping both of these theories in mind, future research needs to focus not only on identifying exact pathophysiology, but also on possibilities for treatment and prevention.

Future studies including uterine (i.e. non-pregnant) biopsies may need to be performed at a preconceptional time point. Identifying immunological, hormonal and vascular pathways important for successful decidualization and consequent implantation may be of great importance. In depth phenotypic and functional assays from specific human immune cell types and vascular components may be of great importance ultimately discovering interactions at fault in defective remodeling.

The function of the maternal immunovascular system and interaction with invading trophoblast cells, although thought to be crucial, is still unclear. We propose that investigating the behavior of these cells in association with specific maternal factors may be important to identify how the mother regulates the process of remodeling before and in their presence. For instance it may be crucial to identify through what pathways specific immune cells modulate this intricate process. Specific macrophage polarization, (regulatory) T cell subset presence and consequent production of (pro- or anti-) inflammation molecules may be involved. Although we know preeclampsia involves a maternal pro-inflammatory phenotype, we need to figure out which cells interact with trophoblast cells and consequently how this influences the composition of the artery wall. Advanced molecular phenotyping of specific maternal cell types in complicated and healthy may show pathways relevant for this type of research. For this we need to establish a large collection of samples to overcome phenotypic heterogeneity and identify common pathways. Although many groups investigate pathology using cells derived from the placenta, this may not be enough. As this thesis shows that maternal uterine environment is extremely important for successful pregnancy, the problematic interplay may lie 'deeper'. It may then be possible, to use this information to perform remodeling and invasion assays through a recent method of 3D printing of spiral artery like vessels.[68]

In addition, other pregnancy complications than preeclampsia need to be taken into consideration as well as they have been similarly associated with placental bed pathology and subsequent cardiovascular risk (i.e. spontaneous preterm birth, placental abruption).

If pre-existent maternal factors are largely at fault, perhaps an increasing role must be attributed to pre-conceptional care (Figure 2A). Ultimately, 'waiting for the stress test to fail', may simply be too late. Family physicians, birth- and public health care professionals, authorities and education all must contribute to increase the awareness of parents-to-be that being healthy,

before and during pregnancy, is necessary to have a healthy baby. As some health issues have been clearly linked to pregnancy outcome (i.e. obesity, smoking) they need to be addressed in order to establish successful, public and individual, preconceptional health. For other factors, we first need to improve current insights (i.e. endothelial dysfunction, inflammatory response) how they contribute to pregnancy complications. Also, we need to identify whether modifying these factors improves both pregnancy-related and long-term outcome.

Alternatively, treatment may be possible during early pregnancy in order to prevent preeclampsia and FGR or to treat it after becoming apparent. Although our current hypothesis poses this to be a difficult moment to intervene as symptoms may occur through several different pathways. In addition, accurate prediction of women at risk would need to be improved in order for this to be a possibility (Figure 2B).

When pregnancy complications are already manifest, treatment without specific pathophysiological knowledge remains a significant challenge. As babies and mothers are at precarious risk for severe morbidity and mortality, studies implementing (systemic) treatment have shown difficult to execute. More knowledge needs to first be gained on pregnancy specific adaptations and mechanisms leading to failure, taking the direct and long-term effects of possible treatment into account. As pregnancy requires extremely intricate and specific changes, results from animal studies are difficult to extrapolate for use in human healthcare.

