MATERNAL PLACENTAL SYNDROMES: PATHOLOGICAL MECHANISMS AND LONG-TERM CONSEQUENCES

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Maternal Placental Syndromes: pathological mechanisms and long-term consequences

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MATERNAL PLACENTAL SYNDROMES: PATHOLOGICAL MECHANISMS AND LONG-TERM CONSEQUENCES

Maternale placenta syndromen: pathologische mechanismen en lange termijn gevolgen
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de universiteit van Utrecht op gezag van rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 25 juni 2015 des middags te 4.15 uur

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TABLE OF CONTENTS

Chapter 1

General Introduction

Hypertensive disorders of pregnancy affect 2-7% of all pregnancies worldwide. (1,2) These disorders include pregnancy-induced hypertension (PIH), preeclampsia, and haemolysis, elevated liver enzymes and low platelets (HELLP) syndrome. Preeclampsia is commonly subdivided into early (delivery before 34 weeks of gestation) and late (delivery after 34 weeks of gestation) onset preeclampsia. In a large population based study the overall preeclampsia rate was 3.1% and the incidence increased sharply with gestation; early- and late-onset preeclampsia rates were 0.4% and 2.7%, respectively. (3) Preeclampsia is clinically defined as a multisystem syndrome by its key signs hypertension and concomitant proteinuria, after 20 weeks of gestation. Hypertension is defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP) as diastolic blood pressure above 90 mmHg and/or systolic blood pressure above 140 mmHg, measured on two or more separate occasions at least 4 hours apart. Proteinuria is diagnosed when urinary protein is above 300 mg per 24 hour or above 2+ at dipstick urinalysis. (4) Maternal complications of preeclampsia are HELLP syndrome, placental abruption, eclampsia, cerebral haemorrhage, liver hematoma or rupture, renal failure, pulmonary edema, coagulopathy and maternal death. The foetus of a preeclamptic mother is at increased risk for intra uterine growth restriction (IUGR), perinatal asphyxia and death. HELLP syndrome, placental abruption, eclampsia and IUGR can also be diagnosed as distinct clinical entities and together with preeclampsia these are collectively known as maternal placental syndromes. Many risk factors for developing preeclampsia have been identified of which chronic hypertension; late maternal age and primiparity are the most studied. (1) Preeclampsia remains one of the leading causes of maternal and perinatal morbidity and mortality worldwide. (5)

PREECLAMPSIA AND SPIRAL ARTERY REMODELLING

Despite extensive research the pathophysiology of preeclampsia is still not completely elucidated. However, it is now widely accepted that failure of remodelling the spiral arteries plays a pivotal role in the development of (in particular early onset) preeclampsia and other disorders of pregnancy. (6) Spiral arteries emerge from the radial arteries that in turn sprout from accurate arteries that lie deep in the myometrial part of the placental bed. (7) Many of these findings were discovered in the early 1960's using hysterectomies and placental bed biopsies. (8) Sampling techniques to study the placental bed are further discussed in length in **chapter 2**. Physiological spiral artery remodelling is now thought to develop in five steps (figure 1). This model is widely accepted although it is by no means fully established. The earliest stage in vascular remodelling (stage 1) consists of endothelial vacuolation and some swelling of individual muscle cells. Invasion of stromal and perivascular tissues by interstitial trophoblast is associated with further

disorganization of the vascular smooth muscle layer (stage 2). Only then endovascular trophoblast appear (stage 3). Trophoblasts become embedded intramurally within a fibrinoid layer, which replaces the original vascular smooth muscle (stage 4). Finally re-endothelialisation occurs (stage 5). (6) Lack of adequate spiral artery remodelling is present in about 52-90% of preeclampsia cases. (9) Impaired spiral artery remodelling-however, is not confined to preeclampsia and IUGR, but is also seen in 19% of pregnancies complicated by preterm birth, abruption of the placenta (58%) and even in 4-7% of normal pregnancies. (9-13)

The placenta itself has also been subject of many research papers over the last decades. (14) Both IUGR and maternal hypertensive disease, in particular early-onset preeclampsia, are characterized by placental pathology associated with hypoxia and reperfusion damage caused by impaired remodeling and/or obstruction of the spiral arteries. (15,16). Common lesions include infarction (17), inflammation (18,19) and le-

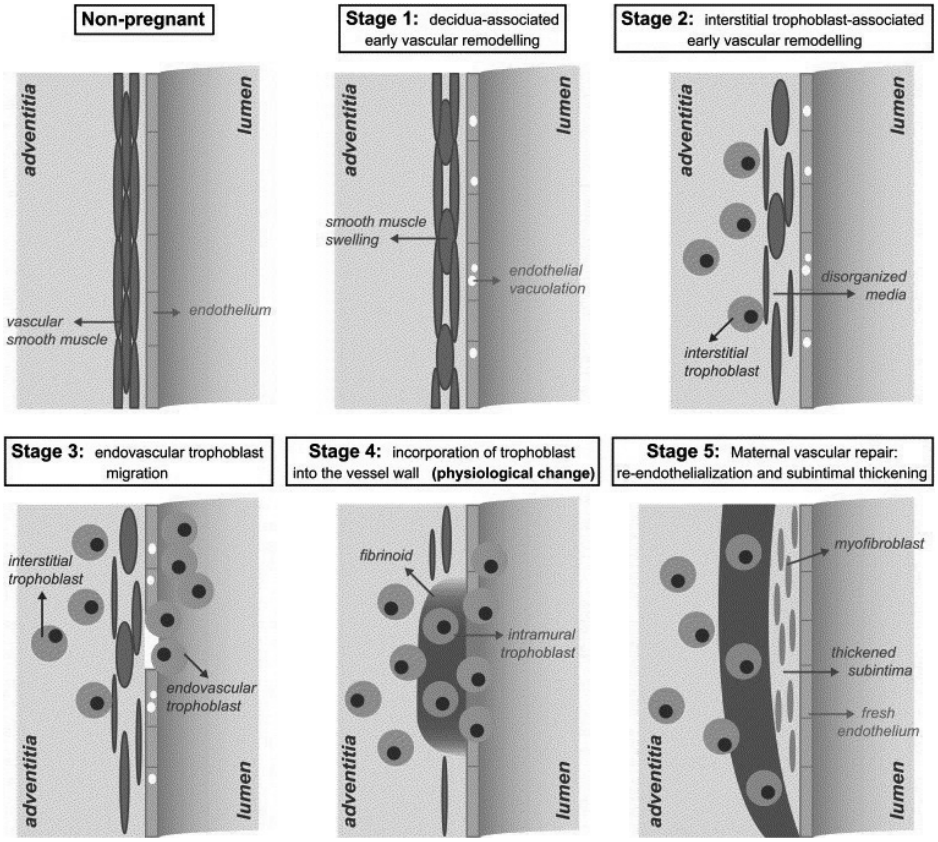

Figure 1. The multistep pathway of remodeling of spiral arteries. Adopted from Pijnenborg et al. (6), copied with permission from the publisher.

sions consistent with chronic ischemia. (19,20) Other lesions like chronic villitis or villitis of unknown etiology (VUE) are seen in normal pregnancy as well and are in fact one of the most common benign inflammatory placental lesions found. We investigated the hallmark features of placental pathology associated with early IUGR and to compare these between placentas from women with or without preeclampsia in **chapter 4.**

Currently the most accepted hypothesis in the development of the clinical syndrome of preeclampsia is the three-step model described by Redman and Sargent. (21) In the first stage the maternal immune system plays a vital role. It is proposed that maternal intolerance to the allogeneic trophoblasts causes a maldevelopment of the spiral arteries due to impairment of trophoblast invasion and/or function. As a result the second stage is characterized by poor placentation leading to small muscular spiral arteries resulting in a high-pressure pulsatile flow in to the intervillous space (figure 2). Subsequently this causes oxidative stress, endoplasmic reticulum (ER) stress and inflammatory stress. (22) The final stage of preeclampsia is caused by a global maternal inflammatory response to the poorly developed and stressed placenta. (21) According to this theory, development of the syndrome of preeclampsia depends on the extent to which poor placentation causes inflammatory signals and the nature of the maternal response to those signals. However, the precise factors that link placental abnormalities to the development of maternal disease remain unknown. It is likely that several maternal factors (genetic, environmental and behavioural) interact with poor placentation to result in the preeclamptic syndrome. (6)

MECHANISMS OF IMPAIRED TROPHOBLAST INVASION

The mechanisms underlying failed trophoblast invasion are likely to be complex. Evidence suggests that several factors are involved, including cell adhesion molecules, matrix metalloproteases (MMPs), apoptosis, nitric oxide and other vasoactive mediators, oxygen tension, cytokines and other immunological factors. (23)

Matrix metalloproteases

MMPs are proteolytic enzymes that play a key role in the trophoblast invasion process by degrading basement membranes and ECM components, and thereby facilitating cell movement through tissues. *In vitro*, the invasive capacity of trophoblasts has been related to their secretion of MMPs. (24) Several types of MMPs have been identified and many have proven to be involved in controlling trophoblast invasion. (25) Lyall summarized the evidence in her review and concluded that overall evidence supports MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and MMP-11 involvement in normal trophoblast invasion of

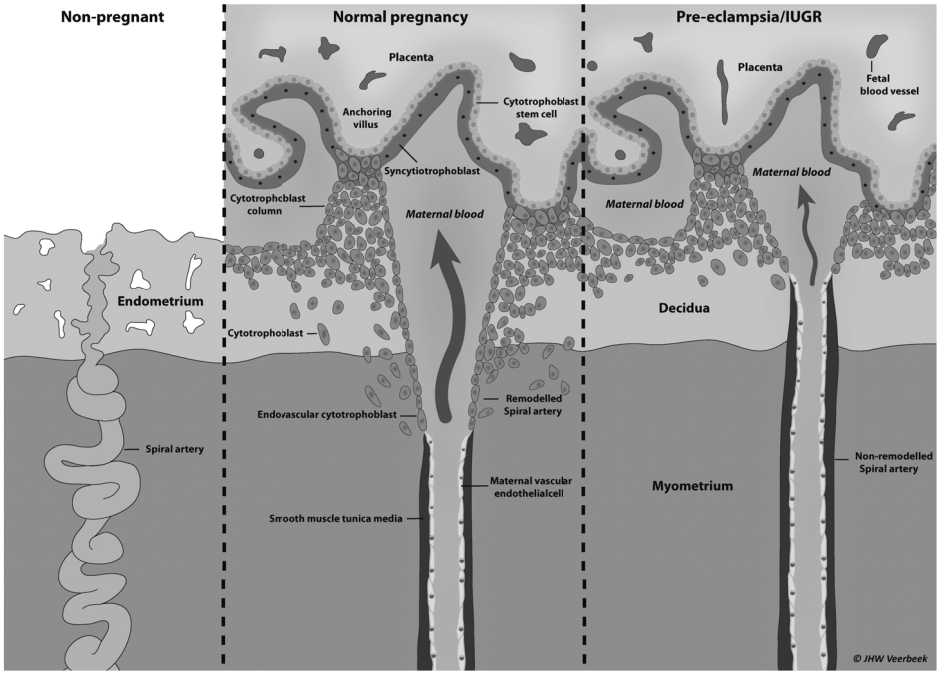

Figure 2. Spiral artery remodelling in non-pregnant, normal pregnancy and pre-eclampsia/IUGR. Notice the reduced blood flow and diameter of the spiral arteries during PE/IUGR.

the decidua. (23) The balance between secretion of MMPs by trophoblasts and decidual cells and their inhibition by tissue inhibitors of MMPs produced by the same cells result in normal invasion. Abnormalities in these processes could lead to excessive invasion such as choriocarcinoma or restricted invasion as in early pregnancy failure, preeclampsia and IUGR. (23) It has been shown for instance that a significant reduction in MMP levels in cases of IUGR is associated with shallow trophoblast invasion. (26) Causes of these impaired processes are largely unknown. However, protein secretion, functionality and activity are negatively influenced by ER stress. (27) We therefore investigated the impact of ER stress on MMP activity and subsequent trophoblast invasion in **chapter 6**.

ER stress

Secretory and membrane proteins are synthesized and post-translationally modified within the ER before entering the Golgi apparatus for final targeting. Fast adaptation of ER capacity to the varying demands of the cell is essential to prevent disturbances in cellular homeostasis. Factors that affect ER integrity or conditions during which ER demand outpaces its capacity, can disrupt ER homeostasis and trigger ER stress. The ER has its own intricate network of signalling proteins that continuously senses and communicates ER status to the cell. When ER homeostasis is disrupted, these proteins coordinate

the so-called unfolded protein response (UPR), a signalling cascade that aims to restore ER homeostasis and relieve the stress. If ER stress cannot be resolved, UPR signalling can activate apoptotic pathways, which leads to removal of fatally injured cells. The primary response initiated by UPR signalling is to inhibit protein synthesis thereby reducing protein load on the ER. (28) Yung et al were the first to demonstrate that ER stress-mediated inhibition of protein synthesis plays a key role in the pathophysiology of IUGR. (27) They showed that inadequate remodelling of the spiral arteries leads to ischemia reperfusion (I/R) injury in the placenta. The placental ischemia resulted in impaired ATP- and Ca^{2+}-dependent protein modifications, leading to protein misfolding and subsequent induction of ER stress and UPR signalling. (22,27,29) In vivo modelling of ER stress is difficult and up to now no model exists. In **chapter 5** we investigated if low-grade ischemia reperfusion (I/R) injury during labour can provide us with a biomodel for ER stress.

Immunological factors

It is thought that inadequate maternal toleration to alloantigens, expressed by foetal trophoblasts, leads to poor placentation and subsequently dysfunctional uteroplacental perfusion, placental oxidative stress and release of pro-inflammatory factors. (21) Therefore, an altered maternal immune response against the semi-allogeneic fetus, in which many components of the immune system (cells, cytokines and molecules) participate, is probably one of the main problems in preeclampsia. Trophoblast invasion partly depends on the interaction between invading trophoblasts and maternal immune cells. (21,30,31) These include macrophages, uterine natural killer (uNK) cells and T-cells, which are discussed in greater detail separately.

The regulatory functions of $FOXP3^+$ CD4 cells (Treg) in pregnancy have been studied extensively by Rowe et al. (32) They showed that there is an accumulation of immune suppressive Treg cells during pregnancy and an accelerated expansion of Treg cells during secondary pregnancy. Interestingly this expansion was driven by proliferation of foetal-specific $FOXP3^+$ cells retained from prior pregnancy, whereas induced $FOXP3^+$ expression and proliferation of pre-existing $FOXP3^+$ cells each contribute to Treg expansion during primary pregnancy. In other words, pregnancy imprints $FOXP3^+$ CD4 cells that sustain protective regulatory memory to foetal antigens. (32) In turn, pregnancy complications including preeclampsia have shown to be associated with blunted maternal Treg expansion in peripheral blood and placental bed. (33,34) Sasaki et al. (34) studied the presence of regulatory T cells in the placental bed. The percentage of these regulatory T cells was lower in the placental bed of preeclamptic cases compared to uncomplicated pregnancies, which could possibly lead to a reduction in the maternal tolerance towards the fetus as a consequence of insufficient regulatory function.

Natural killer (NK) cells have been extensively studied by Trowsdale and Moffet. (30,35) Uterine NK cells (uNK) have shown to be substantially different from circulating peripheral NK cells. Maternal uNK are in direct contact with extra villous trophoblast (EVT) in the decidua (basalis and parietalis). Although it was shown that the multinucleated syncytiotrophoblast does not express HLA class II or I molecules, EVT expresses polymorphic HLA-C and non-polymorphic HLA-E and HLA-G. Binding of EVT to uNK cells is facilitated through binding of uNK cell killer immunoglobulin-like receptors (KIR) to HLA-C and HLA-G on invading EVT. Of great interest is the finding that the combination of maternal KIR-AA genotype (on uNK cells) and foetal HLA-C2 genotype (on EVT) has been shown to result in the highest risk of PE relative to other comparable combinations. (35) Studies on the proportion of uNK-cells in the placental bed have yielded some conflicting results. Stallmach et al. (36) reported an increase in the proportion of uNK cells in cases of preeclampsia associated with IUGR, compared to uncomplicated pregnancies; this increase was not found in cases of preeclampsia without IUGR. Williams et al. (37) on the other hand, found a reduction of uNK cells in placental bed biopsies of preeclamptic patients, further indicating a possible regulatory function for the innate immune system in trophoblast invasion.

Macrophages are derived from blood monocytes and have important immunological functions such as phagocytosis, secretion of cytokines and orchestrating innate and adaptive immune responses. (38) Macrophages are thought to be involved in regulating trophoblast migration through regulating apoptosis as well as maternal tolerance to fetal antigens. (39) Density and infiltration patterns of macrophages in preeclampsia have been investigated most extensively in placental bed biopsies and were reported as increased (40), decreased (41) and equal (42) compared to uncomplicated pregnancies. A plausible explanation for the discrepancy in these results is the use of different markers to detect macrophages. The presence of the maternal immune cells in the placental bed is further discussed and investigated in **chapter 3**.

ACUTE ATHEROSIS

The (mal)function of macrophages is not only studied in light of their role in trophoblast invasion but also play an important role in the pathophysiology of a pregnancy specific lesion: acute atherosis (figure 3).

Acute atherosis can be found in the basal arteries of the placental bed, as well as in the spiral arteries itself (43), and is characterized by fibrinoid necrosis, ultimately throughout the vessel wall; accumulation of lipid in the myointimal cells and macrophages of the damaged arterial wall, giving them a foamy appearance (so-called foam cells); and

infiltration of mononuclear inflammatory cells (mostly lymphocytes and macrophages) in the decidua around the affected vessels. (43-45)

Acute atherosis is often associated with thrombosis, which may lead to placental ischemia and ultimately infarction. (46) As the spiral artery lumen is narrowed by the lesion, it is assumed that acute atherosis contributes to the altered placental perfusion seen in preeclampsia. (47) It has long been recognized that striking similarities exist between acute atherosis and other vascular conditions, such as atherosclerosis in systemic artery disease (44) and arterial lesions in acute rejection of renal transplants. (48) These findings support an immunological basis of preeclampsia, especially now atherosclerosis is more and more considered an inflammatory disease. (49) These findings also generated the hypothesis of a common pathophysiology in acute atherosis and atherosclerosis; especially when the shared risk factors and the predisposition of preeclamptic women to other cardiovascular disorders later in life are taken into account. (31)

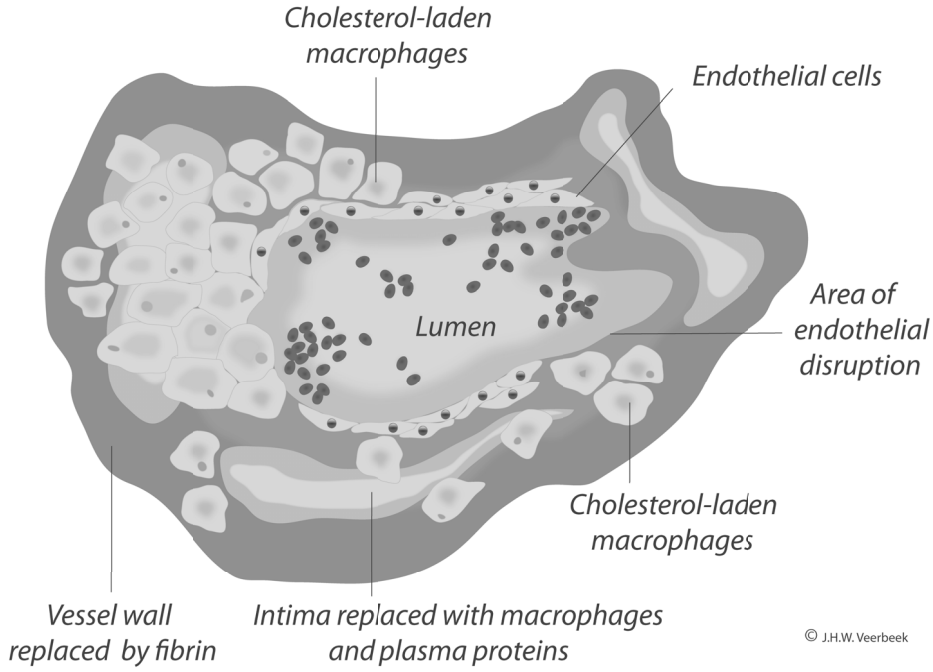

Figure 3. Acute atherosis in decidual arteriole of patient with pre-eclampsia. The vessel wall has been replaced by fibrin and the intima is replaced with plasma proteins and cholesterol-laden macrophages.