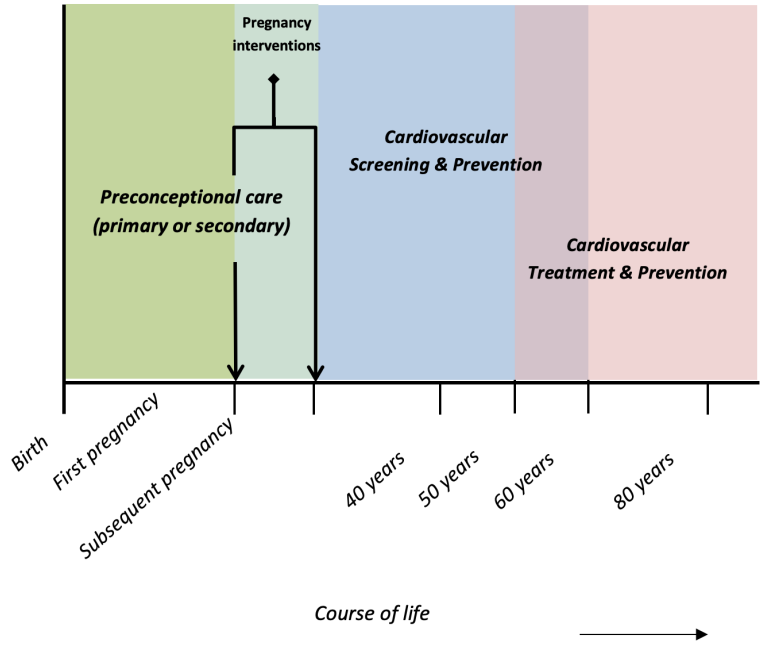

FIGURE 2. Options for prevention, intervention and treatment regarding both pregnancy complications and long-term cardiovascular outcome in the mother.

Additional to preventive measures before and during pregnancy, more long term research is required assessing CVD prevention options for women specifically (Figure 2C and Figure 2D). Our model based assessment implementing lifestyle interventions used a great deal of assumption because data on adherence, quality of life and other behavioral factors were not available. Although these women are young, early treatment or lifestyle interventions needs to be adequately researched and implemented in order to support healthy aging in the mother.

In this thesis we mainly focused on maternal risk, but there is substantial, and increasing, awareness that the long term health of the baby is compromised by complicated pregnancy. Similar research for future health should focus on the children. We do not yet know in what magnitude a hostile environment in utero induces detrimental fetal programming resulting in cardiovascular and metabolic risk so early in life, or whether we can influence these mechanisms. Follow up of these children need to be carried out to see if and when offspring can benefit from early intervention or whether they may benefit from earlier delivery taking into account the significant risk severe prematurity poses.

In conclusion, this thesis provides new information on prevalence of damage and inadequate adaptations of the arteries underlying the placenta and candidate immunovascular mechanisms, when a women experiences preeclampsia/FGR and 'fails the stress test of pregnancy'. In addition, we provide proof that women with previous preeclampsia may benefit from cardiovascular screening and preventive measures before the currently recommended age of 50 years, as irreversible damage to the coronary arteries develops much earlier than in the general population. We hope these results will help unravelling these pregnancy complications and corresponding cardiovascular risk, and ultimately, to provide better care for the many women and children who are affected by preeclampsia.

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A

NEDERLANDSE SAMENVATTING

De zwangerschap zien als een stress test

Zwangerschap vormt een uitvoerige fysiologische uitdaging voor alle orgaansystemen. Met name de cardiovasculaire, endocriene en respiratoire systemen vereisen aanzienlijke aanpassingen om een succesvol resultaat voor moeder en kind te garanderen. Verwacht wordt dat zwangerschapscomplicaties, zoals pre-eclampsie en foetale groeirestrictie (FGR), een predispositie voor toekomstige ziekten onthullen. Vanuit dit oogpunt kan de zwangerschap als een 'stress test' dienen om vrouwen (en kinderen) te identificeren die risico lopen om op latere leeftijd problemen te ontwikkelen zoals bijvoorbeeld hart- en vaatziekten.

Onvoldoende vasculaire aanpassing op de plaats in de baarmoeder die onder de placenta ligt (het placentabed), bekend als 'spiraal arterie remodellering', wordt beschouwd als een belangrijk onderdeel in het ontstaan van deze zwangerschapsproblematiek. Hoewel de precieze pathofysiologie niet bekend is, schrijft de klassieke hypothese een centrale rol toe aan een afwijkende invasief karakter van de binnendringende trofoblastcellen vanuit de placenta. Hierdoor kan de moeder uiteindelijk hoge bloeddruk, eiwitverlies en schade aan de bloedvaten (endotheel dysfunctie) ontwikkelen. Alhoewel niet altijd het geval, gaat pre-eclampsie vaak gepaard met beperking van het groeipotentieel van de baby. De abnormale spiraalarteriën zouden in dat geval leiden tot een onderontwikkelde placenta en abnormale toevoer van voedingsstoffen naar de baby. Na de bevalling laat een pathologische beoordeling van de placenta vaak histologische kenmerken zien die gerelateerd zijn aan ernstige dysfunctie, zoals bijvoorbeeld chronische ontsteking en ischemie.

Immunovasculaire pathologie onderliggend aan pre-eclampsie, foetale groeirestrictie en gezonde zwangerschap

In het **eerste deel** van dit proefschrift beschrijven we onderzoek naar factoren die leiden tot het feitelijke 'falen van de stress test' tijdens de zwangerschap door maternale spiraalarteriën (gelegen onder de placenta) en de omgeving binnen de baarmoeder te bestuderen in normale zwangerschap en in zwangerschappen met complicaties.

In **hoofdstuk 2** beschrijven we het gebruik van een nieuw biopsieafname- en beoordelingssysteem om vaatproblematiek in het placentabed systematisch te kunnen beoordelen. In 29 vrouwen met pre-eclampsie en 29 vrouwen met gezonde zwangerschap laten we zien dat vaatafwijkingen, zoals afwijkende remodellering, vaker voorkomen bij vrouwen met pre-eclampsie. Tevens vinden we bij deze vrouwen vaker tekenen van vaatschade zoals vetophoging en ontsteking (bekend als 'acute atherose') en trombose. Daarnaast zien we dat vrouwen met 'acute atherose' vaker een minder gunstig profiel van risicofactoren voor hart- en vaatziekten in het bloed hebben. Dit lijkt mogelijk een relatie tussen pre-eclampsie en hart- en vaatziekten op deze vroege leeftijd al te weerspiegelen.

Het protocol opgesteld in hoofdstuk 2 wordt vervolgens in **hoofdstuk 3** gebruikt om betrouwbare prevalentie gegevens te produceren omtrent het voorkomen van abnormale

spiraalarterie ontwikkeling en vaatschade bij pre-eclampsie en FGR. Wij laten zien dat abnormale remodellering bij twee-derde van de vrouwen met pre-eclampsie en/of FGR voorkomt en vaak gepaard gaat met afwezigheid van intramurale trofoblastcellen. Dit terwijl de aanwezigheid van trofoblastcellen in de omgeving vaak overvloedig was. Dit ontkracht mogelijk de klassieke hypothese dat afwijkende ontwikkeling van de spiraalarterie het gevolg is van een abnormaal invasief karakter van trofoblastcellen. Eerdere studies hebben aangetoond dat de eerste stadia van spiraalarterie transformatie afhangt van een maternale respons op zwangerschap, zelfs in afwezigheid van trofoblastcellen. Dit alles maakt het waarschijnlijk dat een maternale vasculaire incompetentie reeds voor de zwangerschap aanwezig is en de nodige zwangerschap specifieke aanpassingen hindert, welke gevolgd worden door de ontwikkeling van pre-eclampsie en FGR.

Naast defecte remodellering kwamen laesies van de vaatwand, waaronder ‘acute atherose’ en trombose, vaker voor bij pre-eclampsie en FGR **(hoofdstuk 3).** We concluderen dat acute atherose in bijna 30% van de gevallen voorkomt en dat deze laesies even vaak voorkomen in de decidua (oppervlakkige laag) als in het myometrium (diepere laag) van het placentabed. Acute atherose laat vergelijkbare histopathologische kenmerken met vroege atherosclerose zien (d.w.z. infiltratie van leukocyten, accumulatie van lipiden en depositie van fibrine). Terwijl atherosclerose zich langzaam ontwikkelt gedurende het gehele leven, treedt vasculaire pathologie in de utero-placentaire circulatie relatief snel op tijdens de zwangerschap. Dit zou het onvermogen voor vasculaire aanpassingen van de moeder kunnen benadrukken onder acute of chronische stress en derhalve het pre-existente risico op vaatproblematiek kunnen reflecteren.