CARDIOVASCULAR DISEASE AND HYPERTENSIVE DISORDERS OF PREGNANCY

Cardiovascular disease (CVD) has gained interest in obstetrics in recent years, since large observational studies revealed a remarkable increase in the long-term risk of CVD in women who experienced different types of gestational hypertensive disorders (50-52). These include pregnancy-induced hypertension (PIH) and preeclampsia.

A review of the literature showed an increase of the postpartum risk of CVD events according to the severity of the hypertensive pregnancy disorder, with the highest risk in women who experienced early-onset preeclampsia. (50) Other studies revealed that common modifiable risk factors are already significantly elevated 6 months after a pregnancy complicated by early and late onset preeclampsia, pregnancy induced hypertension. (53-56) Together, these data suggest that the differences in long-term CVD risk between women with a history of a hypertensive pregnancy may be dependent on variation in the underlying maternal CVD risk profiles. In **chapter 9** we further investigate this relationship by comparing CVD risk profiles between three hypertensive disorders of pregnancy: early onset preeclampsia, late onset preeclampsia and PIH.

Ray et al. (52) related several other maternal placental syndromes to the subsequent maternal risk of premature cardiovascular disease, including placental infarction, placental abruption and IUGR. These maternal placental syndromes may be considered as a cluster of pregnancy-related disorders that appear when the cardiovascular system fails to adapt to the increased metabolic, inflammatory and hemodynamic demands during pregnancy and represent the first manifestation of compromised cardiovascular health of the mother. (54,57) Although cardiovascular risk profiles were significantly altered in several of the maternal placental syndromes, this has not been separately studied for women with previous placental abruption. In **chapter 8** we present a case control study of cardiovascular risk factors in women who have and have not experienced placental abruption in a recent pregnancy.

The aforementioned has led to the general accepted hypothesis that pregnancy is a stress test for cardiovascular health (figure 4). (58) Women who experience maternal placental syndromes have failed this stress test. How these pregnancies complications are related to the risk of cardiovascular disease remains to be elucidated. It is speculated that these women might have a maternal phenotype predisposing to cardiovascular disease. (59)

Previous studies not only suggest a role for common CVD risk factors (obesity, dyslipidemia, hyperinsulinemia, chronic hypertension and family history of CVD)(60), but also for markers of the systemic acute-phase inflammatory response (e.g. plasma C-reactive protein (CRP) and fibrinogen levels), as predictors of future CVD after preeclampsia. (61) The relation between CRP and recurrent preeclampsia and other cardiovascular risk

factors is further investigated in **chapter 7**. The growing body of evidence that women with pregnancy complications are at higher risk of developing cardiovascular disease later in life led to the recognition of preeclampsia to be an independent risk factor for cardiovascular disease. The American Heart Association (AHA) updated the guideline for the prevention of CVD in women in 2011 in which they recognized preeclampsia, gestational diabetes and pregnancy-induced hypertension as an independent risk factor for CVD. (62) Furthermore it has recently led to a nationally implemented guideline for cardiovascular risk management after reproductive disorders. (63)

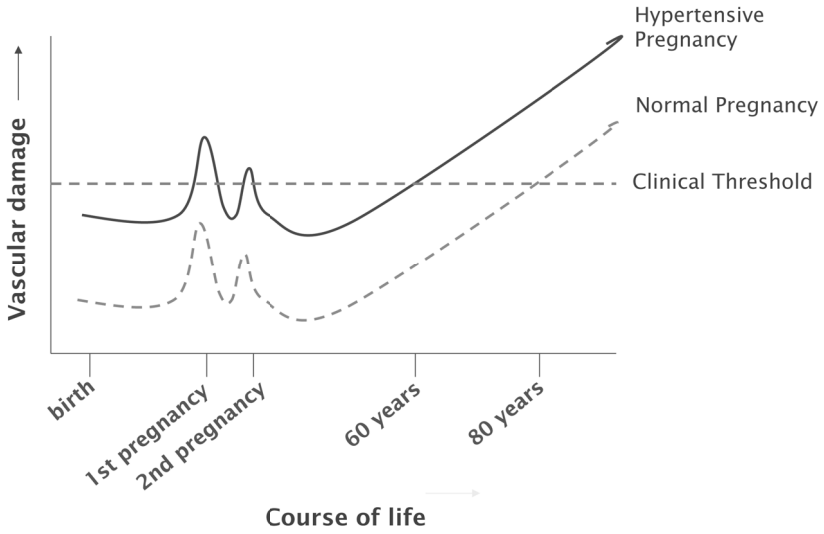

Figure 4. Underlying maternal phenotype becomes identifiable during the stress test of pregnancy. Adopted from Sattar and Greer. (58)

In summary, failed spiral artery remodelling plays a pivotal role in the pathogenesis of hypertensive pregnancy complications also know as maternal placental syndromes. Several mechanisms, such as an exaggerated systemic inflammatory response in the mother, are thought to impair the capacity of trophoblast to remodel maternal spiral arteries. It has been widely acknowledged in recent years that the likelihood of developing cardiovascular disease is increased after a pregnancy complicated by maternal placental syndromes, especially after early onset preeclampsia. Moreover, preeclampsia and cardiovascular disease have numerous common risk factors, including endothelial dysfunction, obesity, hyperglycemia, insulin resistance, diabetes mellitus, hypertension, dyslipidaemia and lipid deposition in vessel walls, suggesting a shared pathogenesis. This thesis, therefore, focuses both on mechanisms of defective spiral artery remodelling and on possible shared pathological pathways in the development of preeclampsia

and cardiovascular disease. Understanding the involvement of the maternal immune system in the placental bed and the placenta during preeclampsia may provide new insights in not only the pathogenesis of preeclampsia, but also into the development of cardiovascular disease. Moreover, we shed new light on modifiable cardiovascular risk factors after several maternal placental syndromes, in order to further personalize (vascular) follow up and prevention in these patients.

AIMS OF THE THESIS

Part I

- To review existing techniques used to sample the placental bed and propose a uniform protocol. **(Chapter 2)**
- To identify key pathological characteristics of placentas from pregnancies complicated by early intrauterine growth restriction, and to examine the correlation of these characteristics with maternal hypertensive disease and umbilical artery Doppler waveform abnormalities. **(Chapter 3)**
- To examine a newly developed scoring system for lesion characteristics of the placental bed which is used to investigate the role of the maternal immune system in normal pregnancy and preeclampsia. **(Chapter 4)**

Part II

- To investigate whether labour induces ER stress in placentas from healthy pregnancies and if exposure to labour presents the opportunity to study placental cellular changes to ER stress in the absence of maternal factors. **(Chapter 5)**
- To identify the role of ER stress in trophoblast migration and its effects on matrix metalloproteases. **(Chapter 6)**

Part III

- To investigate whether C-reactive protein (CRP) and fibrinogen levels in non-pregnant primiparous women with a history of early-onset preeclampsia measured several months postpartum are predictive of recurrent preeclampsia in a next pregnancy. **(Chapter 7)**
- To compare common CVD risk factors between women with a history of placental abruption at 6-12 months after delivery and control women with a history of only uneventful pregnancies. **(Chapter 8)**
- To compare postpartum cardiovascular disease risk factors between women who experienced early onset preeclampsia, late onset preeclampsia and pregnancy induced hypertension. **(Chapter 9)**

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Part I

Placental (bed) pathology in preeclampsia and IUGR

Chapter 2

Biopsy techniques to study the human placental bed

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ABSTRACT

The physiologic transformation of uterine spiral arteries in the human placental bed is essential for a healthy pregnancy. Failure of this transformation, due to deficient trophoblast invasion, is widely believed to underlie pregnancy complications such as preeclampsia, fetal growth restriction, miscarriage and preterm labour. Understanding invasive behaviour and remodelling properties of trophoblasts in the uterine wall is essential in elucidating the aetiology of these pregnancy complications. However, there is a lack of satisfactory specimens of the placental bed to study the mechanisms that control trophoblast invasion. Several techniques can be used to obtain biopsies from the placental bed and sample handling can be executed differently depending on the research question. This systematic review provides an overview of all studies investigating the placental bed and sampling techniques used. Publications describing surgical techniques, specimen handling, complications and/or success rate of the placental bed biopsy procedures were included. The acquisition of placental bed biopsies is a necessary and feasible technique to study abnormalities in the placental bed associated with pregnancy complications. Depending on the technique the likelihood of sampling a spiral artery and trophoblast from the placental bed is 51% to 78% per subject, without significant complications. Caution is needed when interpreting data if the placental bed has been subject to labour. We propose a uniform sampling technique and conservation protocol for the study of the placental bed and provide tools for selection of the appropriate technique to establish future placental bed collections.

INTRODUCTION

Over the last three decades extravillous trophoblast (EVT) dependent spiral artery remodelling has emerged as a vital process in establishing a functional human haemochorial placenta. (1) However, despite extensive research it remains largely unknown how EVT invasion is regulated in vivo and how EVTs facilitate the extensive so called "physiological change" of the spiral arteries of the placental bed. (2) In recent years maternal immune cells such as the abundant uterine natural killer (NK) cells and macrophages have been attributed as key regulators of the placental bed, regulating both EVT invasion and spiral artery remodelling. (3) How failure of deep EVT invasion and spiral artery remodelling contributes to the aetiology of placental disorders such as recurrent miscarriage, preeclampsia, intra uterine growth restriction (IUGR), and preterm birth remains to be elucidated.

Major modifications of the placental bed take place during early pregnancy. (4) The study of early developmental processes in the placental bed *during* pregnancy is not feasible, and many studies have relied on samples of the placenta bed *after* (preterm) delivery of the placenta. However, functional studies on these specimens are very limited and most investigators have resorted to *in vitro* models of trophoblast invasion with the use of modified human trophoblast cell lines. Although these studies have given us a better understanding of the molecular mechanisms regulating invasion and remodelling of the placental bed spiral arteries, the interpretation of data must be executed with caution as they do not reflect the *in vivo* situation. Therefore, representative placental bed biopsies are still needed from both early and late normal and complicated pregnancies, taken by reproducible techniques and coupled to detailed clinical characterisation of the study subjects.

A 'true' placental bed biopsy consists of both decidual and myometrial tissue and contains EVTs. However, in order to study spiral artery remodelling a 'successful' placental bed biopsy must contain at least one spiral artery. Although not the 'true placental bed', spiral arteries and EVTs can also be found in decidual tissue alone. To obtain a successful placental bed biopsy is challenging, because the placental bed only contains around 30-60 spiral arteries. (5) The success rate varies with the biopsy technique used and interpretation of data is heavily dependent on subsequent tissue handling and conservation. Choosing the right technique and sample processing is therefore of pivotal importance.

In this review we provide a comprehensive overview of the techniques employed in current studies of the human placental bed and the limited number of biopsy collections worldwide. Techniques are discussed with their corresponding success rates. Finally, we propose a uniform protocol for sampling the human placental bed and subsequent tissue processing in order to increase the availability of comparable and good quality

specimens with which to unravel the mechanisms of trophoblast invasion and spiral artery remodelling, and their failure in adverse pregnancy outcomes.

METHODS

Studies included were identified by a systematic search of the databases PubMed, Embase and Cochrane. The following search terms were combined using the Boolean operator OR: placental bed, spiral arteries, uteroplacental arteries and preeclampsia. The complete search query is presented in Table 1. The search string was restricted to title and abstract. All papers, written in English, that described studies of the placental bed in humans, using placental bed biopsies or hysterectomy specimens taken after caesarean or vaginal delivery, were included. Case reports, expert opinions, studies concerning placental bed research in animals and reviews that did not describe actual placental bed studies were excluded.

Table 1. Search syntax

pregnan*[tiab] AND ("placental bed"[tiab] OR "placenta bed"[tiab] OR "spiral artery"[tiab] OR "spiral arteries"[tiab] OR "uteroplacental arteries"[tiab] OR "uteroplacental artery"[tiab])

To identify relevant studies not covered by the search, cross-references were also picked up in the review process and screened for relevance. A total of 1027 papers were identified with the search query and after duplicates were removed. After screening title and abstract for the inclusion and exclusion criteria mentioned above, 130 papers remained for full text analysis. Finally, full text screening identified 91 studies that have used placental bed biopsies. Together with 3 additional studies found by cross-references a total of 94 papers were included. Not all papers have reported success rates of the biopsy technique used and some studies have used archived collections of placental bed biopsies, in which case only the original study was included in the analysis of success rates. Twenty-five papers reported success rates of their biopsy technique. (6-30) Successful biopsies were defined as either sampling a myometrial spiral artery with trophoblast or any spiral artery and trophoblast. In Supplemental Figure 1 a flowchart of the selection progress is shown.

If success rates were reported for each studied patient group separately an average rate for that paper was calculated. Average success rates for each technique were calculated including only papers that reported success rate per case (and not per biopsy). A weighted mean was calculated to include sample size in the equation. Statistical analyses were performed using SPSS (release 20.0; Chicago, IL).

RESULTS

Historical overview placental bed research

From meticulous examinations of the human placenta *in situ* in the 16th century, it was concluded for the first time that there is no direct vascular connection between mother and foetus. (31) This finding presented indirect evidence for the existence of a vascular bed in the uterine wall adjacent to the placenta: the 'placental bed'. The Hunter brothers published the first illustrations of the placental bed. Using coloured wax injected into specimens of human uteri with placentas *in situ* they revealed the first direct macroscopic evidence for spiral arteries. (32)

With the discovery of the microscope and its rapid advances, trophoblasts were identified in the second half of the 19th century. Not long thereafter the invasive nature of the trophoblast was first appreciated. (1) The advances in early placenta bed research were attributed to the availability of hysterectomy and post-mortem specimens of uteri with *in situ* placentas. Several of these specimens are preserved in histological collections and often accessibility is limited. The biggest collections of early pregnancy material are stored in the Boyd collection at the Centre for Trophoblast Research, University of Cambridge (33) and the Carnegie Collection at the Human Developmental Anatomy Center, Washington DC. (34) The Carnegie collection consists mainly of the specimens preserved by Hertig and Rock who carefully collected very early embryos and implantation sites from hysterectomies. The placenta-in-situ samples of the Boyd collection are overrepresented by low-lying placentas that may have caused the need for hysterectomy. Although no tissue blocks remain the collection holds *in situ* placenta hysterectomies from various gestational ages that are partly digitalized and accessible on the website of the Centre for Trophoblast Research. (http://www.trophoblast.cam.ac.uk).

Ground breaking work on spiral artery remodelling in the placental bed has undoubtedly been performed by Brosens et al. (7,8,35,36) and Pijnenborg et al. (4,37,38) The early histological studies Pijnenborg conducted on the placental bed revealed a higher density of extravillous trophoblasts (EVTs) in the proximity of spiral arteries with morphological signs of remodelling. The specimens that were used for these studies came from an impressive collection of 48 intact uteri with pregnancies ranging from 8 to 18 weeks gestational age. Advances in obstetric care and surgical techniques have made pregnant hysterectomy a rare procedure. Therefore, obtaining new hysterectomy samples is very uncommon, let alone in complicated pregnancies. However, the growing body of evidence implicating abnormalities of the placental bed in the genesis of pregnancy complications, such as preeclampsia, increases the need for representative samples to study the placental bed. (39)

"Modern" placental bed biopsy techniques

In the early 1950's, Hertig (40) and Zeek and Assali (41) among others described changes in decidual vessels that were recognized as having acute atherosis. However, the authors acknowledged the fact that the material was not adequate and that further study of maternal vessels deeper in the placental bed was required. In 1958 Dixon and Robertson introduced a technique to obtain representative placental bed material suitable for histological examination of such deeper myometrial maternal vessels. (42) Using a punch biopsy technique they were the first to collect myometrial parts of the spiral arteries apart from the existing hysterectomy and post-mortem uterus specimens. Since that time several techniques for the sampling of the placental bed have been used which we will discuss in the following section.

Punch biopsy at caesarean section

Since its introduction in 1958, the *punch biopsy* technique has been widely used. Gerretsen et al studied the physiological changes of spiral arteries in normal pregnancies and a variety of pregnancy complications. (12) The authors used the technique to obtain 1 sample of the placental bed of 175 patients. In 42% of the cases they found at least one spiral artery and trophoblasts. Robson et al sampled 139 patients during caesarean section and collected successful biopsies in 45% of the normal pregnancies and 52% of the pregnancies complicated by preeclampsia. (26) Eight biopsies were taken under direct vision of the placental bed after placental removal and intravenous administration of 10IU oxytocin to facilitate uterine contraction. Taking multiple biopsies from the placental bed seemed not to enhance the chances of successfully sampling the bed.

Our group recently started a new biobank study in which the punch biopsy technique during caesarean section has shown comparable success rates. Collecting 280 biopsies from 70 patients we managed to sample the placental bed containing at least one spiral artery and EVT in 57% of the cases (unpublished data). Our proposed standardized protocol will be discussed in detail later.

The studies that reported the likelihood of sampling a spiral artery and trophoblasts from the placental bed with punch biopsy at caesarean section are shown in Table 1. With this technique an average success rate of 51% is feasible.

Transcervical punch biopsy

The punch biopsy technique was not only used in the setting of caesarean sections. Gerretsen *et al.* also employed a comparable technique in 1981 after vaginal delivery and reported a success rate of 33%. (43) Success was defined as sampling tissue containing interstitial trophoblasts and spiral arteries at the endometrial-myometrial junction. In 1990 Michel *et al*. were the first to describe the ultrasound-guided placental bed biopsy. (44) Focusing on leucocytes in the first trimester they did not report the number of spiral

arteries in their biopsies. By excluding the cases with insufficient myometrial vessels it is impossible to assess the success rate. The experiences from Robson and colleagues in sampling under ultrasound guidance were discussed in their publication in 2002. (26) They report success rates with both transcervical techniques as well as biopsies under direct vision during caesarean section as discussed earlier. Currently this is the largest series of early placental bed biopsies reported in the literature. Biopsies were attempted in 313 women who underwent termination of pregnancy (TOP) before 20 weeks of gestation and in 104 women in need of evacuation of retained products of conception (ERPC) after foetal death between 7 and 21 weeks of gestation. In short, after induction of general anaesthesia an ultrasound scan was performed for placenta localization and estimation of gestational age. After evacuation of the uterine contents a biopsy forceps was introduced under ultrasound guidance to the site of the presumed placental bed. Four biopsies were then taken from the central placental bed. With this procedure 17% to 77% of the biopsies were successful in sampling a spiral artery. There was a significant positive correlation between gestational age and the likelihood of obtaining at least one spiral artery. The latter was probably related to the difficulty to clearly visualize the placental bed with ultrasound in very early pregnancy. There were no complications recorded that could be related to the biopsy procedure.

This biobank of placental bed biopsies has been extensively used to study spiral artery remodelling and related processes. They contributed, for example, to our knowledge on the role of adhesion molecules (45), nitric oxide (46), transforming growth factor beta (45), interleukin-6 and 8 (47), and vascular smooth muscle cell apoptosis (48) in trophoblast invasion and spiral artery remodelling.

Studies that reported the likelihood of sampling a spiral artery and trophoblasts from the placental bed with punch biopsy after vaginal delivery, TOP or ERPC are shown in Table 2. With this technique an average success rate of 54% is feasible.

Scissors or scalpel biopsy technique

Robertson and colleagues published their 30-year experience from three centres in sampling the placental bed in 1986. (49) Centres in Leuven Belgium, London United Kingdom and Dublin Ireland have collected placental bed samples with curved scissors or scalpel, a technique that is reported to sample true placental bed in more than 70% of the cases. However, it is not clear if success was defined as the presence of spiral arteries or presence of interstitial trophoblasts alone. Biopsies were taken at caesarean section under direct visualisation of the placental bed. After digitally marking the centre of the placenta, the placenta itself was peeled away and the supposed placental site and surroundings were carefully inspected. With curved scissors a disk of 1.5 cm in diameter was removed from the central part of the placental bed. A depth of 0.5 cm proved to be more than sufficient to obtain the myometrial sections of spiral arteries. The same

dimensions can be obtained when a surgical scalpel is used to collect a wedge-shaped biopsy. The resulting defect was closed with absorbable sutures. There were no reports of biopsy-related complications and this technique has been widely used ever since. A variant of the scalpel biopsy technique was published by Pijnenborg et al. (24) A 22-gauge marking needle was thrust through the serosal side of the uterus through the centre of the placental bed that was manually localized previously. A second needle was inserted from the decidual side into the uterus next to the marking needle that could then be removed. With a sharp scalpel a cone of 1-2 cm wide and 1cm deep was obtained. In 68% of hypertensive women the biopsies contained at least one spiral artery, whereas normotensive women were accurately sampled in 35% of the cases. The difference between success rates was not addressed in the paper.