Naast vaatproblematiek, onderzoeken we in **hoofdstukken 2 en 3** ook de aanwezigheid van het afweersysteem in en rondom de spiraalarteriën in het placentabed. De aanwezigheid van meer soorten immuuncellen benadrukt de belangrijke rol die het maternale inflammatoire systeem waarschijnlijk speelt bij het remodelleren van de spiraalarteriën. Onze onderzoeken zijn echter opgezet om grote verschillen in perivasculaire en lokale ontsteking te identificeren en omvatten immunohistochemische markers voor algemene cel populaties die ons geen specifieke informatie geven. Derhalve rechtvaardigen deze onderzoeken toekomstig specifiek onderzoek naar subsets, fenotype en functie van deze cellen in de zwangere baarmoeder.

In **hoofdstuk 4** hebben we vastgesteld dat de baby eerder en relatief kleiner wordt geboren wanneer pre-eclampsie en/of FGR met onderliggende abnormale spiraalarterie ontwikkeling gepaard gaat. Hoewel de toename van morbiditeit en mortaliteit in de perinatale periode waarschijnlijk te wijten is aan een lagere zwangerschapsduur bij de geboorte, stellen we de hypothese voor dat baby’s die worden geboren na ineffectief geremodelleerde spiraalarteriën in de baarmoeder meer gecompromitteerd zouden kunnen zijn. Het vermoeden bestaat dat een problematische zwangerschap (d.w.z. chronische hypoxie) mechanismen in de foetus kan ‘programmeren’, met gevolgen voor de gezondheid en ziekte later in het leven. De gezondheid van deze kinderen op lange termijn dienen in toekomstig onderzoek worden

gevolgd om de relatie tussen pathofysiologische mechanismen en het vermogen om zich normaal te ontwikkelen te bepalen.

In **hoofdstuk 5** beschrijven we nieuwe technieken waarmee we vasculaire endotheelcellen uit het placentabed op transcriptieniveau hebben bestudeerd. We vonden aanwijzingen voor onder andere endotheeldysfunctie en veranderde inflammatoire mechanismen. We identificeerden differentieel tot expressie gebrachte genen die er op wijzen dat het aspecifieke afweersysteem, de activatie van bloedplaatjes en endotheelcellen anders functioneren bij vrouwen met vroege pre-eclampsie in combinatie met FGR. Multiplex immunoassay bij deze vrouwen bevestigde activatie van maternale inflammatoire en endotheliale cellen in de symptomatische fase van deze zwangerschapscomplicaties. Deze studie is, voor zover wij weten, een van de eerste onderzoeken die deze technieken gebruikt om relevante genen in dit celtype te identificeren en verdient verder onderzoek om de exacte relevantie en toekomstige klinische mogelijkheden te identificeren.

In **hoofdstuk 6** onderzochten we of overgewicht en obesitas voorafgaand aan de zwangerschap placentaire afwijkingen veroorzaakt tijdens een ongecompliceerde zwangerschap. We vonden dat vrouwen met een toenemende BMI van zwaardere baby's en zwaardere placenta's bevallen en vaker tekenen tonen van ernstige chronische villitis en versnelde villeuze rijping in de placenta. Omdat veel traditionele risicofactoren voor hart- en vaatziekten geassocieerd worden met de ontwikkeling van zwangerschapscomplicaties, is de toenemende hoeveelheid vrouwen met een ongezonde levensstijl en een hoge BMI zorgelijk. Bovendien vonden we een substantieel effect van het krijgen van één of meerdere kinderen op het gewicht van moeder en kind. Dit geeft aan dat het hebben van (meer) kinderen, zelfs als de zwangerschap ongecompliceerd verloopt, implicaties heeft voor gezondheidsrisico's in de toekomst. Tevens versterkt dit onze hypothese dat vrouwen zo gezond mogelijk de zwangerschap in zouden moeten gaan en vraagt derhalve om aandacht voor preconceptiezorg en het vergroten van kennis hiervan bij de algemene populatie om zwangerschapscomplicaties te kunnen voorkomen.