The studies that reported the likelihood of sampling a spiral artery and trophoblasts from the placental bed with scalpel or scissors are shown in Table 3. With this technique an average success rate of 58% is feasible.

Vacuum suction technique

More recently Harsem evaluated a new technique collecting decidual tissue by vacuum suction for functional and morphological studies described earlier by Staff et al. (15,50) Quality criteria included the presence of spiral arteries and extravillous trophoblasts. However, they do not fit the definition of 'true placental bed' as discussed earlier. Morphological features of the obtained biopsies were compared with archived placental bed material and basal plate sections. During caesarean section 5IU oxytocin was given intravenously after delivery of the baby to ensure uterine contraction. The placenta was then manually located and gently removed from the uterine wall. Placental bed tissue was collected by applying suction force directly on a nylon net, which in turn was flushed with 500 mL of sterile saline to remove blood. A random portion was selected for morphological studies. In the evaluation study 51 women undergoing caesarean sections and vacuum suctions were included and compared to 33 placental bed punch biopsies and 33 placental plate specimens sampled from the maternal side of the delivered placenta. In 86% of the decidual suction samples one or more spiral arteries were found. Placental bed biopsies and placental plate biopsies contained spiral arteries in 61% and 48% respectively. In the long term follow up of 38-60 months after vacuum suction there were no biopsy related complications reported. (15)

The studies that reported the likelihood of sampling a spiral artery and trophoblasts from the decidua with vacuum suction are shown in Table 4. With this technique an average success rate of 78% is feasible although the chances of obtaining myometrial spiral arteries are very small (13%; one study).

Other techniques

Several other techniques have been used to sample the placental bed. Most of the studies did not report any success rates merely because the aim was to investigate the metabolism or the immune system in relation to localisation within the foetal placental unit. For instance, Schafer et al. collected decidual material from the placental bed during caesarean section by wiping the uterine cavity with a tampon. The number of spiral arteries or interstitial trophoblasts is not discussed. (51)

Transcervically obtaining spiral arteries from the placental bed by curettage after vaginal delivery has proven not to be successful. Only one out of 46 (2%) biopsies was successful in sampling an appropriate portion of spiral artery. The technique was therefore abandoned by Gerretsen et al. (43) Stallmach et al. used curettage of the placental bed under direct vision during caesarean section to sample the placental bed. (52) Their success rates seem to be significantly higher with an average of 6,2 spiral arteries per sample. However, no reports were made on samples without spiral arteries. Interestingly, although no incisions were made in the uterine wall, biopsies 'usually' contained myometrium.

It is beyond the scope of this review to discuss all studies that have sampled decidual tissue and basal plate on the delivered placenta. Although Khong and Chambers showed that *en face* specimens from the basal plate of the delivered placenta could sample spiral arteries with a 56.4% success rate we focussed on techniques of direct sampling of the placental bed. (53)

LOCALIZATION OF THE PLACENTAL BED

For accurate sampling of the 'true placental bed' it is the crucial to localize the placental bed either by ultrasound or under direct visualization. The transcervical biopsy procedure is accurately described in the publications by Robson et al. (26) It has proven difficult to visualize the early pregnancy placental bed with ultrasound before 8 weeks of gestation. Only 17% of those samples contained spiral arteries. By thrusting the biopsy forceps through the *in situ* placenta to the placental bed it was usually possible to obtain two or three biopsies in the study of Dixon and Robertson. (42) This technique very accurately localized the placental bed, but did not necessarily result in a high success rate. The method of Pijnenborg et al (24) involved insertion of a 22-gauge needle from the serosal side of the uterus after localizing the centre of the placenta as previously described. Finally, biopsies can be taken under direct visualization of the placental bed during caesarean section. Before the procedure the placental site should be identified using ultrasound, as routine practice. Immediately after delivery of the baby the centre of the placenta is manually localized and gently separated from the underlying myome-

trium by controlled cord traction. From our own experience it is possible to see a slightly depressed placental site, different from the smooth surrounding decidua. This is easily palpable and should allow the obstetrician to make the distinction between the two. When the placenta is located on the anterofundal site of the uterus the uterine wall can be exteriorized by slightly inverting the incision side.

SAFETY, COMPLICATION RATES AND POST-BIOPSY PROCEDURES

Although based on expert opinion, when sampling the placental bed from complicated pregnancies caution should be taken when preeclampsia is further complicated by coagulopathy. Especially when the pregnancy is complicated by the haemolysis, low platelets and elevated liver enzyme (HELLP) syndrome, one should assess platelet count before surgery. Platelet count <100.000/mm^3 is included in the classification of HELLP syndrome. (54) In our protocol discussed below we set the limit of platelet count to 50.000/mm^3 with normal ranges being 150.000/ul-400.000/mm^3. The limit was set in accordance with local protocols indicating that administration of platelets in patients requiring caesarean section was necessary if platelet count is <50.000/mm^3.

During caesarean section all procedures are as normal. It is at the obstetrician's discretion to abandon the biopsy procedure in case of any complications like excessive blood loss, hemodynamic problems, etc. Robson et al. (26) as well as Harsem et al. (15) have described the use of oxytocin after delivery of the foetus; 5 and 10IU were administered intravenously. In our own study we have used 5IU oxytocin in order to increase the chances of sampling a spiral artery and reducing blood loss. After delivery of the placenta and confirmation that all uterine contents were removed, uterine angles were first secured to ensure patient safety and a reduction of blood loss before the sampling procedure commenced.

From our experience is has never been necessary to use sutures to close any defects in the uterine wall after punch biopsy. It must be emphasized that it is not of vital importance to sample the myometrium in great depth. Serial sections of hysterectomy specimens demonstrated that the radial arteries divided approximately 0.5 cm beneath the endometrium (i.e., myometrial junctional zone) into 2 or 3 arteries with physiologic changes or transformation. (55) Hence, shallow biopsies no deeper than 5 mm are sufficient and reduce the need for sutures and possible complications. Using the scissors and scalpel biopsy technique it is reported that absorbable sutures are often needed. However, no complications have been reported in 30 years of experience. The few published adverse events could not be accredited to the biopsy procedure. (49)

SAMPLE HANDLING

In an attempt to optimize sample collection of placental (bed) studies, very recently an extensive review has been published on sample handling and tissue processing. (56) In short, biopsies should be processed as soon as possible. Depending on the substrate or organelle of interest there may or may not be any delay in processing the samples. For instance there is no change of levels of heat shock proteins within a time period of 45 minutes. However hypoxemia occurs already after 7-10 min in delivered placentas, resulting in dilation of several organelles already within 5 minutes. (57)

Fixation of the obtained biopsies in an appropriate fixative is a crucial step for long-term conservation. Placental bed samples are small in size (0.5 cm^3-1.5 cm^3), so caution should be taken with the duration of fixation. Burton et al. recommend that placental samples of the same dimensions should be fixed in paraformaldehyde no longer than 12 hrs. when used for immunohistochemistry. (56) It is advisable to always snap-freeze some of the biopsies for protein, DNA, RNA and metabolomics studies. Ideally one would have the fixative available in the operating theatre in order to reduce the interval between sampling and conservation to a minimum.

After fixation, accurate orientation of the biopsies for proper cutting of paraffin or frozen tissue sections is essential. Biopsies should ideally be positioned in such way that perpendicular sections from decidual surface to the myometrial base can be obtained. However, macroscopically decidua and myometrium are difficult to distinguish. Figure 1 shows an overview picture of a well-orientated placental bed biopsy obtained with the punch biopsy technique. Taking biopsies with the scalpel and scissors technique will result in samples that are easy to orientate as well. Some authors have recommended placing the scalpel or scissor biopsy with the myometrial aspect down on a piece of filter paper for orientation. (49) Organizing effective logistics is very important in creating a representative and consistent biobank. This includes accurate registration of patient characteristics and clinical data. Due to many possible-confounding factors as medication use, labour and severity of clinical disease, data can be easily misinterpreted. Nelson and Burton (58) have listed possible confounders in a recent paper encouraging authors to register basic clinical characteristics in placental (bed) research.

EVALUATION OF PLACENTAL BED BIOPSY

When evaluating the success of the placental bed biopsy immunohistochemistry staining can be used for cytokeratin 7 (CK7) and preferable Periodic Acid Shiff (PAS) after diastase. CK7 identifies EVTs and PAS after diastase can be used to demonstrate fibrinoid depositions in the wall of transformed spiral arteries. Success can be defined in

different ways. We propose an uniform protocol by dividing specimens in 3 categories as described previously (26): (1) specimens without trophoblast and spiral artery; (2) specimens with only trophoblast but no spiral arteries; and (3) specimens with both trophoblast and spiral artery (=successful biopsy).

In Tables 1, 2, 3 and 4 the success rates per technique are shown as deduced from papers that have reported their success rates in sampling the placental bed.

COLLECTION PROTOCOL

The following protocol is derived from our experience and based on publications on placental bed biopsies discussed in this review. The bullet action points are arranged in chronological order and can be incorporated into a local Standard Operation Procedure. It is recommended that the presence of all materials used are checked and replenished regularly in order not to delay the processing of the biopsies. The protocol is mainly applicable for biopsy techniques during caesarean section. However, many of the actions are also applicable to transcervical punch biopsies after delivery.

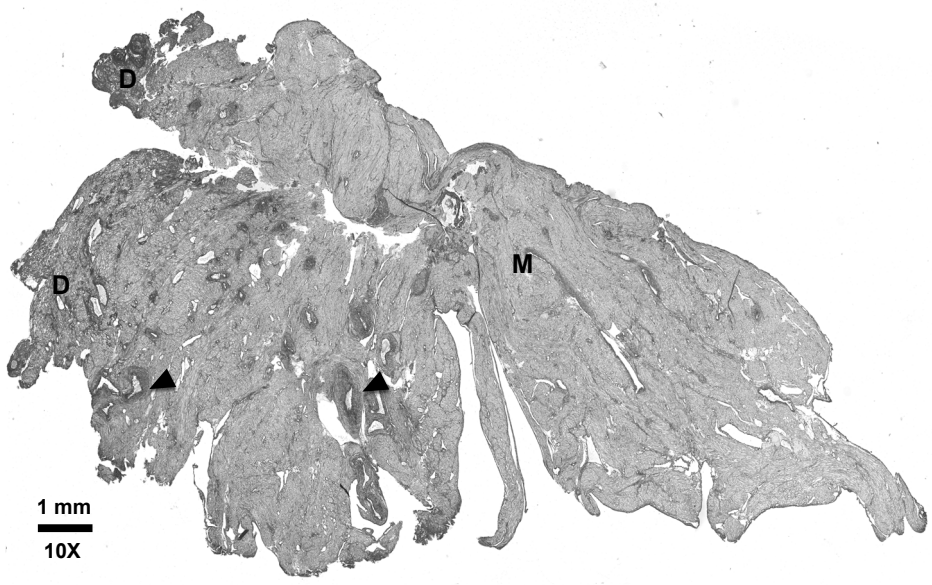

Figure 1. Well-orientated biopsy perpendicular embedded and sectioned from decidual surface to the myometrial base (PAS after diastase stain). Arrowhead: non-remodeled spiral artery; M: myometrium; D: decidua.

Preparations

1. Obtain informed consent according to the declaration of Helsinki before start of the procedure, in acute situations this must have been done beforehand.
2. Localize placenta (bed) by (trans-abdominal) ultrasound
3. Make sure (sterile) biopsy materials are present in the operating theatre
4. Check platelet count (> 50.000/ul)
5. Collect clinical data in structured manner i.e. standardized list of pregnancy and patient characteristics.

During caesarean section

6. After delivery of the foetus give 5-10IU oxytocin intravenously.
7. Identify the outer angles of the incision in the uterus and clamp with haemostatic forceps to reduce bleeding
8. Ascertain location of placenta manually and remove by controlled cord traction
9. Confirm removal of all foetal tissues from the uterus
10. Manually and/or visually identify the location of the placental bed. It is possible to observe a slightly depressed placental site, different from the smooth surrounding tissue covered by decidua parietalis.
11. Depending on preferred technique:
    - Punch biopsy: Introduce the biopsy forceps in to the uterine cavity directing it to the uterine wall. Whenever possible angle the forceps to 45 degrees with the uterine wall. Place the other hand on the serosal side of the uterus just underneath the placental site in order to prevent perforation. Take 4-8 biopsies placed on a small bandage gauze.
    - Scalpel/Scissors biopsy: Identical as punch biopsy, but use curved scissors or scalpel. Remove a disk of 1,5 cm in diameter from the central part of the placental bed or a wedge shaped sample of 1,5 cm in length. Both no deeper than 0,5cm. Further details are described elsewhere. (49)
    - Vacuum suction biopsy: Identical procedures as punch biopsy, but applying suction force directly on a nylon net placed on the placental bed. Further details are described elsewhere. (15)
12. Biopsy site is inspected for bleeding, if necessary an absorbable suture can be used to close the defect. The suture technique is at the obstetricians' discretion. The remainder of the procedure can be completed as normal.

Processing biopsy

13. Directly rinse collected specimens in sterile saline to remove excessive blood and blood clots.

14. Place specimens in desired fixative preferably within 5-10 minutes, e.g., on the operating table itself to prevent any delay.
15. Snap-freeze immediately in liquid nitrogen or transfer to fixative such as fresh 4% buffered paraformaldehyde overnight. If regulations do not allow liquid nitrogen in the operating theatre, the sluice room will suffice to process the samples.
16. For embedding in Optimal Cutting Temperature (OCT) compound transfer to 20-30% sucrose overnight at 4°C
17. Register the biopsy-fixation time and store in biobank
18. Embed in paraffin or OCT

FUTURE PERSPECTIVES

In this review we have presented a comprehensive overview of studies with placental bed biopsies. Unfortunately many studies did not report the likelihood of sampling a spiral artery, but only presented data of preselected biopsies containing a spiral artery and trophoblasts. Currently it is possible to obtain successful biopsies in 17% up to 70% of the cases, with an average of approximately 50% depending on the technique used. Success rates seem to be dependent on experience of the obstetrician in sampling the placental bed. (59) Careful localization, visualisation and inspection of the placental bed is very important and experience will enhance the chances of success. Interestingly, taking multiple biopsies appears not to increase the chance of obtaining spiral arteries and trophoblasts from the placental bed. Gerretsen et al obtained a portion of a spiral artery from the deciduomyometrial junction in 42% of the cases with a single biopsy. (43) Whereas Bulmer et al had a comparable success rate of 47% per case after taking 8 biopsies using only a slightly different biopsy forceps. (26) Moreover, in our own study we show a success rate of 57% per case when 4 biopsies were obtained. The number of biopsies taken should be adjusted to the quantity appropriate for the research question and use of multiple processing techniques.

Researchers interested to collect placental bed biopsies must choose their technique carefully. Depending on the research question and tissues needed the less invasive decidual suction methods may well suffice for their research purpose. For instance, acute atherosis lesions are more likely to be found in the decidual parts of the spiral artery. (60) Therefore the suction technique or even basal plate sampling should be used instead of punch biopsy or scalpel biopsy. If the research question encompasses remodelling of the deeper myometrial spiral arteries one should consider techniques that sample the placental bed up to 0.5 cm of depth (scalpel and scissors or punch biopsy). Although no complications were reported, the scissors and scalpel technique often needs closure of the created defect in the uterine wall as opposed to the punch biopsy technique.

Therefore the punch biopsy with its excellent success rates and reproducibility is the recommended choice for (myometrial) placental bed sampling.

Several factors influence interpretation of the placental bed biopsy. Orientation is very important and sections from decidual surface to the myometrial base should be obtained. Secondly, it is stressed that samples must be thoroughly rinsed in PBS/saline to remove excess blood before placing the biopsy in fixative of choice, preferably within 5 minutes from sampling. Further, the placental bed that has been exposed to labour must be used with care as many biological processes may be affected by the acute hypoxia and reperfusion of the placental bed during contractions. For instance, recent studies have shown that endoplasmic reticulum stress and oxidative stress are higher in laboured placentas. (61,62)

The greatest challenge in advancing this field further is the inability to obtain high-quality tissue samples from the first and second trimester in on-going pregnancies. Failure of spiral artery remodelling occurs between 8-14 weeks of gestation and possibly even later. (4) The only materials currently available are remainders of chorionic villous samples collected largely for serum screening related risk for chromosomal abnormalities. (63) However, newer techniques in detecting foetal cells in the maternal circulation (i.e. Non Invasive Prenatal Testing (NIPT)) may make invasive villous sampling obsolete in the nearby future. (64) Other techniques should be explored to overcome this problem. Although prediction models for preeclampsia still suffer from low sensitivity and specificity, recent studies indicate that early preeclampsia can be accurately predicted in the first trimester of pregnancy using maternal characteristics, biomarkers and Doppler flows of the uterine artery. (65,66) Obtaining these parameters before termination of pregnancy might enable us to stratify for women at high risk of developing (early) preeclampsia if their pregnancy would have continued. We therefore encourage researchers to continue collecting placental bed samples in terminations of (early) pregnancy and when possible to use prediction models for preeclampsia to stratify for women that are at higher risk of developing preeclampsia combined with judging the level of spiral artery remodelling. (67) However, the predictive nature of such studies will still result in a demand for placental bed biopsies sampled in term (complicated) pregnancies.

Standardisation of placental bed biopsy procedures will improve the quality and comparability of studies using placental bed biopsies and allow large collections of samples to be generated. We have proposed a standard in this paper. Placental bed samples, when combined with functional experiments on primary and/or immortalized trophoblasts, should give us further insight in the mechanisms of trophoblast invasion and subsequently in the pathology of pregnancy complications such as preeclampsia, IUGR, preterm birth and placental abruption.