Maternale cardiovasculaire gezondheid na pre-eclampsie

Lange termijn epidemiologische studies hebben aangetoond dat pre-eclampsie geassocieerd is met een verhoogd risico op hart- en vaatziekten. Hoewel we ons al meer dan tien jaar bewust zijn van deze associatie, is preventieve gezondheidszorg nog steeds in veel landen slecht geïmplementeerd, zo ook in Nederland. Daarnaast wordt in de huidige richtlijnen (preventieve) behandeling alleen geadviseerd indien het 10-jaars absoluut risico op hart- en vaatziekten (d.w.z. Framingham risicoscore) meer is dan tien procent. Aangezien deze risicoscores sterk afhankelijk zijn van leeftijd, worden preventieve maatregelen daarmee vrijwel nooit geadviseerd in de eerste 10-20 jaar na pre-eclampsie. Met andere woorden, de huidige cardiovasculaire screening en preventie is gericht op vrouwen van middelbare leeftijd en is waarschijnlijk niet geschikt voor vrouwen met zwangerschapscomplicaties in de voorgeschiedenis. In het **tweede deel** van dit proefschrift onderzoeken we de ontwikkeling

van hart- en vaatziekten nadat een vrouw de 'stress test' gefaald heeft (d.w.z. pre-eclampsie in de voorgeschiedenis). Met andere woorden, we onderzoeken factoren die kunnen helpen identificeren wie een verhoogd risico heeft op hart- en vaatziekten, wanneer schade ontstaat en of tijdige screening en interventies een positieve impact kunnen hebben als een vrouw pre-eclampsie heeft ervaren.

Om verder te specificeren wie voordeel zou kunnen hebben van vroegtijdige screening en preventie, presenteren we in **hoofdstuk 7** een systematische review en meta-analyse omtrent het risico op hart- en vaatziekten bij vrouwen die in meer dan één zwangerschap pre-eclampsie hebben ervaren. We vonden dat vrouwen met pre-eclampsie in meer dan één zwangerschap consequent een hoger risico op hart- en vaatziekten hebben in vergelijking met vrouwen met pre-eclampsie in één zwangerschap, gevolgd door vrouwen met uitsluitend ongecompliceerde zwangerschappen in de voorgeschiedenis. Dit ondersteunt het concept dat zwangerschap als een 'stress test' kan worden gezien voor cardiovasculaire gezondheid. Het is waarschijnlijk dat vrouwen met een slechtere vasculaire constitutie, en dus een hoger risico op hart- en vaatziekten, opnieuw problemen ondervinden in de tweede zwangerschap terwijl de meeste andere vrouwen vervolgens ongecompliceerde zwangerschappen hebben (ongeveer 85%). Een oorzakelijk verband kan echter niet volledig worden verworpen. Als pre-eclampsie blijvende schade aan het maternale cardiovasculaire systeem veroorzaakt, zal herhaling tijdens een volgende zwangerschap het cardiovasculaire risico verder verhogen. Ongeacht het mechanisme, is het optreden van recidief pre-eclampsie een duidelijke indicator die screening en preventieve maatregelen rechtvaardigen. Om vast te stellen wanneer zichtbare cardiovasculaire schade ontstaat, hebben we coronaire CT-scans gemaakt bij vrouwen met pre-eclampsie in de voorgeschiedenis.

In **hoofdstuk 8** beschrijven we dat vrouwen met pre-eclampsie in de voorgeschiedenis gemiddeld 5 jaar eerder kransslagaderverkalking ontwikkelen dan vrouwen met een ongecompliceerde obstetrische voorgeschiedenis, namelijk rond de leeftijd van 45. De 10-jaars risicoscore was consequent hoger bij vrouwen met pre-eclampsie in de voorgeschiedenis, maar overschreed zelden de behandelingsdrempel van tien procent, waaruit blijkt dat deze score voor deze vrouwen waarschijnlijk niet toereikend is. Tevens vonden we dat de meeste regulier gemeten cardiovasculaire risicofactoren vaker voorkomen bij vrouwen met pre-eclampsie in de voorgeschiedenis. Alhoewel we in onze analyses geen effect vonden tussen het totale cholesterolniveau, de systolische bloeddruk of het hebben van diabetes in de associatie tussen leeftijd en toegenomen aderverkalking, is dit een sterke aanwijzing dat reguliere cardiovasculaire screening en preventieve maatregelen adequaat moeten worden uitgevoerd bij deze vrouwen. Dit onderzoek onderbouwt een advies dat preventieve maatregelen zouden moeten geïmplementeerd vóór de leeftijd van 45 jaar.