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Supplemental data chapter 2

Supplemental table 1. Punch biopsy at caesarean section

Author	Patient population	Complications	Number of patients	Number of biopsies taken	Technique used	Successful biopsy: myometrial spiral artery and trophoblast (%)	Successful biopsy: any spiral artery and trophoblast (%)
De Wolf et al (1980)	1. Pregnancies complicated by IUGR but without hypertensive disease.	None	5	1	Punch biopsy	N/A	80
Gerretsen et al (1981)	1. Normal pregnancies 2. Preeclampsia 3. Variety of complications (i.e. fetal infection, diabetes, drug abuse, etc)	None	175	1	Punch biopsy	N/A	42
Gosseye et al (1992)	1. Normal blood pressure 2. Moderate hypertension 3. Chronic essential hypertension 4. Preeclampsia	None	38	≥1	Punch biopsy	N/A	63
Harsem et al (2004)	Not described	None	33	1	Punch biopsy	61	61
McFayden et al (1986)	1. Elective or emergency operation 2. Severe maternal hypertension or IUGR	None	109	1	Punch and Scalpel (mixed)	N/A	47
Robson et al (2002)	1. Normal pregnancy 2. Preeclamspia	None	139	8	Punch biopsy	39	47
Veerbeek et al (2014)	1. Normal pregnancy 2. Preeclampsia	None	68	4	Punch biopsy	N/A	57
Weir (1981)	1. Normal pregnancy	None	32	1	Punch biopsy	N/A	84
					Success rate (%)	**43***	**51***

[#] Success rate calculated per biopsy

* Weighted means were calculated including only studies that reported success rate per case

PIH: Pregnancy Induced Hypertension; APS: Anti Phospholipid Syndrome; IUGR: Intra Uterine Growth Restriction

Supplemental table 2. Punch biopsy at top/erpc (Vaginal)

Author	Patient population	Complications	Number of patients	Number of biopsies taken	Technique used	Successful biopsy: myometrial spiral artery and trophoblast (%)	Successful biopsy: any spiral artery and trophoblast (%)
Ball et al (2006)	1. TOP 2. Sporadic early miscarriage	None	252	3 to 4	Punch biopsy	41	56
Carbillon et al (2005)	1. TOP and missed abortions	None	5	≥1	Punch biopsy	0	0
Gerretsen et al (1981)	1. Normal pregnancies 2. Preeclampsia 3. Variety of complications (i.e. fetal infection, diabetes, drug abuse, etc)	None	55	1	Punch biopsy	N/A	33
Robson et al (2002)	1. TOP 2. ERPC	None	417	4	Punch biopsy	35	55
Talaulikar et al (2012)	1. TOP	None	10	≥1	Punch biopsy (Hysteroscopy)	40	70
					Success rate (%)	**37***	**54***

* Weighted means were calculated including only studies that reported success rate per case

TOP: Termination of Pregnancy; ERPC: Evacuation of Retained Products of Conception; IUGR: Intra Uterine Growth Restriction

Supplemental table 3. Scalpel or curved scissors biopsy at caesarean section

Author	Patient population	Complications	Number of patients	Number of biopsies taken	Technique used	Successful biopsy: myometrial spiral artery and trophoblast (%)	Successful biopsy: any spiral artery and trophoblast (%)
Brosens et al (1977)	1. Normal weight foetuses 2. IUGR foetuses	None	108	≥1	Scalpel or curved scissors (Robertson et al)	N/A	73
Dommisse et al (1992)	1. Placental abruption	None	18	1	Scalpel (10mmx5mm)	N/A	67
Frusca et al (1989)	1. Normal pregnancy 2. Preeclampsia	None	38	≥1	Scalpel	42#	42#
Hanssens et al (1997)	Paper not accessible	?	201	1 to 2	Scalpel	46	N/A
Katabuchi et al (2003)	1. Normal pregnancy 2. Preeclampsia	None	26	1 to 2	Scalpel (15x5 mm; Robertson et al)	N/A	75#
Madazli et al (2000)	1. Normal pregnancy 2. Preeclampsia	None	60	2 to 3	Scalpel, (10x10x10mm)	N/A	64
McFayden et al (1986)	1. Elective or emergency operation 2. Severe maternal hypertension or IUGR	None	109	1	Punch and Scalpel (mixed)	N/A	47
Meekins et al (1994)	1. Normal pregnancy 2. Preeclampsia	None	45	2 to 3	Scalpel or curved scissors (Robertson et al)	90#	N/A
Naicker et al (2003)	1. Normal pregnancy 2. Hypertensive pregnancy	None	76	1	Curved scissors (Robertson et al)	N/A	72

Supplemental table 3. (Continued)

Author	Patient population	Complications	Number of patients	Number of biopsies taken	Technique used	Successful biopsy: myometrial spiral artery and trophoblast (%)	Successful biopsy: any spiral artery and trophoblast (%)
Olofsson et al (1993)	1. Normal pregnancy 2. IUGR 3. IUGR and PIH or preeclampsia 4. Preeclampsia	None	55	1	Scalpel	N/A	63
Pijnenborg et al (1991)	1. Normal pregnancy 2. Hypertensive pregnancy	None	82	1	Scalpel conisation	N/A	52
Reister et al (1999)	1. Normal pregnancy 2. Preeclamspia	None	59	1	Scalpel (10x5x5 mm)	N/A	27
Stone et al (2006)	1. Normal pregnancies 2. APS	None	36	2	Curved scissors (Robertson et al)	N/A	60
Vuorela et al (2006)	1. Normal pregnancy 2. Preeclampsia	None	35	1	Scalpel	50	50
					Success rate (%)	**47***	**58***

[#] Success rate calculated per biopsy

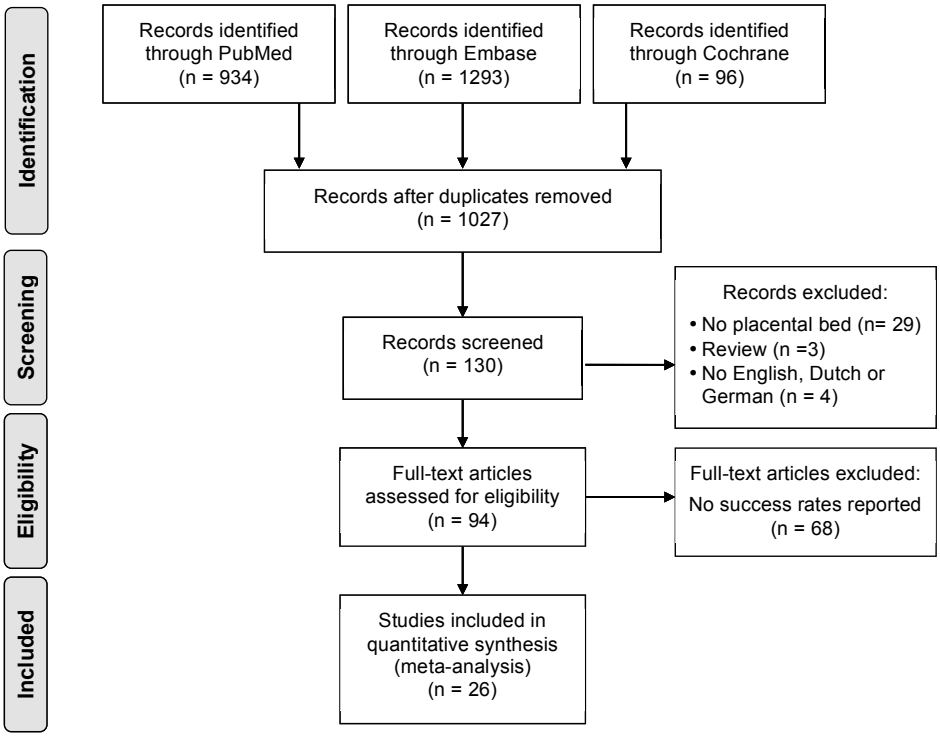
* Weighted means were calculated including only studies that reported success rate per case

PIH: Pregnancy Induced Hypertension; APS: Anti Phospholipid Syndrome; IUGR: Intra Uterine Growth Restriction

Supplemental table 4. Vacuum suction at caesarean section

Author	Patient population	Complications	Number of patients	Number of biopsies taken	Technique used	Successful biopsy: myometrial spiral artery and trophoblast (%)	Successful biopsy: any spiral artery and trophoblast (%)
Harsem et al (2007)	1. Normal pregnancies 2. Preeclampsia 3. Diabetes and superimposed preeclamspia	None	102	1	Vacuum suction	N/A	74
Harsem et al (2004)	Not described	None	51	1	Vacuum suction	13	86
					Success rate (%)	-	**78***

* Weighted means were calculated



Supplemental figure 1. Flowchart of search.

Chapter 3

Spiral artery remodeling and maternal cardiovascular risk: the SPAR study

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ABSTRACT:

Women with a history of placental bed disorders, including preeclampsia (PE) and intrauterine growth restriction (IUGR) have an increased long-term risk of cardiovascular disease (CVD). Similarities between atherosclerosis and pathology observed in placental bed spiral arteries in pregnancies affected by PE and IUGR, such as acute atherosis and defective remodeling, suggest a common pathophysiological pathway underlying both disorders. The aim of this study was to investigate vascular and inflammatory lesions in the placental bed of women with PE and normal pregnancy using a new systematic approach to characterize lesions of the placental bed. Placental bed punch biopsies were performed following Caesarean section and systematically studied to assess vascular pathology, remodeling, and the presence of T-cells, NK cells and macrophages. In addition we tested modifiable CVD risk factors immediately post-partum. We found fewer spiral arteries with complete remodeling in women with PE than in the control group (21% versus 68%; P=0.008). Women with PE showed a lower presence of T-cells in both decidua and myometrium. With our newly developed scoring system we were able to identify several types of maternal immune cells and estimate the extent of their presence in a reproducible way. Preliminary findings suggest a correlation between acute atherosis and higher triglycerides and LDL. Systematic study of vascular pathology in uterine spiral artery biopsy samples in relation to CVD risk factors may provide valuable insight into the link between cardiovascular health and placental bed disorders.

INTRODUCTION

Preeclampsia (PE) is a common hypertensive disorder of pregnancy and is associated with several foetal and maternal complications, including eclamptic seizures, intrauterine growth restriction (IUGR) and intrauterine foetal death in some cases. (1) Our group and others have shown that these women show different cardiovascular disease (CVD) risk profiles postpartum as compared to 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 PE 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 (SA) in the placental bed is thought to be involved in the development of PE and other hypertensive disorders of pregnancy. Lack of adequate SA remodeling, associated with incomplete trophoblast invasion, has been described as the key pathophysiological defect in PE, and is present in about 52-90% of cases. (9) Impaired SA remodeling, however, is not confined to PE and IUGR, but is also seen in 19% of pregnancies complicated by preterm birth and even in 4-7% of normal pregnancies. (9-11)

Besides abnormal spiral artery remodeling, women with PE also show other characteristic placental vascular abnormalities described as "acute atherosis". (12,13) 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, foam cells and the presence of macrophages. (14,15) It has been suggested that the presence of these lesions correlate with an increased risk of cardiovascular disease (CVD). (16) The most acknowledged hypothesis regarding this relation proposes a mutual pathophysiological pathway causing both diseases. (17) Similar to the pathophysiology of atherosclerosis, it is likely that the inflammatory response (to the invading foetal trophoblast) has a central role in regulating SA remodeling and may be an important culprit in the development of hypertensive disease in pregnancy. (18) Biobanking 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. (13,16) 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 CVD risk factor screening. Aims of this study were: [1] to develop a structured scoring system for lesion characteristics of the placental bed pathology; and [2] to study the involvement of local immune cells in spiral artery remodeling in normal and pre-eclamptic pregnancy. In addition, as a preliminary analysis, we explored 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 an ongoing prospective cohort-control study conducted in a single tertiary Perinatology Center at the University Medical Center Utrecht (UMCU), the Netherlands. Patients are recruited from the antenatal clinic or maternity ward when consented for a caesarean section because of PE. Preeclampsia is defined as (new) onset of hypertension (≥140/90 mmHg) and significant proteinuria (≥300mg/24h) after 20 weeks of gestation, according to the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP). (19) Controls are women eligible for an elective Caesarean section after an uneventful pregnancy and without any major underlying pathology (mostly for breech position, and women with previous Caesarean section declining a trial of labour).

IUGR is defined as an ultrasonically estimated foetal weight below the 10th percentile. (20) Hemolysis Elevated Liver enzymes Low Platelet syndrome (HELLP) is defined by the following laboratory findings in serum; aspartate aminotransferase > 50 U/L or alanine aminotransferase >50 U/L, lactate dehydrogenase > 600 U/L, platelet count < 100x109/L and evidence of hemolysis. (21) All patients receive study information and sign informed consent forms prior to participation. This study was reviewed and approved by the local Institutional Ethical Review Board (IERB) of the University Medical Center Utrecht, reference number: 11-503. The study adheres to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001.

Placental bed biopsies and immunohistochemistry

At the time of Caesarean section, after the neonate is delivered, the central part of the placental bed is manually located on the uterine myometrial wall. Four placental bed biopsies are taken using cervical biopsy forceps (Aesculaap, ER063R). Samples were fixed in 4% buffered paraformaldehyde for storage and transport. After dehydration the biopsies are embedded in paraffin wax for histological examination according to routine laboratory protocols. Our certified Pathology Laboratory, according to standard operating procedures, further processes placental bed biopsies. Sections are cut at 3µm thickness and mounted onto glass slides. Sections from each biopsy are stained with haematoxylin and eosin and the PAS after diastase stain (using the DAKO coverstainer, Belgium) using a standardized protocol to enable basic orientation.

For this study, we developed a placental bed scoring system, based on available literature on spiral artery pathology and a large study on systematic scoring of atherosclerotic lesions. (22,23) 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 cluster-

ing, of cells by two independent observers blinded for outcome (PN, EvV, JV). When interpretations differed, a third observer was consulted. As part of our scoring protocol, all sections (6-8 per patient in total) were stained for specific cell types using a Ventana Benchmark Ultra (Roche, Switzerland). CK7 staining (clone OV-TL 12/3, Biogenex, USA) was used to identify extravillous trophoblast (EVT) cells. Immune cells were identified by CD56 (clone 123C3, Neomarkers, USA) and CD3 (DAKO coverstainer, Belgium) staining, to identify natural killer (NK) cells and T-cells, respectively. CD68 (clone KP1, Novocastra, United Kingdom) staining was used to identify the presence of macrophages. 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 EVT, were used to score the number of immune cells. In the biopsies that also contained a spiral artery, the developmental state of spiral arteries was assessed, as well as the presence of acute atherosis, defined as aggregates of lipid laden macrophages in the artery wall, thrombosis and the degree of intima proliferation. Definitions of these specific lesions are shown in Supplemental 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 analysed by standard protocols used by the Laboratory of Clinical Chemistry and Hematology of the UMCU. Concisely, hemoglobin, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL), glucose, homocysteine, thyroid stimulating hormone (TSH) and high sensitivity C-reactive protein (hsCRP) were measured using a Vitros analyser (Ortho, Mulgrave, Australia) or DxC800 analyzer (Beckman Coulter, Brea, USA). Low-density lipoprotein cholesterol (LDL) was calculated using the Friedewald formula.24 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 hsCRP and 2.4% for fasting glucose levels. In addition, several of these measurements were dichotomized to previously described cut-off 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 foetal birth weight and birth weight percentile were recorded directly after birth.

Statistical analysis

Baseline characteristics were presented as medians (interquartile ranges [IQR]) 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 Chi-squared or Fisher's Exact tests for continuous and categorical variables, respectively. The placental bed scoring is displayed in 2 x 3 tables to give an overview of the data. CVD risk factors were compared by means of T test. Cut off values deducted from common practice were used and compared between the groups. Finally CVD risk factors of women with acute atherosis were preliminary compared by means of t-test. Statistical analyses were performed using SPSS (release 20.0; Chicago, IL). P-values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics

We included 29 women with PE and 29 normal pregnant controls in this study. Maternal and neonatal baseline characteristics are presented in Table 1. The percentage of nul-

Table 1. Maternal and pregnancy characteristics within the study population.

Parameter	Control n=29	PE 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(21.4-27.2)	0.320
Caucasian, %	28 (96.6%)	25 (86.2%)	0.352
Smoking, %	0(0%)	3 (10.3%)	0.105
Nulliparity, %	8 (27.6%)	22 (75.9%)	**0.001**
Pregnancy outcome			
GA at delivery (days)	275(273-278)	212 (202-223)	**<0.001**
Birth weight (grams)	3730 (3402-3958)	1135(913-1395)	**<0.001**
IUGR, %	0 (0%)	16 (55.2%)	**<0.001**
HELLP, %	0(0%)	11 (37.9%)	**<0.001**
Systolic blood pressure (mm Hg)	125(120-130)	180(170-193)	**<0.001**
Diastolic blood pressure (mm Hg)	80(70-80)	110(105-116)	**<0.001**
General and obstetric history			
Pre-existent hypertension	0 (0%)	5 (17.2%)	0.052
Pre-existent DM	0 (0%)	1 (3.4%)	1.000
Hypertensive pregnancy complication	1(5%)	4(80%)	**0.002**

Values are presented as median (interquartile range; IQR) or as number (%) for continuous and categorical variables respectively. P values calculated through Mann-Whitney-U test for continuous variables and Fisher exact test for categorical variables. PE indicates preeclampsia; BMI, Body Mass Index; HELLP, Hemolysis Elevated Liver enzymes Low Platelet syndrome; GA, gestational age at delivery (days); IUGR, Intra-Uterine Growth restriction; DM, diabetes mellitus. N/A: not applicable. Obstetric history is shown for all multiparous women.

liparous women was higher in the PE group compared with controls (75.9% vs. 27.6% respectively, p=0.001). In 11 cases (37.9%), PE was complicated by HELLP syndrome. Gestational age (GA) and birth weight differed significantly between the PE and the control group (212 vs. 275 days and 1135 vs. 3730 grams, respectively, p<0.001). As expected, the maximum systolic and diastolic blood pressure level in pregnancy was higher for preeclamptic women than for women with a normal pregnancy outcome (180 vs. 125 and 110 vs. 80, respectively, p<0.001).

Postpartum CVD and inflammatory markers

CVD and inflammatory markers in serum, measured on the first day postpartum, are presented in Table 2. We found no differences between groups for hemoglobin, fasting blood glucose, TSH, total cholesterol and hsCRP levels. Compared to controls, women with PE had higher creatinine and homocysteine levels (p<0.001 for both). Median triglyceride levels were slightly higher and HDL lower in the PE group, but this was not

Table 2. Cardiovascular disease risk markers measured postpartum in women with pre-eclampsia and controls.

SPAR laboratory data	**Control n=29**	**PE 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, µmol/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, µmol/L	4.5(3.4-5.3)	7.0(5.2-9.5)	**<0.001**
Cut-off 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 (>12µmol/L)	5(17.2%)	19(65.5%)	**<0.001**

Values are presented as median (interquartile range; IQR) or as number (%) for continuous and categorical variables respectively. P values calculated through Mann-Whitney-U test for continuous variables and Fisher exact test for categorical variables. PE indicates preeclampsia; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TSH, thyroid stimulating hormone; HsCRP, High sensitivity c-reactive protein; BMI, body mass index.

statistically significant (p=0.077 and p=0.059 respectively). Adjustment for maternal age, BMI and smoking by multivariable regression did not change the results (data not shown). When using clinical cut-off points for CVD risk factor levels, we more often found low HDL levels in PE patients than in controls (48.3% vs. 20.7%, p=0.026). Similar, PE patients were more likely to have high homocysteine levels (65.5% vs 17.2%, p<0.001).

Placental bed biopsies

Table 3 shows characteristic findings of the placental bed biopsies containing both decidual and myometrial segments of spiral arteries. In the PE group, we excluded 12 samples where the biopsy sample appeared not to be obtained from the site of the placental bed, since no trophoblasts 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 T cell presence, we observed significantly more moderate to heavy staining of CD3 positive cells in the placental bed tissue of the control group compared to the PE group (p=0.006 and p=0.047 respectively). The number of CD68 positive cells in the decidua was distributed similar for both groups. We found moderate to heavy presence of macrophages, i.e. CD68 positive cells, in the myometrium of most patients in both the PE and control group, with a trend towards more heavy clustering of macrophages in PE pregnancies (5/17 cases versus 1/19 controls; P=0.10) with similar findings for macrophages surrounding spiral arteries. The incidence of CD56 positive cells (NK cells) in the decidua and myometrium show no difference for women women PE and controls.

Table 3. Characteristic pathology of the placental bed in women with pre-eclampsia and controls.

Placental bed characteristics	**Control (n=19)**	**PE (n=17)**	**p-value**
T-cell presence (decidua)			**0.041**
Minimal	0 (0%)	0 (0%)	
Moderate	9 (47,4%)	14 (82,4%)	
Heavy and/or clustering	10 (52,6%)	3 (17,6%)	
T-cell presence (Myometrium)			**0.037**
Minimal	2 (10,5%)	6 (35.3%)	
Moderate	7 (36,8%)	7 (41,2%)	
Heavy and/or clustering	10 (52,6%)	4 (23,5%)	

Table 3. (Continued)

Placental bed characteristics	Control (n=19)	PE (n=17)	p-value
Macrophage presence (decidua)			0.829
None to minimal	1 (5,3%)	1 (5,9%)	
Moderate and/or clustering	11 (57,9%)	7 (41,2%)	
Heavy and/or clustering	7 (36,8%)	9 (52,9%)	
Macrophage presence (myometrium without SA)			0.052
None to minimal	3 (15,8%)	0 (0%)	
Moderate and/or clustering	15 (78,9%)	12 (70,6%)	
Heavy and/or clustering	1 (5,3%)	5 (29,4%)	
Macrophage (myometrium with SA)*			0.160
None to Minimal	9(47.4%)	4(28.6%)	
Moderate and/or clustering	8(42.1%)	8(57.1%)	
Heavy and/or clusters	0(0%)	2(14.3%)	
N/A	2(10.5%)	0 (0%)	
Macrophage (around spiral arteries)*			0.741
None to Minimal	7(36.8%)	4(28.6%)	
Moderate and/or clustering	10(52.6%)	6(42.9%)	
Heavy and/or clusters	1(5.3%)	2(14.3%)	
N/A	1(5.3%)	2(14.3%)	
Natural killer cells presence (decidua)			0.415
None	0(0%)	0(0%)	
Minimal	1 (5.3%)	3 (17,6%)	
Moderate	11 (57,9%)	10 (58,8%)	
Heavy and/or clusters	7 (36,8%)	4 (23,5%)	
Natural killer cells presence (myometrium)			**0.032**
None	0(0%)	0(0%)	
Minimal	3 (50,8%)	1 (5,9%)	
Moderate	9 (47,4%)	15 (88,2%)	
Heavy and/or clusters	7 (36,8%)	1 (5,9%)	
Remodeling*			**0.008**
None	0(0%)	3 (21.4%)	
Minimal	1(5.3%)	4(28.5%)	
Moderate	5(26.3%)	4(28.5%)	
Complete	13(68.4%)	3(21.4%)	
Thrombosis*	0(0%)	1(7.1%)	0.424
Atherosis*	1(5.3%)	2(14.2%)	0.561
Intima proliferation*	16(84.2%)	7 (50%)	0.057

Data are presented as numbers (percentages) and p values are calculated by Fisher exact tests. PE: pree-clampsia, SA: spiral artery * only in cases with biopsies containing spiral artery Control: n=19 and PE: n=14

Incomplete SA remodeling was more common in the PE group when compared to the control group with 3 (21.4%) vs. 13 (68.4%) cases, respectively (p=0.008). Presence of atherosis and thrombosis in the placental bed SA was similar for women with PE and controls. Intima proliferation was less often present in the PE group, however this difference was not statistically significant (50% vs 84.2%, p=0.057).