Tenslotte hebben we in **hoofdstuk 9** een modelgebaseerd micro-simulatieonderzoek uitgevoerd met gegevens uit de bovenstaande CT-scan studie. Dit model gebruikt de onderzoek data van risicofactoren, die zowel kort na de zwangerschap (ongeveer 6 maanden) als na 10-20 jaar follow-up zijn verzameld. Het model implementeert vervolgens

cardiovasculaire risico screening bij 2000 vrouwen met pre-eclampsie in de zwangerschap, vanaf de leeftijd van 30 jaar en herhaalt deze elke 5 jaar. Vrouwen ontvingen daarbij leefstijlinterventies wanneer het 10-jaars risico op hart- en vaatziekten boven een bepaalde drempelwaarde lag. Resultaten laten zien dat deze strategie waarschijnlijk voordelig is, de kwaliteit van leven verhoogt en kosteneffectief is. Het gebruik van een 10-jaars risicodrempel van 5% was de meest waarschijnlijk kosteneffectieve strategie met een toegenomen kosteneffectiviteitsratio van € 3882/QALY, resulterend in 0,06 QALY's per vrouw. Een aantal zaken dienen te worden meegenomen in de interpretatie van deze resultaten. Ten eerste, een simulatiemodel geeft een schatting van het potentieel en geen feitelijke data. Ten tweede, dat ons model een toename van de individuele kwaliteit van leven laat zien, maar dat we moeten bedenken dat 0,06 QALY uiteindelijk tot enkel 20-25 dagen van leven in perfecte gezondheid opbrengt per individu. Jonge vrouwen zijn mogelijk niet bereid om screening en leefstijladvies te volgen voor deze kleine winst. Het is echter waarschijnlijk dat de voorgestelde leefstijlinterventies additionele gezondheidswinst zou kunnen opleveren in het voorkomen van andere ziektes die nu (nog) niet zijn meegenomen (bijv. verminderen van COPD bij stoppen met roken).

Conclusie

De resultaten van dit proefschrift bieden nieuwe informatie over de prevalentie van vaatschade en ontoereikende remodellering van spiraalarteriën onderliggend aan de placenta. Tevens identificeren we nieuwe immunovasculaire mechanismen die ten grondslag zouden kunnen liggen aan het ontstaan van pre-eclampsie/FGR en het 'falen van de stress test'. Daarnaast leveren we bewijs dat vrouwen met pre-eclampsie in de voorgeschiedenis kunnen profiteren van cardiovasculaire screening en preventieve maatregelen vóór de huidige aanbevolen leeftijd van 50 jaar, omdat irreversibele schade aan de kransslagaders eerder optreedt dan in de algemene populatie. We hopen dat deze resultaten zullen bijdragen aan het ontrafelen van deze zwangerschapscomplicaties en het bijbehorende cardiovasculaire risico om uiteindelijk betere zorg te kunnen bieden aan de vele vrouwen die de belemmeringen van pre-eclampsie ondervinden.