Next, we explored associations between CV 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, the preliminary analysis suggests a possible relation between higher levels of triglycerides and LDL cholesterol in women with acute atherosis lesions compared to 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.

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 CV risk markers immediately postpartum, at 6-12 months and then biennial CV follow-up. In this first analysis, we present results of systematic evaluation of the placental bed biopsy samples using a scoring system with pre-defined histopathological criteria, and show novel evidence for several differences in vascular and inflammatory characteristics of the placental bed in pregnancies affected by PE as compared to controls, including incomplete myometrial SA 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 positive T-cells that were present in lower numbers in women with PE. Preliminary analysis of CVD risk factors,

assessed immediately postpartum, revealed low HDL levels and high homocysteine levels in women with PE. 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 dyslipidaemia and placental bed vascular pathology. However, these findings are still preliminary and need to be further confirmed in a sufficient-sized study sample to include CV 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 sign of PE. (25,26,27) The role of the maternal cardiovascular and inflammatory system in this process is unknown. Our small but well-defined cohort-control study provides new data on the histopathology of the placental bed and several biochemical CV risk factors directly postpartum. With this approach, the SPAR study has the potential to provide a unique insight into the role of maternal CV health in relation to normal and pathological spiral artery development.

The most striking pathological finding in this study is the lower presence of CD3 positive T cells in PE affected pregnancies. Although this needs to be explored in further experimental work, it is likely that these absent T cells consist mostly of regulatory T-cells (Treg) as recently demonstrated by Staff et al. (28) Tregs are known to act as a suppressor to several inflammatory cells that otherwise exacerbate the immune response. Therefore, Tregs play an important role in preventing excessive immune reactions involved in inflammatory disease, including atherosclerosis. (18, 28-30) In view of our findings, it is interesting to note the small study of Sasaki et al who indeed found that the population of T regulatory cells in placental bed biopsies is lower in preeclampsia. (31) In addition, we found a trend towards an increased presence of macrophages. Macrophages are thought to be involved in regulating trophoblast migration through regulating apoptosis as well as maternal tolerance to fetal antigens. (32) An accumulation of macrophages within the vascular wall is also a hallmark of atherosclerosis. In a recent review it was shown that the relative proportion of macrophage subsets within the plaque might be more important for plaque stability than the total numbers of macrophages. (33) Future studies are needed to clarify the role of subsets of immune cells within the placental bed. In addition to spiral artery sampling, we assessed circulating CVD risk markers in serum drawn one day postpartum and found higher homocysteine and creatinine levels the PE group. Higher creatinine levels are likely explained by increased vascular permeability in patients with PE that also causes generalized peripheral edema, and leads to renal hyperfiltration and relative haemoconcentration, which a common feature of this disease. (34) 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. (35,36,37) When we used clinical cut-off values to stratify biochemical risk parameters we found an increased amount of patients with low HDL in the PE group.

Two recently published papers link acute atherosis in decidua basalis and decidua parietalis with severity of PE, adverse perinatal outcomes and a higher predicted CVD and thrombotic risk several months after pregnancy. (38,39) Adding to these studies, we were also able to demonstrate atherosis lesions in about 8% of *myometrial* spiral arteries, with no statistical difference between PE cases and controls, although our sample size is still too small to draw firm conclusions. It has been suggested that these vascular lesions primarily occur in the decidual part of the SA and could therefore explain why they are substantially less present in myometrial biopsies in this study. (16) It has been proposed that acute atherosis lesions, irrespective of pregnancy outcome, predispose to CVD later in life. (28) However, studies to relate placental bed pathology to subsequent cardiovascular risk factor profile and/or incident CVD events have not yet been conducted. It is interesting to note that, despite the small sample size, our preliminary findings suggest an association between atherosis lesions in the placental bed and subsequently measured maternal levels of established CV markers, including increased levels of triglycerides and LDL cholesterol. This has not been demonstrated before and needs to be further investigated in the full SPAR cohort analysis.

Strengths of the SPAR study include detailed and reliable phenotyping of PE group according to consensus criteria19, the reliable and safe protocol for biopsy sampling, use of a predefined scoring system and assessment of pathological features by a clinical pathologist unaware of the diagnosis, and systematic follow-up postpartum with serial assessment of CVD health and risk factor status. In our study, we were able to successfully sample the placental bed and obtain a spiral artery in 56.9% of biopsies, reaching a relatively high success rate compared to previously noted rates ranging between 42%-47% when looking at successfully identifying spiral arteries in a similar placental bed punch biopsy studies at Caesarean sections. (23) There were no adverse events or complications related to the biopsy procedure.

Some limitations of this study need to be addressed. Firstly, the sample sample size does not yet allow for detailed or stratified analysis. Secondly, CV risk factor levels when assessed immediately postpartum, may have been influenced by medication; severity of disease and, the postoperative fluctuations after the caesarean section procedure, which could have influenced CV risk markers e.g. lipids glucose and blood pressure. (40) This should become clearer as the results of the follow-up programme for cardiovascular evaluation 6 months after pregnancy become available, thereby reducing a possible transient effect of pregnancy on cardiovascular disease risk factor levels. Thirdly, it is relevant to note that PE cases had early onset disease and required a preterm caesarean section <34 weeks of gestation. Control women, on the other hand, were mostly term elective caesarean section. Therefore, these results of this do not represent the entire

spectrum of patients affected by PE, and an effect of gestational age on spiral artery pathology between 34 weeks and term pregnancy cannot be excluded.

With this study, we established a new cohort and biomaterial resource that will allow for systematic phenotyping of the placental bed in normal and complicated pregnancies, and relate findings to underlying maternal and foetal health. Findings presented in this paper, e.g. 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. Systematic evaluation of placental bed vascular and inflammatory lesions in women with a history of preeclampsia may prove to be a valuable tool to identify pathways that explain how the shared predisposition of women to pre-eclampsia and subsequent CV risk translates to placental disorders.

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Supplemental data chapter 3

Supplemental table. coring tool used to define maternal immune cell presence in placental bed biopsies

Placental bed marker	Definition	FOV
T-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).	
T-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).	
Macrophage 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).	
Macrophage 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).	
Macrophage (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
Macrophage (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
Natural killer cells 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)
Natural killer cells 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)

Supplemental table. (Continued)

Placental bed marker	Definition	FOV
Remodeling present?		
None	No physiological changes of the SA circumference	N/A
Minimal	Focal physioclogical changes <1/3 of the SA circumference, some arterial miscle still present.	
Moderate	Focal physioclogical changes >1/3 but <2/3 of the SA circumference, , some arterial miscle still present.	
Complete	Complete circular physiological changes of the SA.	
Thrombosis	Presence of thrombosis in the arterial lumen.	N/A
Atherosis	Aggregates of lipid laden macrophages invading the artery wall.	
Intima proliferation	Intraluminal fibrosis	

FOV : Field of view as used by the pathologist.

Chapter 4

Placental pathology in early intrauterine growth restriction associated with maternal hypertension

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ABSTRACT

Introduction: To identify key pathological characteristics of placentas from pregnancies complicated by early intrauterine growth restriction, and to examine their relations with maternal hypertensive disease and umbilical artery Doppler waveform abnormalities.

Methods: Single-center retrospective cohort study of singleton pregnancies with abnormal umbilical artery Doppler flow patterns resulting in a live birth <34 weeks of a baby with a weight <10th percentile for gestational age. Umbilical artery end diastolic flow was classified as being either present or absent/reversed (AREDF). Data were stratified into intrauterine growth restriction with or without hypertensive disease and pathological characteristics were compared between these various conditions according to predefined scoring criteria.

Results: Among 164 placentas studied, we found high rates of characteristic histopathological features that were associated with intrauterine growth restriction, including infarction (>5% in 42%), chronic villitis (21%), chronic chorioamnionitis (36%), membrane necrosis (20%), elevated nucleated red blood cells (89%), increased syncytial knotting (93%), increased villous maturation (98%), foetal thrombosis (32%) and distal villous hypoplasia (35%). Chronic inflammation of foetal membranes and syncytial knotting were more common in women with concomitant hypertensive disease as compared to women with normotensive IUGR ($p < 0.05$). Placentas from women with umbilical artery AREDF were more likely to show increased numbers of nucleated red blood cells and distal villous hypoplasia ($p < 0.05$).

Discussion: Placentas of women with early IUGR show high rates of several histological aberrations. Further, concomitant maternal hypertension is associated with characteristic inflammatory changes and umbilical artery AREDF with signs of chronic hypoxia.

INTRODUCTION

Intrauterine growth restriction (IUGR) is one of the leading causes of perinatal mortality and morbidity. (1,2) Although maternal tobacco use, infections and genetic disorders are known to cause IUGR in some cases, impaired placentation is thought to underlie most cases of severe IUGR. (3-6) Both early IUGR and maternal hypertensive disease, in particular early-onset preeclampsia, are characterized by placental pathology associated with hypoxia and reperfusion damage caused by impaired remodeling and/or obstruction of the spiral arteries. (3,5) Common lesions include infarction (7), inflammation (8,9) and lesions consistent with chronic ischemia. (9,10) Maternal hypertensive disease and/or Doppler abnormalities may coincide with early IUGR, and both are associated with poorer perinatal outcome. (11,12) However, it is not known whether maternal hypertensive disease and/or Doppler abnormalities in early IUGR are associated with more extensive or different placental pathology.

We conducted a single-center retrospective cohort study of women who delivered at a single perinatal center before 34 weeks of gestation after a pregnancy complicated by early IUGR, with the purpose to analyze characteristics of placental pathology according to predefined histopathological scoring criteria. The aim of this study was to identify hallmark features of placental pathology associated with early IUGR and to compare these between placentas from women with or without concomitant maternal hypertensive disease and 2 subclasses of abnormal umbilical artery Doppler waveform patterns.

MATERIALS AND METHODS

We performed a retrospective cohort study (*Utrecht Cohort*) that identified all 185 preterm singleton IUGR neonates (gestational age (GA) <34 weeks and birth weight <10th percentile, according to the most recent Dutch population growth charts (13)), who were delivered between January 1st 1997 en 31 December 2004 at the University Medical Center Utrecht, the Netherlands. Clinical data on perinatal outcome have been published before (14). For the purpose of this study, we chose to analyze only placentas of women who were delivered by Cesarean section (n = 164) without induction of labor with the aim to minimize the potential effect of labor itself on placental histology (e.g. inflammation, membrane necrosis) and excluded cases with apparent maternal infection, preterm premature rupture of membranes, chromosomal abnormalities and/or major congenital abnormalities, foetal deaths and multiple pregnancies. Further, we excluded women for whom results of umbilical artery Doppler ultrasound examination were normal or were unavailable (total n = 21), including only IUGR with weight <p10 and abnormal Doppler patterns. Maternal hypertensive disease was defined as

the presence of preeclampsia and/or the Hemolysis, Elevated Liver enzymes and Low Platelets (HELLP) syndrome. Pre-existent hypertension was defined as known hypertension prior to pregnancy that required treatment. Preeclampsia was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP) as a *de novo* rise in systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg in the second half of pregnancy and proteinuria ≥300 mg/24 h. (15) HELLP syndrome was diagnosed according to previously published criteria based on the following laboratory abnormalities: AST >50 U/L or ALT >50 U/L, LDH >600 U/L, platelet count $<100 \times 10^9$/L and evidence of hemolysis (16). Finally, umbilical artery end-diastolic flow (EDF) was classified as being either present or absent/reversed (AREDF). Clinical Doppler ultrasound data (Pulsatility Index) were collected retrospectively and were verified by chart review, but without knowledge of the placental pathology. The Institutional Review Board (IRB) of the University Medical Center Utrecht approved the study and concluded that no informed consent was needed due to the reviewing nature of the study.

All placentas were examined macroscopically and microscopically as part of routine clinical care. Placentas were weighed (without umbilical cord and membranes) and the percentage of macroscopic lesions on cut surface was determined. The ratio between infant birth weight and placental weight (fetoplacental weight ratio) was calculated. Subsequently, two samples of the umbilical cord, foetal and placental membranes and at least two samples of macroscopically normal placental tissue were taken from a central part of a placental cotyledon and additional samples of lesions. Placentas were immediately fixed in 4% neutral buffered formalin for at least 24 h and then dehydrated and embedded in paraffin wax for histology according to routine laboratory protocols.

To determine whether placentas showed signs of pathology 4-micron paraffin sections were dewaxed and standard hematoxylin and eosin staining was performed. For the present study, archived placental slides were reviewed by an expert perinatal pathologist (PN), blinded to the clinical outcome, to include the following predefined histopathological characteristics as suggested by Redline et al. (17-19): acute chorioamnionitis, acute funisitis, chronic villitis, chronic chorioamnionitis, necrosis of membranes, nucleated (foetal) red blood cells (NRBCs), syncytial trophoblast knotting, maturation of placental parenchyma, foetal thrombosis and distal villous hypoplasia. Detailed descriptions of the criteria used to classify the placental findings are summarized in Table 1. (10,17-21) Because some of these findings depend on gestational age (e.g. maturation of placental parenchyma progresses during gestation (9)), information on gestational age at delivery was disclosed to the pathologist before review. A simple scoring scheme was adopted to grade the histopathological findings as either normal or abnormal for gestational age. In the supplementary table clinical characteristics of pregnancies for placentas studied are displayed as suggested by Nelson and Burton. (22)

Statistical analyses were performed using the Statistical Package for the Social Sciences version 20.0 (IBM Corp.). Data were stratified into two pre specified study groups, i.e. (1) on the basis of the presence or absence of concomitant maternal hypertensive disease and (2) on the basis of the presence or absence of AREDF at Doppler examination. Differences between study groups were analyzed by the chi square test (Fisher's exact test if the expected count was <5) for categorical variables or the Mann–Whitney *U* test for continuous variables. We considered differences to be statistically significant at $P < 0.05$.

Table 1. Definitions for gross and histopathological placental findings (adapted from Redline et al (17-20))

Placental finding	Definition
Placental weight	Weight of the placenta measured without umbilical cord and membranes
Fetoplacental weight ratio	The ratio of fetal to placental weight
Infarction	Presence of infarction in the placental disc measured as a percentage of total placental volume
Acute chorioamnionitis	More than a few scattered neutrophils in the chorionic plate or membranous chorionic connective tissue and/or the amnion (19).
Acute funisitis	Aggregates of neutrophils in the wall of the vein and / or arteries without or with expansion in Wharton's jelly. Stage 1: chorionic vasculitis/umbilical phlebitis Stage 2: umbilical arteritis Stage 3: umbilical perivasculitis (19)
Chronic villitis of unknown etiology	Mononuclear cells infiltrating Low grade: less than 10 villi involved per focus in 1 slide (focal) High grade: 10 or more villi per focus involved in more slides (multifocal) (20)
Chronic chorioamnionitis	Lymphocytic infiltration of the membranes, defined as clusters of >50 lymphocytes in the chorion.
Membrane necrosis	Presence of necrosis in the membranes defined as decidual necrosis
Elevated NRBC	Circulating nucleated erythrocytes present in less than two capillaries in a random 10x field. Severely elevated NRBC compromised circulating nucleated erythrocytes present in more than two capillaries in a random 10x field.
Increased syncytial knotting	Syncytial knotting: Syncytial nuclei forming a multinucleated protrusion from the villous surface of terminal villi present in all slides from macroscopically normal placental parenchyma. Increased knotting: marked increased comparable to guideline from Loukeris et al. (21)
Increased villous maturation	Maturation of chorionic villi in the fetal lobule matched with gestational age: appropriate or more advanced than stated age
Fetal thrombosis	Diagnosed as at least 5 avascular fibrotic villi without inflammation or mineralization or if adherent thrombi in stem vessels were present.
Distal villous hypoplasia	Villous pattern characterized by slender, elongated, poorly branched and poorly capillarized villi with an apparent increase in the intervillous space. (10)

RESULTS

The demographic characteristics of the study population are shown in Table 2. We found no differences for maternal age, race, and gestational age at delivery and birth weight. Women with a pregnancy complicated by early-onset IUGR who had developed maternal hypertension were less likely to smoke during pregnancy than women with normotensive IUGR (2% versus 15%). Furthermore, nulliparity was more common when IUGR occurred in the presence of maternal hypertensive disease.

Placental pathology was found in all the placentas from pregnancies complicated by IUGR. Commonly observed histopathological characteristics included infarction (>5% in 42%), chronic villitis (21%), chronic chorioamnionitis (36%), membrane necrosis (20%), elevated nucleated red blood cells (89%), increased syncytial knotting (93%), increased villous maturation (98%), foetal thrombosis (32%) and distal villous hypoplasia (35%). Next, we performed subgroup analysis of pregnancies with early IUGR with concomitant maternal hypertensive disease, compared to pregnancies with normotensive early IUGR. As shown in Table 3, placental weight and fetoplacental weight ratio was similar for both study groups. Chronic chorioamnionitis was more common in IUGR complicated by maternal hypertensive disease as compared to IUGR without hypertension (41% vs. 20%). Placentas of infants with IUGR born form hypertensive pregnancies showed a higher rate of increased knotting and were more likely to show increased maturation compared to normotensive IUGR pregnancies (96% vs. 83% and 100% vs. 93% respec-

Table 2. Demographic characteristics of the study population.

		Preterm IUGR	
	Preterm IUGR (n = 164)	Normotensive (n = 40)	Hypertensive disease† (n = 124)
Mean age (SD)	29.75 (5.2)	29.10 (6.0)	29.95 (4.9)
Race			
White (%)	159 (97)	40(100)	119 (96)
Black (%)	1 (0.6)	0(0)	1(0.8)
Other (%)	4 (2.4)	0(0)	4(3.2)
Smoking (%)	9 (5.5)	6(15)	3(2.4)*
Nulliparous (%)	126 (76.8)	25(62.5)	101(81.5)*
Chronic hypertension (%)	21 (12.8)	0 (0)	21(16.9)*
Pre-gestational diabetes (%)	1 (0.6)	1(2.5)	0(0)
Gestational age (weeks)	30.3 (30.0-30.6)	31 (30.4- 31.6)	30.3 (29.8-30.5)
Birth weight (grams)	894 (861-927)	868 (796-941)	901 (863-938)

Data are presented as means with SD or 95% CI or percentages. IUGR: intra uterine growth restriction. *$P<0,05$. † maternal hypertensive disease implies the presence of concomitant pre-eclampsia and/or HELLP syndrome

tively) although very high percentages of increased knotting and maturation were seen in all study groups. We found no differences for infarction, membrane necrosis, elevated nucleated red blood cells, foetal thrombosis and distal villous hypoplasia. Only few placentas showed signs of chorioamnionitis and acute funisitis (0–5%), all of whom were in the normotensive IUGR group.

In Table 4 we present data of our second subgroup analysis based on the umbilical artery Doppler data, to compare pregnancies with positive EDF to pregnancies with AREDF. We found no differences in any of the demographic characteristics. AREDF was associated with lower placental weight, but a similar fetoplacental weight ratio. Furthermore, severely elevated nucleated red blood cells and distal villous hypoplasia were

Table 3. Characteristic placental histology in preterm IUGR with and without maternal hypertensive disease.

	Normotensive IUGR (n=40)	**IUGR with hypertensive disease† (n = 124)**
Placental weight (grams)	188 (171-205)	197 (187-206)
Fetoplacental weight (ratio)	4.81 (4.4- 5.2)	4.75 (4.5-5.0)
Infarction (%)	75	83
Infarction >5% (%)	43	42
Acute chorioamnionitis (%)	5	0
Acute funisitis (%)	5	0
Chronic villitis (%)	20	22
Chronic chorioamnionitis (%)	20	41*
Chronic chorioamnionitis with concomitant chronic villitis (%)	38	29
Membrane necrosis (%)	18	21
Elevated NRBC (%)	93	88
Severely elevated NRBC (%)	20	19
Increased knotting (%)	83	96*
Increased maturation (%)	93	100*
Fetal thrombosis (%)	40	30
Distal villous hypoplasia (%)	35	36
Maternal vascular malperfusion [a]	90	97
Inflammation [b]	38	51

Data are presented as means with 95% CI or percentages. IUGR: intra uterine growth restriction. NRBC: nucleated red blood cells *P< 0.05.

† maternal hypertensive disease implies the presence of concomitant pre-eclampsia and/or HELLP syndrome**.**

[a] Maternal vascular malperfusion': placenta with any of the following lesions; increased knotting, distal villous hypoplasia or infarction >5%.

[b] Inflammation: placenta with any of the following lesions related to an altered inflammatory response; chronic chorioamnionitis, chronic villitis, acute funisitis or acute chorioamnionitis.

more common in placentas from pregnancies with AREDF as compared to placentas with positive EDF (31 vs. 9% and 44 vs. 27%, respectively), but the prevalence of other histopathological characteristics (including infarction, thrombosis, villous hypoplasia and inflammation) was similar for both groups.

Table 4. Placental histology in preterm IUGR with present versus absent or reversed end-diastolic umbilical artery flow.

	Positive EDF (n=82)	**Absent/reverse EDF (n=82)**
Placental weight (grams)	206 (193-218)	184 (172-192)*
Fetoplacental weight (ratio)	4.6 (4.4-4.9)	4.9 (4.6-5.2)
Infarction (%)	82	81
Infarction >5% (%)	43	41
Acute chorioamnionitis (%)	0	2
Acute funisitis (%)	0	2
Chronic villitis (%)	22	21
Chronic chorioamnionitis (%)	33	39
Membrane necrosis (%)	21	20
Elevated NRBC (%)	88	90
Severely elevated NRBC (%)	9	31*
Increased knotting (%)	94	92
Increased maturation (%)	99	98
Fetal thrombosis (%)	31	34
Distal villous hypoplasia	27	44*
Maternal vascular malperfusion [a]	45	50
Inflammation [b]	95	96

Data are presented as means with 95% CI or percentages. IUGR: intra uterine growth restriction; EDF: end-diastolic flow. NRBC: nucleated red blood cells

[a] Maternal vascular malperfusion': placenta with any of the following lesions; increased knotting, distal villous hypoplasia or infarction >5%.

[b] Inflammation: placenta with any of the following lesions related to an altered inflammatory response; chronic chorioamnionitis, chronic villitis, acute funisitis or acute chorioamnionitis. *P<0.05

DISCUSSION

In this study, we provide more insight into the prevalence, distribution and type of placental pathology among women with clinically recognizable subgroups of early IUGR, i.e. presence or absence of maternal hypertensive disease and/or abnormal umbilical artery Doppler findings. We found high numbers of several key histopathological characteristics in placentas of all subgroups that are not expected to be present in normal

placental tissue (23) and showed several placental characteristics to be specific to IUGR complicated by hypertensive disease or AREDF.

In comparison to previous studies on placental pathology in IUGR (5,7,24-26), strengths of our study includes its well-defined cohort design, its use of comprehensive predefined scoring criteria with adjustments for gestational age and its sample size, which allowed for stratified analysis of relevant clinical subgroups. Some smaller studies have focused on pregnancies with IUGR and abnormal umbilical artery (UA) flow (27,28) but very little is known about differences in placental pathology between early IUGR with or without concomitant maternal hypertensive disease. (29,30) To our knowledge this is the first study of IUGR placentas that stratified for concomitant PE and/or HELLP and excluded pregnancy-induced hypertension. The retrospective design of our study might cause selection bias and missing data. However, we were able to identify and re-analyze the placental pathology specimens from all IUGR-placentas that were delivered in our institute during the study period.

Findings from our and previous studies show that IUGR placentas may have pathological lesions of two major categories, i.e. (1) those related to reduced vascular supply of nutrients and oxygen due to maternal vascular malformation *(*e.g. *infarction, villous hypoplasia, increased syncytial knotting and elevated NRBCs)* and (2) those related to an altered inflammatory response (e.g. *chorioamnionitis, villitis and acute funisitis*). Similar to other reports we found substantial placental infarction (>5% of placental volume) in about half of women with early IUGR as well as multiple thrombotic occlusions in foetal vessels in about a third. (7, 24-26) Interestingly though, placental infarction appeared not to be associated with AREDF, nor with presence or absence of maternal hypertensive disease. Similar to previous reports, we did find more subtle signs of chronic ischemia and poor nutrient supply (i.e. severely elevated NRBCs and the presence of distal villous hypoplasia) in placentas from pregnancies with IUGR and umbilical artery AREDF as compared to pregnancies with normal EDF. (29, 31-34) This is likely to represent the severity of the IUGR and in our study this was also associated with reduced placental weights in the AREDF group, although without a significant difference in the fetoplacental weight ratio. (35)

We observed marked inflammatory changes (e.g. chronic villitis, chronic chorioamnionitis) in many placentas from pregnancies complicated by early IUGR. Chronic villitis or villitis of unknown etiology (VUE) was seen in 1 in 5 cases independent of the presence of EDF or maternal hypertensive disease. A recent review on this phenomenon generally found studies that included term placentas only. VUE is known to become more common at term with numbers up to 33% (36). However, in preterm groups like our study group 20% is rather high and uncommon. Although not completely understood, chronic villitis has been associated with anatomic and functional placental insufficiency, most likely by an immunopathological process that leads to placental hypoplasia and

subsequent foetal growth restriction (37). We also found higher levels of chronic chorioamnionitis from pregnancies with early IUGR and maternal hypertensive disease, as compared to normotensive IUGR pregnancies. To our knowledge, this finding has not been reported before. Chronic chorioamnionitis is a somewhat elusive phenomenon that was originally described by Gersell et al. (38) who found this unusual variant of chorioamnionitis in a small series of 17 cases with different pregnancy outcomes including preterm birth, IUGR and a number of other pregnancy complications. Since then, only one small study five IUGR placentas confirmed these findings and identified CD3+, CD8+ and to a lesser extent CD4+ lymphocytes (39). Preterm preeclampsia is widely believed to be associated with an underlying innate pro-inflammatory phenotype and excessive production of several inflammatory cytokines and chemokine's. (39,40) We speculate that higher levels of chronic chorioamnionitis observed in pregnancies complicated by early IUGR with hypertensive disease may reflect differences in the maternal immune response to the placental pathology. Possibly, as earlier suggested by Kim et al. (41), by a more pronounced allograft rejection and graft-versus-host disease in the membranes in women with concomitant hypertensive disease.

In summary, we found high rates of several characteristic placental pathologies in placentas from pregnancies complicated by preterm IUGR. These seem to be related to reduced perfusion, chronic hypoxia, poor nutrient supply and inflammation. Further, hallmarks of reduced placental perfusion and chronic hypoxia appeared to be mostly related to severe disease (with abnormal umbilical artery Doppler waveform patterns), whereas chronic chorioamnionitis was more common in pregnancies with concomitant maternal hypertensive disease, which may reflect the more pronounced role of the maternal immune system in early IUGR cases complicated by preeclampsia. In conclusion, placental pathology in early IUGR appears to reflect distinct clinical features including maternal hypertensive disease and severity of placental insufficiency.

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Supplemental data chapter 4

Supplemental table 1. Clinical characteristics of pregnancies for placentas studied.

	Normotensive IUGR (n=40)	**IUGR with hypertensive disease (n=124)**
Gravidity *Median (25-75%;range)*	2.00(1.00-3.00;7)	1.00(1.00-2.00;5)
Parity *Median (25-75%;range)*	0.00(0.00-1.00;3)	0.00(0.00-0.00;3)
Gestational age(GA) *(wks) Average (SD)*	31.00(1.73)	30.11(1.72)
Maternal age (years) *Average (SD;range)*	29.10(6.0)	29.96(4.9)
Race *(%)*		
Black, n=	0(0)	1(0.8)
White, n=	40(100)	119(96)
Mediterranean, n=	0(0)	3(2.4)
Other, n=	0(0)	1(0.8)
Unknown, n=	0(0)	0(0)
Prenatal medications *(%)*		
None, n=	27(67.5)	35(28.2)
Iron, n=	1(2.5)	0(0)
Other (list):		
Antihypertensiva, n=	0(0)	66(53.2)
+ anticoagulants, n=	2(5)	5(4.0)
+ prednisone, n=	0(0)	0(0)
+ calciumcarbonate,n=	0(0)	1(0.8)
+ thyrax, n=	0(0)	1(0.8)
+ tocolyse, n=	0(0)	0(0)
+ antibiotics, n=	0(0)	1(0.8)
+ cardura	0(0)	1(0.8)
+ anti-asthmatics	0(0)	1(0.8)
Antidepressants, n=	1(2.5)	0(0)
Tocolytics, n=	1(2.5)	1(0.8)
Anticoagulants, n=	3(7.5)	4(3.2)
+ insulin, n=	1(2.5)	0(0)
+ antipsychotic, n=	1(2.5)	0(0)
Prednison, n=	1(2.5)	0(0)
Antiepileptics, n=	0(0)	1(0.8)
Anti-asthmatics	2(5)	1(0.8)
Unknown, n=	0(0)	6(4.8)
Drugs *(%)*		
Cigarettes, n=	6(15)	3(2.4)
Alcohol, n=	0(0)	0(0)
Other (list): n=	0(0)	0(0)
Unknown, n=	0(0)	0(0)

Supplemental table 1. (Continued)

	Normotensive IUGR (n=40)	IUGR with hypertensive disease (n=124)
Previous prenatal admission(s) *(%)*		
Yes, n=	15(37.5)	
No, n=	25(62.5)	
Diagnoses if yes:		
Fetal distress, n=	5(12.5)	
PE, n=	0(0)	
HELLP, n=	0(0)	
PROM, n=	1(2.5)	
IUGR, n=	1(2.5)	
EUG, n=	1(2.5)	
IUVD, n=	4(10)	
NVO, n=	1(2.5)	
Preterm labor, n=	1(2.5)	
Unknown, n=	0(0)	
Blood pressures <140/90 mm Hg *(%)*		
Yes, n=	38(95)	0(0)
No, n=	0(0)	108(87.1)
Unknown, n=	2(5)	16(12.9)
Screened for diabetes *(%)*		
Yes, n=	40(100)	124(100)
No, n=	0(0)	0(0)
Unknown, n=	0(0)	0(0)
Antibiotics in labour *(%)*		
None, n=	0(0)	1(0.8)
Penicillin, n=	0(0)	0(0)
Other: (list)		
Erythromycine, n=	1(2.5)	1(0.8)
Unknown, n=	39(97.5)	122(98.4)
Beta strep status *(%)*		
Positive, n =	0(0)	0(0)
Negative, n=	1(2.5)	1(0.8)
Unknown, n=	39(97.5)	123(99.2)
Antenatal steroids *(%)*		
Yes, n=	24(60)	84(67.7)
No, n=	14(35)	38(30.6)
If yes, week GA *Average (SD)*	29.14(1.70)	28.79(1.70)
unknown GA	9(22.5)	32(25.8)

Supplemental table 1. (Continued)

	Normotensive IUGR (n=40)	IUGR with hypertensive disease (n=124)
Unknown, n=	2(5)	2(1.8)
Magnesium sulfate *(%)*		
Yes, n=	0(0)	54(43.5)
No, n=	39(97.5)	68(54.8)
Unknown n=	1(2.5)	2(1.8)
Anesthesia *(%)*		
Epidural, n=	0(0)	2(1.8)
Spinal, n=	27(67.5)	63(50.8)
Narcotics, n=	0(0)	0(0)
General, n=	5(12.5)	26(21.0)
Other/none n=	0(0)	0(0)
Unknown, n=	8(20)	33(26.6)
Cervical ripening agent *(%)*		
Prostaglandin E1, n=	0(0)	0(0)
Prostaglandin E2, n=	0(0)	0(0)
Mechanical, n=	0(0)	0(0)
Other: list		
None, n=	40(100)	124(100)
Labor *(%)*		
Yes, n=	0(20)	0(0)
No, n=	40(100)	59(100)
Unknown, n=	0(0)	0(0)
Delivery mode *(%)*		
Vaginal delivery: n=	0(0)	0(0)
C-section, repeat, no labor: n=	5(12.5)	9(7.4)
C-section, repeat, with labor: n=	0(0)	0(0)
C-section, primary, no labor: n=	34(85)	115(92.6)
C-section, primary, with labor: n=	0(0)	0(0)
C-section, details unknown: n=	1(2.5)	0(0)
Maternal oxygen given at delivery *(%)*		
Yes, n=	0(0)	0(0)
No, n=	0(0)	0(0)
Unknown, n=	40(100)	124(100)
Birth weight *(g) Average(SD)*	868.3(227.5)	902.4(210.8)
Placental weight *(g) Average(SD)*	187.8(53.3)	197.1(54.1)
Baby's sex		
Female, n=	17(42.5)	51(41.1)
Male, n=	23(57.5)	73(58.9)

Supplemental table 1. (Continued)

	Normotensive IUGR (n=40)	**IUGR with hypertensive disease (n=124)**
Unknown, n=	0(0)	0(0)
Delivery to processing (min) *Average;(SD)*		
Unknown, n=		
	40(100)	124(100)
Pre-gestational diabetes *(%)*		
Yes, n=	1(2,5)	0(0)
No, n=	39(97.5)	124(100)
Unknown, n=	0(0)	0(0)
Chronic hypertension *(%)*		
Yes, n=	0(0)	21(16.9)
No, n=	40(100)	103(83.1)
Unknown, n=	0(0)	0(0)
Nulliparous *(%)*		
Yes, n=	25(62.5)	101(81.5)
No, n=	15(37.5)	23(18.5)

Part II

Endoplasmic reticulum stress and the placental bed

Chapter 5

Endoplasmic reticulum stress is induced in the human placenta during labour

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ABSTRACT

Placental endoplasmic reticulum (ER) stress has been postulated in the pathophysiology of pre-eclampsia (PE) and intrauterine growth restriction (IUGR), but its activation remains elusive. Oxidative stress induced by ischaemia/hypoxia-reoxygenation activates ER stress in vitro. Here, we explored whether exposure to labour represents an in vivo model for the study of acute placental ER stress. ER stress markers, GRP78, P-eIF2α and XBP-1, were significantly higher in laboured placentas than in Caesarean-delivered controls localised mainly in the syncytiotrophoblast. The similarities to changes observed in PE/IUGR placentas suggest exposure to labour can be used to investigate induction of ER stress in pathological placentas.

INTRODUCTION

Insufficient remodelling of spiral arteries is thought to underlie several pregnancy complications, such as pre-eclampsia (PE), intrauterine growth restriction (IUGR) and preterm labour (1-3). Secondary to this maldevelopment, placental malperfusion may result in a repetitive hypoxia-reperfusion type of injury resulting in oxidative stress, which can compromise normal function of cellular components including mitochondria and the endoplasmic reticulum (ER) (4,5).

The ER is the organelle responsible for the synthesis and post-translational modification of secretory and membrane proteins, prior to delivery to the Golgi apparatus for final targeting. The ER has its own intricate network of signalling proteins that continually senses and communicates ER status to the cell. When ER homeostasis is perturbed, these proteins coordinate the Unfolded Protein Response (UPR), a signalling cascade that aims to restore ER homeostasis and relieve the stress or induce apoptosis if this process fails (6).

Three highly conserved signalling pathways are activated in the UPR, including PRKR-like endoplasmic reticulum kinase (PERK) which in turn phosphorylates eukaryotic initiation factor 2 subunit α (eIF2α) and inhibits non-essential protein synthesis; activating transcription factor 6 (ATF6) which up-regulates ER chaperones (GRP78 and GRP94) to increase folding capacity; and inositol requiring protein 1 (IRE1) which in turn activates X-box binding protein 1 (XBP-1) and TRAF2 for up-regulating phospholipid biosynthesis, promoting mis-folded protein degradation and provoking inflammatory response (6,7). These three signalling pathways are usually activated in a sequential manner dependent on the severity of ER stress stimulation (8,9).

We recently showed that ER stress might contribute to the pathophysiology of early-onset PE and IUGR, but not late-onset PE (10). Our laboratory has previously demonstrated that exposure of placentas to labour can provide a useful in vivo model for studying cellular changes induced by oxidative stress seen in the pre-eclamptic placenta. In this study we further tested whether ER stress can also be induced by the labouring process in placentas from healthy pregnancies and the potential use a in vivo model to study placental cellular changes to ER stress in the absence of maternal factors.

METHODS

A total of 16 placental samples were used for the study, including 8 caesarean section controls and 8 labour placentas. All placentas were delivered at term by standard vaginal delivery or by elective non-labouring caesarean section (CS) from normotensive healthy singleton pregnancies. Both groups had no history of cigarette smoking, diabetes, autoimmune diseases, thrombophilic conditions or complicated pregnancies.

Detailed description of sample collection was published previously (11). Briefly, samples were collected in the University College Hospital in London where local ethics committee approved of the study and all patients signed informed consent. For each placenta, small pieces of tissue from separate lobules were randomly taken and rinsed in PBS to remove excess blood, blotted dry and snap-frozen in liquid nitrogen within 10 min of delivery; the samples were stored at −80 °C.

For immunohistochemistry, one full-thickness section placed into 10% buffered formalin for 12–24 h before embedding in paraffin wax according to standard procedures. Sections were cut at 6 μm thickness. After dewaxing and blocking of endogenous peroxidases, the sections were incubated with non-immune serum for 1 h. Rabbit polyclonal to GRP78/BiP (ab21685, Abcam, Cambridge UK) was diluted 1:3000 and incubated overnight at 4 °C. Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) and SigmaFast diaminobenzidine (Sigma–Aldrich) were used to detect binding. Sections were lightly counterstained with haematoxylin. GRP78 intensity was scored in a semiquantitative fashion scoring the intensity of syncytial staining in 10 random fields at 25× magnification. The staining intensity was graded as 0 (no staining), 1+ (weak), 2+ (moderate), or 3+ (strong). The H-score was calculated using the following formula; H-Score = (% at 0) * 0 + (% at 1+) * 1 + (% at 2+) * 2 + (% at 3+) * 3.

Western blotting was performed as described elsewhere (9).

Statistical analyses were performed using the Prism GraphPad verson 6.0. Differences between study groups were analysed by the two-tailed Mann–Whitney U test and plotted as Box & whiskers with 10–90 percentile. We considered differences to be statistically significant at $p < 0.05$.

RESULTS

Average duration of labour was 12.3 h (SD ± 3.8) and caesarean controls (CS) were free from labour. Mean placental weight (604 g vs. 571 g; $p = 0.43$) and birth weight (3489 g vs. 3270 g; $p = 0.59$) were not statistically different between the two groups.

Western blotting showed significantly higher protein levels of GRP78, XBP-1 and p-eIF2α (Fig. 1A and B) in placentas exposed to labour compared to CS controls. A trend ($p = 0.083$) was seen in the ratio of P-p38 to p38, which was higher in the laboured group (Fig. 1C and D).

Immunohistochemistry showed strong reactivity for GRP78 in laboured placentas, but not in the CS controls. Quantitation of immunoreactivity of GRP78 by H-score indicated an approximate 3 fold increased in its intensity (Fig. 2D). The majority of the positive staining was found in the syncytiotrophoblast while no or only weak staining was observed in stromal and endothelial cells (Fig. 2 A and B).