LIST OF PUBLICATIONS

Human placental bed endothelial cell transcriptomics and systemic biomarker profiling reveals inflammatory endothelial activation in early onset preeclampsia
L Brouwers*, J Wienke*, M Mokry, PGJ Nikkels, TE Vogelvang, A Franx, F van Wijk, BB van Rijn
Manuscript in preparation

Impact of preventive screening and lifestyle interventions in women with a history of preeclampsia: model-based micro-simulation study
GR Lagerweij, **L Brouwers,** KGM Moons, L Benschop, AHEM Maas, A Franx, BB van Rijn, H Koffijberg *on behalf of the CREW Consortium*
Manuscript in preparation

Spiral artery remodeling in relation to preterm birth and associated adverse neonatal outcome in preeclampsia and fetal growth restriction
L Brouwers, KCE Drechsel, S de Gier, TE Vogelvang, PGJ Nikkels, A Franx, BB van Rijn, F Groenendaal
Manuscript in preparation

Early onset of coronary artery calcification in women with previous preeclampsia
L Brouwers*, L Benschop*, GA Zoet, C Meun, E Boersma, RPJ Budde, BCJM Fauser, CMJ de Groot, AHEM Maas, BK Velthuis, A Franx, E Steegers, BB van Rijn, JE Roeters van Lennep *on behalf of the CREW Consortium*
Submitted

Prevalence of impaired placental bed spiral artery remodeling in preeclampsia and fetal growth restriction
L Brouwers, S de Gier, TE Vogelvang, JHW Veerbeek, A Franx, PGJ Nikkels, BB van Rijn
Submitted

Recurrence of pre-eclampsia and the risk of future hypertension and cardiovascular disease: a systematic review and meta-analysis
L Brouwers, AJ van der Meiden-Roest, C Savelkoul, TE Vogelvang, AT Lely, A Franx, BB van Rijn
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L Brouwers, MPH Koster, GCML Page-Christiaens, H Kemperman, J Boon, IM Evers, MD Bogte, MA Oudijk
American Journal of Obstetrics and Gynecology 2014

DANKWOORD

"There is no "I" in team"
[unknown]

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COVER ART

De cover of this thesis was inspired on the artwork of my uncle Bas Verhoeven†. He made this many years ago, not knowing how close it would be to the subject of my thesis. I am deeply grateful that I was able to use it in the design process of this thesis. Unfortunately both him and three of my grandparents are no longer with us, but I am thankful they were.

CURRICULUM VITEA

Laura Brouwers was born on August 30th 1988 at the Diakonessenhuis in Utrecht, the Netherlands. She grew up in Utrecht with her parents and brother. After graduating from high school at the Montessori Lyceum Herman Jordan in Zeist in 2006, she first played cricket in Brisbane, Australia (2007) and Cape Town, South Africa (2008). She started medical school in 2008 in Utrecht, the Netherlands while continuing her cricket career for the Dutch National Women's team. During her third internship she discovered her passion for Obstetrics and Gynecology. This was followed by an extracurricular research internship with Dr. M.A. Oudijk investigating intrahepatic cholestasis in pregnancy. Furthermore, this lead to multiple internships in perinatology, including a research internship under supervision of Dr. J.H.W. Veerbeek and Dr. M.P.H. Koster on spiral artery remodeling in preeclampsia (the SPAR study). A final tropical medicine internship in the labor wards of Mulago Hospital, Kampala, Uganda, confirmed her intentions to pursue a career in Obstetrics and Gynecology. After completing her medical degree in 2015 she started a combined position as a fertility doctor and an Obstetrics and Gynecology resident (not in training) at the Diakonessenhuis in Utrecht, supervised by Dr. N.W.E. Schuitemaker and Dr. T.E. Vogelvang. This position was combined with a PhD research position at the Wilhelmina Kinderziekenhuis under supervision of Prof. A. Franx, Dr. B.B. van Rijn, Dr. T.E.Vogelvang, and Dr. P.G.J. Nikkels, resulting in the current thesis. After two and a half years she received a fulltime research contract with the Dutch Heart Foundation in order to finish both the SPAR study and the research projects executed by the CREW consortium regarding cardiovascular disease after preeclampsia. In 2017 she received the SRI international training grant in order to be trained in the laboratory of Dr. N. Illsley and Dr. S. Zamudio at the Hackensack University (New Jersey, USA) where she learned advanced and innovative laboratory techniques. Recently, she started her specialization training in Obstetrics and Gynecology at the ElisabethTweesteden Ziekenhuis (ETZ) in Tilburg, the Netherlands under supervision of Dr. I. van Rooij and Dr. J. Smeenk.