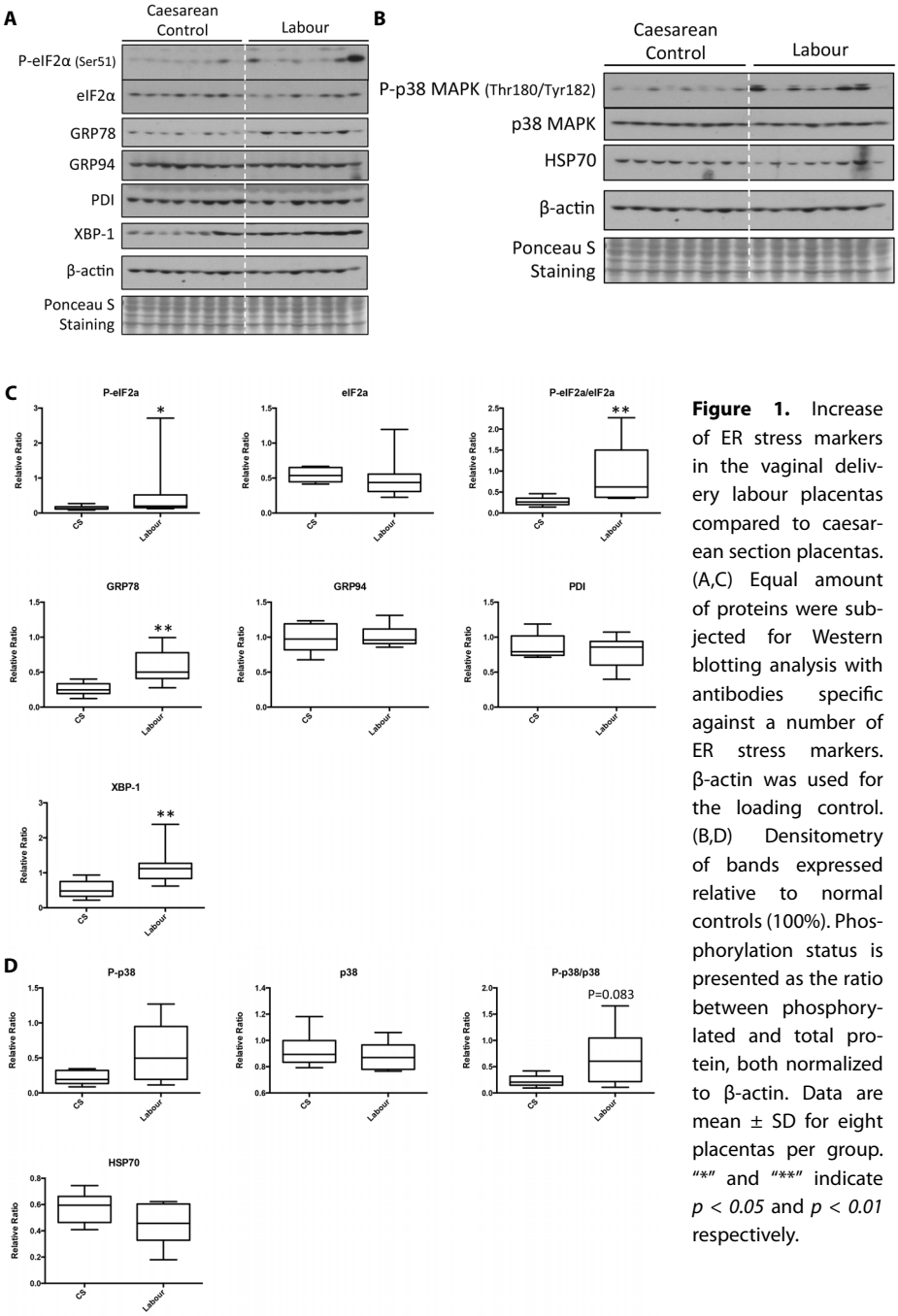

Figure 1. Increase of ER stress markers in the vaginal delivery labour placentas compared to caesarean section placentas. (A,C) Equal amount of proteins were subjected for Western blotting analysis with antibodies specific against a number of ER stress markers. β-actin was used for the loading control. (B,D) Densitometry of bands expressed relative to normal controls (100%). Phosphorylation status is presented as the ratio between phosphorylated and total protein, both normalized to β-actin. Data are mean ± SD for eight placentas per group. "*" and "**" indicate $p < 0.05$ and $p < 0.01$ respectively.

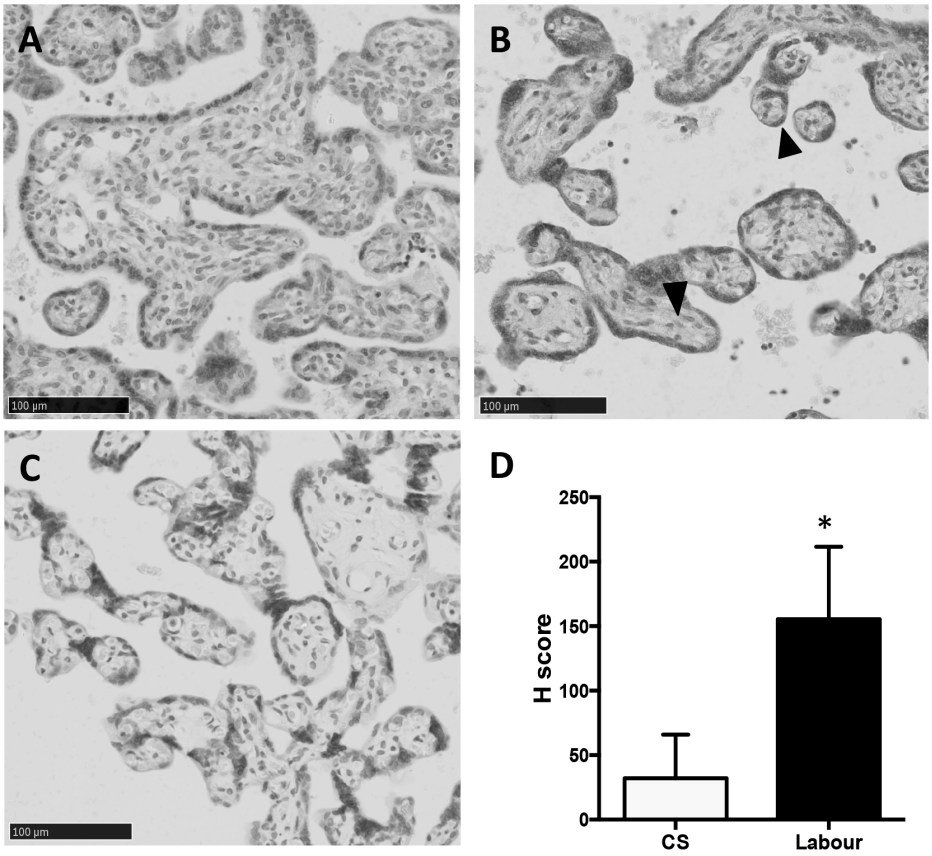

Figure 2. Higher GRP78 immunoreactivity in laboured placenta. A) Caesarean control; B) Labour; C) No primary antibody negative control; D) Semi-quantitation of GRP78 immunoreactivity in placentas using H-score. Data presented as mean ± SD, $n = 8$. * indicates $p < 0.001$. Arrowhead shows positive staining of GRP78 in syncytium. Scale bar = 100 µm.

DISCUSSION

In this study we provide evidence that placental ER stress can be induced by the process of labour, as indicated by the elevated levels of ER stress markers, including GRP78, XBP1 and P-eIF2α. The majority of ER stress induced by labour was found in the syncytiotrophoblast, consistent with the localization of ER stress observed in placentas from cases of IUGR and early-onset PE (9).

Placental ER stress has been implicated in many pregnancy disorders, including small-for-gestational age at high altitude, IUGR, early-onset PE and gestational diabetes, but not in late-onset PE ((9,12) and HWY, unpublished data). ER stress response pathways can be induced by a variety of stimuli, including viral infection, hypoxia, oxidative

stress and nutrient deprivation. We have previously demonstrated that oxidative stress induced by ischaemia/hypoxia-reoxygenation is a strong inducer of ER stress *in vitro* in human choriocarcinoma cells (8,9). Although the exact mechanisms are unclear, there are at least two potential pathways by which the oxidative and ER stress may be linked. Firstly, reduction of intracellular ATP levels during oxidative stress could inhibit SERCA channel activity in the ER membranes, resulting in perturbation of Ca^{2+} homeostasis and loss of function of the protein folding (PDI) enzymes. Secondly, formation of disulfide bonds during folding is an oxidative process and the PDI enzymes require molecular oxygen as an electron acceptor. A short electron transport chain is present within the ER, and, as in mitochondria, leakage can occur generating reactive oxygen species (ROS). Under normal conditions, approximately 25% of ROS generated in a cell arise within the ER, and this will be increased with attempts to refold misfolded proteins. Activation of PERK increases the synthesis of glutathione and also promotes the nuclear translocation of Nrf2, regulating antioxidant gene expression. Nonetheless, if these responses are overwhelmed, a feedback loop that further enhances ER stress will be formed.

Exposure to labour has been demonstrated to be a useful tool to study placental responses to oxidative stress induced by a physiological insult *in vivo* (13). Our results indicate that it may also be used to study acute responses in ER stress signalling pathways. Placental oxidative and ER stress have been implicated in the pathophysiology of early-onset PE. However, elucidating their role in this syndrome is confounded by the presence of the maternal pro-inflammatory milieu, making it difficult to distinguish between cause and effect. Exposure to labour may therefore present the opportunity to study placental cellular changes to oxidative and ER stresses in the absence of maternal factors.

An alternative explanation is that placental stresses induce labour. We have previously reported that induction of oxidative stress in the mouse placenta is associated with upregulation of cyclooxygenase enzymes, and proposed this possibility (14). ER stress response pathways can also cause increase secretion of pro-inflammatory cytokines (7) that are known to promote labour. Separating cause and effect is impossible in the human placenta, and further studies in animal models are required.

Our results further suggest that caution should be taken when interpreting gene expression or other data obtained from placentas exposed to labour, for they may be subjected to stress-induced protein synthesis inhibition and other downstream consequences of ER stress.

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Chapter 6

Endoplasmic reticulum stress reduces MMP activity in HTR8/SVneo trophoblast cells: implications for spiral artery pathophysiology in preeclampsia and IUGR

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In preparation

ABSTRACT

Spiral artery remodelling plays a pivotal role in the development of (hypertensive) disorders of pregnancy such as preeclampsia and intrauterine growth restriction. The mechanisms that cause the failure of extravillous trophoblast-dependent remodelling of the spiral arteries have not been fully elucidated, although they are likely to involve the interplay between several factors. Matrix metalloproteases (MMPs) have been shown to mediate invasion of trophoblasts *in vitro,* and significant reduction in levels of matrix metalloproteases is found in cases of intrauterine growth restriction associated with shallow trophoblast invasion. The secretion and functionality of many proteins are negatively influenced by endoplasmic reticulum (ER) stress. The aim of this study was to determine the effects of ER stress on metalloprotease activity and subsequent trophoblast invasion *in vitro*. We found significantly higher levels of ER stress markers when HTR8/SVneo cells were treated with tunicamycin and thapsigargin. MMP zymogen activity was significantly reduced under ER stress, while protein levels of MMPs appeared increased intracellularly and unchanged in conditioned media. Furthermore, invasiveness of trophoblasts through Matrigel was significantly reduced. Loss of activity of MMPs was unlikely to be caused by alterations in the inhibitor TIMP-1 because under ER stress intracellular levels were unchanged, and levels in conditioned media were reduced. In this study, we provide evidence that ER stress affects trophoblast invasion, possibly by facilitating MMP-2 and -9 mis-folding. Our data suggest a possible role for ER stress in causing the deficient trophoblast invasion observed in IUGR and preeclampsia.

INTRODUCTION

Despite many efforts to unravel the pathophysiology and improve clinical care, pre-eclampsia and intrauterine growth restriction (IUGR) remain leading causes of maternal and perinatal morbidity and mortality worldwide. (1) Further, there has been a growing body of evidence in the last decade suggesting that preeclampsia is not a disorder confined to pregnancy but has long-term (cardiovascular) health consequences as well. (2) Although the pathophysiology is not completely elucidated, it is now widely accepted that failure of extravillous trophoblast-dependent remodelling of the spiral arteries plays a pivotal role in the development of preeclampsia and IUGR. (3) Lack of adequate spiral artery remodelling is present in about 52-90% of preeclampsia cases and myometrial segments of the arteries are more adversely affected in pathological pregnancies than the decidual segments. (4) The mechanisms underlying insufficient trophoblast invasion are likely to be complex, and evidence suggests that several factors are involved, including cell adhesion molecules, apoptosis, nitric oxide and other vaso-active mediators, oxygen tension, cytokines and matrix metalloproteases (MMPs). (5)

MMPs are proteolytic enzymes that play a key role in the trophoblast invasion process by degrading basement membranes and extracellular matrix components, thereby facilitating cell movement through tissues. In *in vitro* experiments, the invasive capacity of trophoblasts has been related to their secretion of MMPs. (6) A significant reduction in MMP levels has been reported in cases of IUGR associated with shallow trophoblast invasion. (7) Several types of MMPs have been identified and overall evidence supports MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and MMP-11 involvement in normal trophoblast invasion of the decidua. (5) The balance between secretion of MMPs by trophoblasts and decidual cells, and their inhibition by tissue inhibitors of MMPs (TIMPs 1-3) produced by the same cells results in the control of normal invasion. Abnormalities in these processes could lead to excessive invasion such as placenta accreta, choriocarcinoma or restricted invasion as in early pregnancy failure, pre-eclampsia and fetal growth restriction. (5) Causes of these impaired processes are largely unknown. However, it has been shown that protein secretion and activity are negatively influenced by endoplasmic reticulum (ER) stress. (8) ER stress is increasingly recognised as the basis of many neurological and metabolic diseases, but its involvement in disorders or reproduction is only beginning to be explored. (9)

The ER is mostly known for synthesizing and the post-translational modification of secretory and membrane proteins before entering the Golgi apparatus for final targeting. When ER homeostasis is disrupted, the so-called unfolded protein response (UPR) is activated, a signalling cascade of three highly conserved pathways that aim to restore ER homeostasis and relieve the stress. (10) The primary response initiated by UPR signalling is to inhibit protein synthesis, thereby reducing protein load on the ER. This is mediated

by phosphorylation of eukaryotic initiation factor 2 subunit α (eIF2α) by PRKR-like endoplasmic reticulum kinase (PERK). Furthermore, induction of activating transcription factor 6 (ATF6) up-regulates ER chaperones (GRP78 and GRP94) to increase folding capacity and inositol requiring protein 1 (IRE1) phosphorylation in turn activates X-box binding protein 1 (XBP-1) for up-regulating phospholipid biosynthesis, promoting mis-folded protein degradation and provoking inflammatory response. (11)

Recently, it has been shown that the activation of the UPR is higher in placentae from early-onset preeclampsia compared to control second-trimester and term placentae. (12) These data show that ER stress might contribute to the pathophysiology of the syndrome of preeclampsia, in particular early-onset preeclampsia. Whether ER stress reduces MMP activity and subsequently trophoblast invasion is currently unknown.

In this study we tested whether ER stress impacts on MMP activity and results in a subsequent reduction in the invasiveness of trophoblast cells. We therefore investigated the effect of ER stress in trophoblast invasion *in vitro* using HTR8/SVneo cells and the ER stress inducers tunicamycin and thapsigargin. To determine the activity of MMPs in ER-stressed trophoblasts we performed a zymogen assay. Finally we tested if ER stress causes impaired trophoblast invasion in an *in vitro* cell invasion assay.

MATERIALS AND METHODS

Cell culture

Dr. E.D. Post Uiterweer, University Medical Center Utrecht, the Netherlands, kindly provided the HTR8/SVneo trophoblast cell line. Cells were cultured in RPMI 1640 medium (Invitrogen Ltd, Paisley, UK) supplemented with 10% heat-inactivated FBS (Invitrogen), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a 5% CO_2 atmosphere. Upon reaching confluence, cells were rinsed with PBS. 2.5ml 0.05% Trypsin-EDTA (Invitrogen) was added for 10 min at 37°C to disrupt cell adhesions and cells were resuspended in medium and centrifuged at 1500 rpm at room temperature for 5 min. The cell pellet was resuspended and cell density calculated with a haemocytometer. The suspension was diluted to 20 x 10^4 cells/ml with medium and cells were seeded in a 6-well plate in a total volume of 2ml/well for subsequent assays.

ER stress induction

Plated cells were cultured for 48 h to reach confluence. Medium was removed and cells were rinsed once with serum-free medium before incubating with 2 ml of serum-free medium containing serial dilutions of tunicamycin (Sigma- Aldrich) and thapsigargin (Sigma- Aldrich). The optimal concentration found was used for further experiments. Incubation with the optimal concentration was 24 h to ensure ER stress detectable with

western blot but with minimal cell death (<10%). Three independent experiments were performed in each condition. Plated cells were then treated with the optimal concentration and cell lysates and medium were collected for further analysis with western blotting and zymogen assay. The collected medium was concentrated to 100µl using a VivaSpin.

Western Blotting

Cells were washed with ice-cold PBS and cell lysis buffer was added containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and complete mini proteases inhibitor cocktail (Roche Diagnostics, East Sussex, UK). After scraping the cells we transferred the mixture to a microfuge tube. After pipetting up and down ~20 times, cells were maintained on ice for 20 min with occasional vortexing and centrifuged at 10,000 *g* for 5 min. Bicinchoninic acid (Sigma-Aldrich) was used to determine protein concentration using 2 µl of cell lysate. Equivalent amounts of protein were resolved by SDS-PAGE, blotted onto a nitrocellulose membrane and incubated with primary antibodies to GRP78-BiP (ab21685 Abcam, Cambridge UK), P-eIF2α (33985, Cell Signaling), GRP94 (ab18055, Abcam, Cambridge UK), MMP2 (ab86607 Abcam, Cambridge UK), MMP9 (ab7299, Abcam, Cambridge UK) Timp1 (ab1827 Abcam, Cambridge UK) and β-actin (Sigma-Aldrich). Ponceau S staining (Sigma-Aldrich) was used to evaluate equal loading. The blots were analysed by enhanced chemiluminescence (ECL) (GE Healthcare, UK) using Kodak X-OMAT film (Sigma-Aldrich). Films were scanned and intensities of the bands were analysed using Image J analysis software (Freeware). (8)

Zymogen assay

Conditioned media were subjected to native gel electrophoresis on ice in a 10% SDS polyacrylamide gel containing 0.1% gelatin (Sigma-Aldrich). Three independent experiments were performed in each condition. After resolving, the gel was incubated in a zymogram renaturing buffer containing 2.5% Triton X-100 (Sigma) with gentle shaking for 1 h at room temperature. For hydrolysing the gelatin the gel was transferred into zymogen buffer containing 50 mM Tris HCl, 200 mM NaCl, 10 mM $CaCl_2$ for overnight incubation at 37°C. After incubation the gel was stained with PAGE-BLUE (Fermentas, UK) before washing in water until MMP activity appeared as clear bands against a dark blue background. The intensity of the bands was quantified using Image J analysis software.

Trophoblast invasion assay

The transwell invasion inserts of a FluorBlock Matrigel coated 24-multiwell insert system (Cat. No. 354165, BD Biosciences, pore size 8 µm) were prehydrated according to manufacturers instructions with 100µl of serum-free medium containing ER stress inducers

with the same optimal concentration. Confluent HTR8/SVneo cells were washed with PBS, trypsinized and centrifuged. The pellet was resuspened in serum free RPMI medium containing the optimal concentration of ER stress inducers. The cell suspension was diluted and 100.000 cells were transferred to the insert. Medium containing 10% serum was added to the lower wells. After 24 h incubation at 37°C in a 5% CO_2 atmosphere the medium was discarded and cells on the lower surface of the filter were fixed and permeabilized with ice-cold methanol. After washing with PBS and MilliQ water the cells were stained with 0.6 µg/ml SYTOX-green (S33025, Nuclear labelling kit for fixed cells, Invitrogen). The number of cells that had migrated through the matrigel insert were visualized with a Leitz DM1L microscope (Leica Microsystems, Wetzlar, Germany). With a 5x objective the number of invaded cells was counted in 9 random fields nearly covering the whole filter.

Statistics

Statistical analyses were performed using the Statistical Package for the Social Sciences version 21.0 (IBM Corp.). Prism GraphPad version 6.0 was used to generate the graphs. Differences between study groups were analysed by the two-tailed Mann-Whitney U test or Student's *t*-test, when appropriate. We considered differences to be statistically significant at $P< 0.05$.

RESULTS

The level of the ER chaperone protein GRP78 was used to evaluate the effect of the ER stress inducers on HTR8/SVneo cells after a dose-response study using the ER stress inducers, tunicamycin and thapsigarin. The sublethal dose of the ER stress inducers which maximized activation of ER stress and was chosen for later study is shown in Figure 1A. For tunicamycin this was found to be 0,078 µg/ml and 0,125 µM for thapsigargin. These concentrations resulted in less <10% cell death equivalent to serum free treated control condition (data not shown). To further investigate the pathways of the UPR activated by the induced ER stress, other ER stress markers were studied using western blots. We observed significantly higher levels of P-eIF2α, GRP78 and GRP94 in treated group versus non-treated groups (Figure 1B-G).

MMP activity is reduced in ER stress treated HTR8/SVneo cells

MMP activity is thought to be crucial in the invasive capacity of trophoblasts, especially MMP-2 and -9. Therefore, we examined the protein levels of MMP-2 and -9 in the cell lysates, as well as the levels secreted by the trophoblast into the media. The levels of MMP-2 were found to be significantly higher in cell lysates of the ER stress-induced

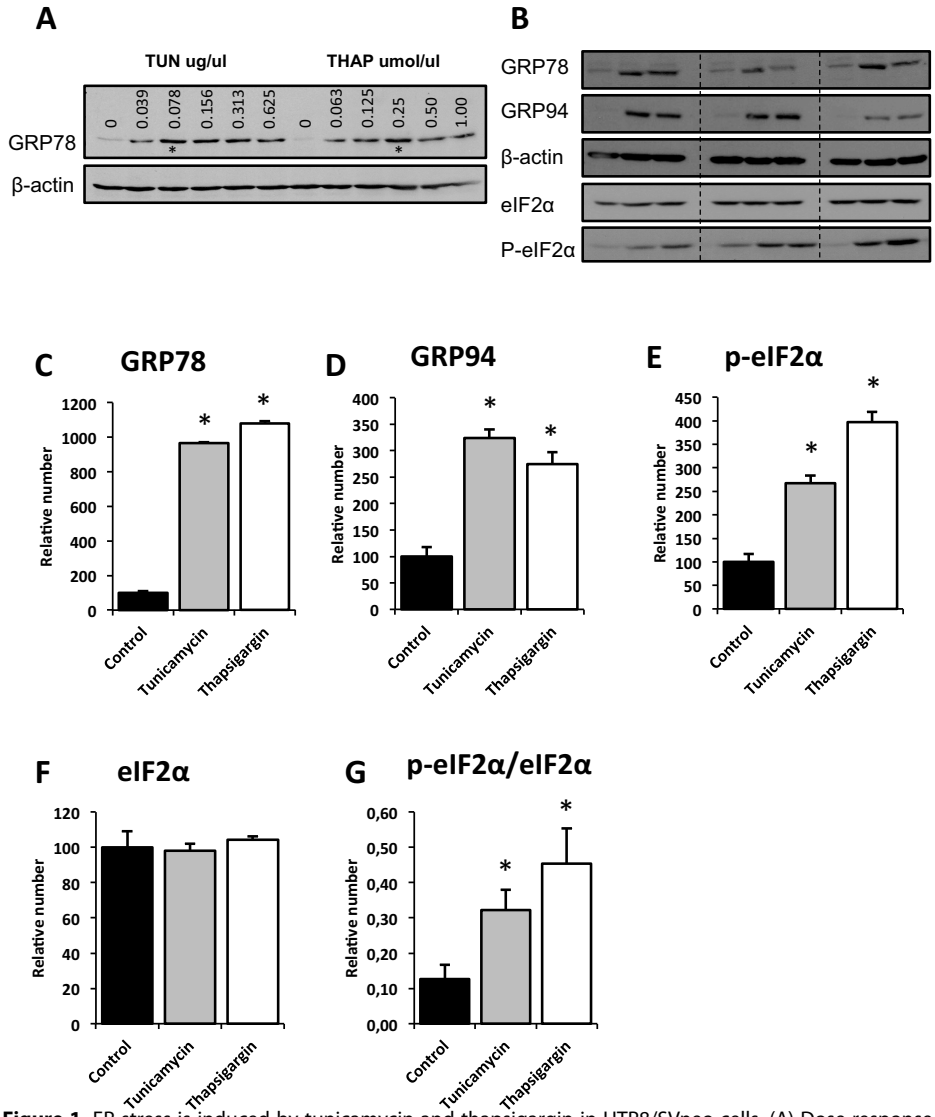

Figure 1. ER stress is induced by tunicamycin and thapsigargin in HTR8/SVneo cells. (A) Dose response curve, * indicating sublethal concentration. (B) Equal amount of proteins were subjected for Western blotting analysis with antibodies specific against GRP78, GRP94, P-eIF2α and eIF2α. β-actin was used for the loading control. (C-G) Densitometry of bands expressed relative to serum free treated controls (100%). Phosphorylation status is presented as the ratio between phosphorylated and total protein, both normalized to β-actin. Data are mean ± SD for three experiments per group. "*" indicates $p < 0.05$

groups (figure 2A). Levels of MMP-9 in the conditioned media showed no differences between the groups (Figure 2A). Despite repeated attempts, the antibodies were unable to recognize MMP-9 in the lysates and MMP-2 in the conditioned media.

Levels of TIMP-1 showed no changes in the cell lysates, but were lower in the media of ER stress-induced HTR8/SVneo cells (Figure 2B).

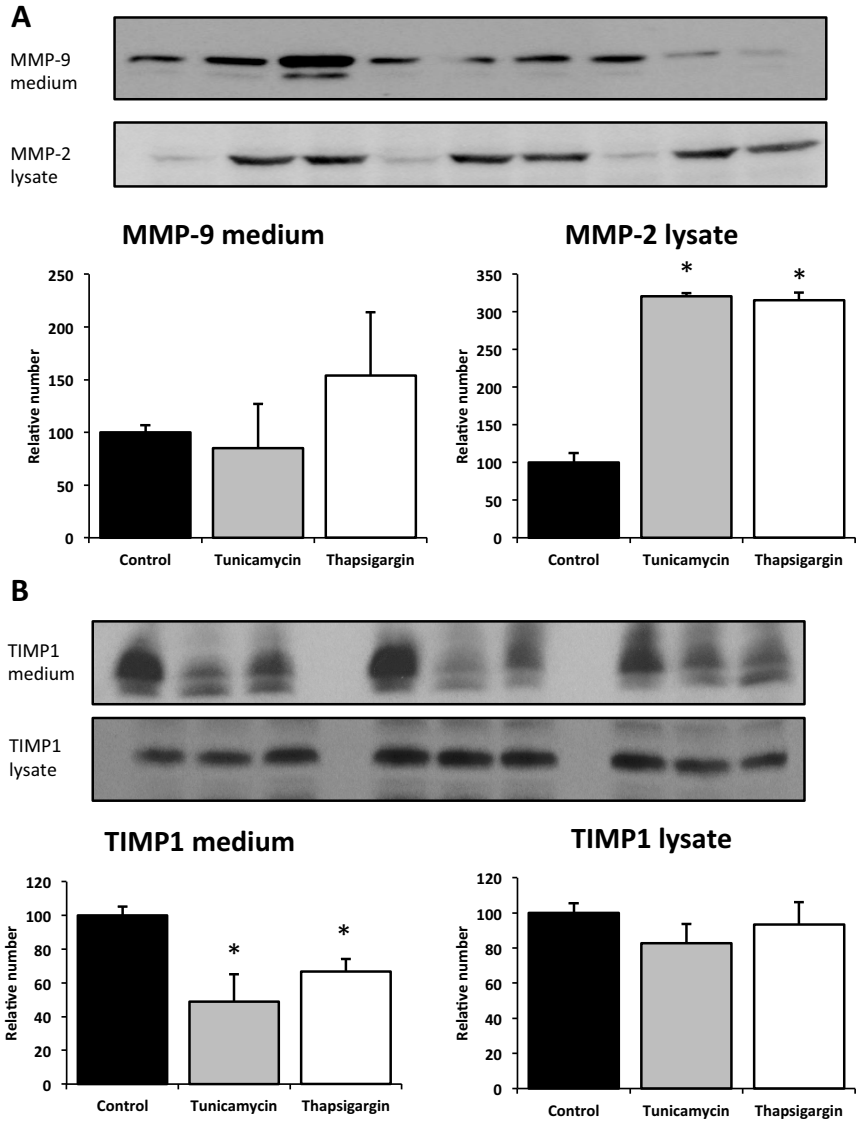

Figure 2. MMP-9 and 2; and Timp1 levels in conditioned medium and cell lysate. (A,B) Equal amount of proteins were subjected for Western blotting analysis with antibodies specific against MMP-9, MMP-2 and TIMP1. Densitometry of bands expressed relative to serum free treated controls (100%). Data are mean ± SD for three experiments per group. "*" indicates $p < 0.05$

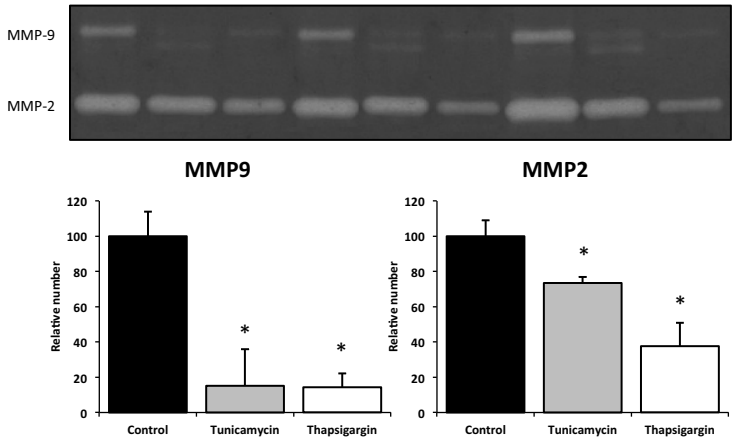

Figure 3. Zymogen activity of MMP-9 and MMP-2. Conditioned media was subjected to a zymogen assay and densitometry of bands was expressed relative to serum free treated controls (100%). Data are mean ± SD for three experiments per group. "*" indicates $p < 0.05$

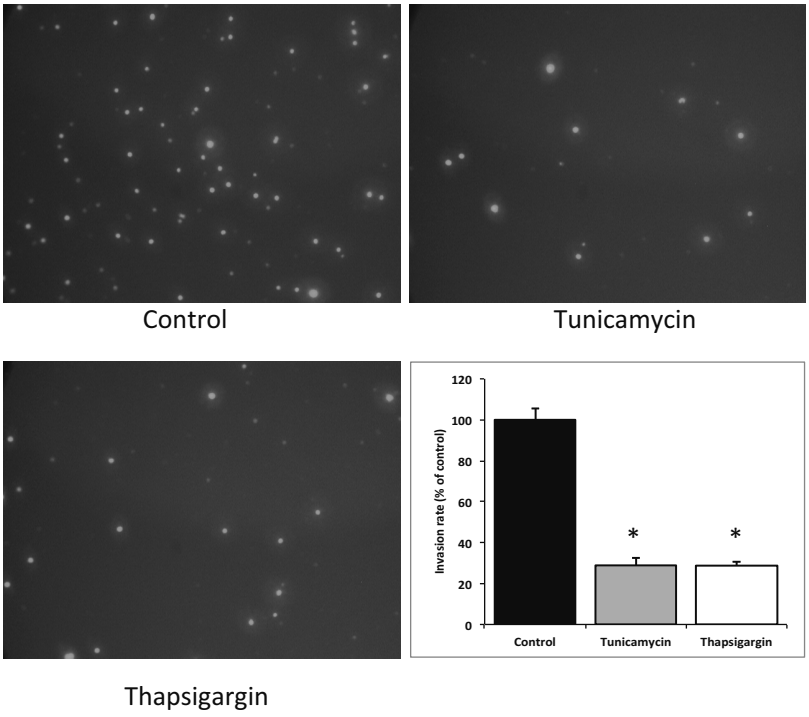

Figure 4. Matrigel invasion assay with ER stress induced HTR8/SVneo cells. Cells were plated on the upper surface of the membrane and counted as they appeared on the under surface. The percentage of migrating cells was expressed relative to the serum free treated control (100%). Data are mean ± SD for three experiments per group. "*" indicates $p < 0.05$

The ER is important in post-translationally modifying proteins and ER stress can lead to mis-folding and subsequent defective function of proteins. To measure the proteolytic capacity of secreted MMP-2 and -9 following ER stress, the conditioned medium was collected for zymogen assay using gelatin as substrate. ER stress treated cells showed significantly lower proteolytic activity compared to serum-free treated cells (Figure 3).

Invasiveness of trophoblast is significantly impaired under ER stress.

Invasive properties of trophoblasts are thought to be vital process in establishing a functional human haemochorial placenta. (3) Therefore, we investigated the effects of ER stress induced low MMP-2 and -9 activity on invasiveness of trophoblast cells. This was performed using a Matrigel invasion assay, in which cells were plated on the upper surface of the membrane and counted as they appeared on the under surface. The technique and data are shown in Figure 4. There were significantly fewer cells that migrated under ER stress conditions compared to control conditions.

DISCUSSION

To our knowledge this is the first study to investigate the inhibitory role of ER stress in the invasiveness of trophoblasts. In this study, we provide direct evidence that ER stress affects trophoblast invasion, possibly through inhibition of MMP-2 and -9 activity. The exact mechanisms for suppression of MMP-2 and -9 activity under ER stress require further investigation. However, our data suggest that they are unlikely to involve reduced MMP expression as MMP-2 protein level was increased upon ER stress. Inhibition of MMP secretion is also unlikely to be the case as MMP-9 extracellular protein concentrations remained unchanged. Finally, the potential inhibition from TIMPs is also unlikely as extracellular TIMP-1 concentrations were reduced upon ER stress. Taken together, these data suggest that ER stress could be involved in regulating migration and invasion of trophoblast cells through modulating MMPs activity but unlikely through their inhibitors TIMPs.

Invasion of trophoblast into myometrium underlying the implantation site is of pivotal importance to successful pregnancy. Failure of this process is a commonly seen in the placental bed of pregnancies complicated by IUGR, preeclampsia, placental abruption and preterm birth. (13-15) Defective deep placentation, in which the deeper lying myometrial parts of the spiral arteries are not remodelled, seems to be a key factor in complicated pregnancies. (15) Despite extensive research, the reasons why extravillous trophoblasts fail to remodel maternal spiral arteries into high capacity and low resistance vessels remain unclear.

The regulation of MMP functions in the placental bed seems to be crucial for successful implantation and may operate at various levels. (16,17) For instance, Caniggia et al showed that an oxygen tension switch around 10-12 weeks of pregnancy may influence MMP activity and subsequent invasion which seemed to be mediated by a reduction of HIF1α expression, and downregulation of trophoblast TGFβ_3 expression. (18) Research on the expression of MMPs in preeclampsia is, however, limited, especially *in vivo*. Lian et al. (19) and Reister et al. (20) demonstrated that the expression of MMP-1, MMP-3 and MMP-7 in extravillous trophoblasts was significantly lower in placental bed biopsies of preeclamptic cases than in those of uncomplicated pregnancies. However, the placental bed samples were taken at term and it is highly unlikely that the levels of MMP found reflect those of early pregnancy. Moreover, it is known that as pregnancy progresses MMPs are differentially expressed. (21) Studies have shown expression of MMP-2 and -9 is most strongly localized in the placental bed very early in pregnancy. (22) In our study we therefore focused on MMP-2 and -9. We have previously shown that ER stress-mediated inhibition of protein synthesis plays a key role in the pathophysiology of IUGR. (23) In the current study we found that under ER stress trophoblasts increase the levels of MMP-2 in cell lysates. Surprisingly, the zymogen assay showed that the activity of MMP-2 is significantly lower, implying that there is a loss of functionality rather then a loss of abundance. However, without data on MMP-2 levels in the conditioned media we cannot make definitive conclusions. The loss of zymogen activity is also reflected in the reduced capability of the trophoblast cells to invade a matrigel coated transwell system (Figure 4). All secreted and membrane-bound proteins undergo post-translational modification within the ER, such as disulphide bond formation and glycosylation. However, under ER stress these processes are compromised, resulting in protein mis-folding and potentially adversely affecting their bioactivity as well.

TNFα and Interferon (IFN) γ have been shown to reduce the activity of pro-MMP-2 as well. (24) Interestingly, both cytokines are also capable of inducing ER stress and activate the UPR. (25,26) Also, we have previously shown that ER stress can be induced in BeWo cells by TNFα, albeit at a mild level. (27) Of interest, is the dominant immune cell population in the decidua; the uterine NK (uNK) cell which is known to exert its effector functions through cytokines like TNFα and IFN γ. (28) The findings of Hiby et al. further support the concept that immune interactions between trophoblast and uNK cells are important in spiral artery remodeling. These authors showed that trophoblast cell MHC class I molecules (HLA-C) can interact with Ig-like receptors (KIRs) on uNK cells, and that certain KIR/HLA-C combinations are predictable of poor obstetric outcome i.e. PE and IUGR. (29) Nevertheless, it remains unclear if indeed aberrant activation of uNK cells (or other maternal immune cells) early in pregnancy results in higher levels of cytokines and subsequently leads to ER stress on extravillous trophoblasts.

Finally, the activity of MMPs is tightly regulated by TIMPs. They inhibit MMP activity by binding to zinc binding sites of active MMPs. (30) We showed that expression of TIMP1 was not changed under ER stress conditions. Excreted levels, however, were significantly lower in conditioned media of ER stressed cells. We can therefore conclude that TIMP1 is probably not involved in downregulating the activity of MMP-2 and -9. However, as earlier stated, multiple TIMPs have been discovered and can be differentially expressed throughout pregnancy. Further research is therefore needed to determine the role of this family in modulating trophoblast invasion under conditions of ER stress.

Unfortunately it is not possible to test our hypothesis *in vivo*. The lack of early pregnancy samples from on-going pregnancies is a major problem for research into placental development and events within the placental bed. Our studies have shown that ER stress is significantly higher in preterm preeclampsia, as consequence of compromised spiral artery remodelling, indicating that ER stress might contribute to the syndrome of early-onset preeclampsia. It is however highly unlikely that the placental ER stress found at term reflects the level of ER stress early in pregnancy. Furthermore, caution must be taken when interpreting data from *in vitro* studies, as they do not reflect actual *in vivo* situation. Finally it must be noted that we only used one immortalized trophoblast cell line. Other cell lines and primary cultures must be included in order to draw definite conclusions. Nonetheless, isolation procedures of primary cells can cause ER stress as well; caution must be taken with the use of these cultures. Unfortunately we were not able to detect MMP-9 in the cell lysate and MMP-2 in the conditioned media. Our positive control for MMP-2 (cell lysate) did show a clear band. This finding might be explained by the fact that the antibody used is made with a recombinant fragment derived from within residues 380 - 655 of human MMP-2. Possibly due to mis-folding or altered glycosylation this epitope is not recognized in the secreted form.

From our data we propose that ER stress could be involved in failure of spiral artery remodeling and thereby be causative of several pregnancy complication. How ER stress is induced in trophoblast remains to be elucidated. Future research should also focus on trying to alleviate ER stress with orally active chemical chaperones. This could provide potential future treatment options. The effectiveness of chaperones that relieve the cell from ER stress has been shown previously in a mouse model of type 2 diabetes, where ER stress was reduced and glucose homeostasis restored. (31) Although proven to be clinically safe, the teratogenity of such drugs remains to be investigated.

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Part III

Cardiovascular risk factors after pregnancy disorders

Chapter 7

C-reactive protein and fibrinogen levels as determinants of recurrent preeclampsia: a prospective cohort study

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ABSTRACT

Objective: Women with a history of early-onset preeclampsia have an increased risk of recurrent preeclampsia and are more prone to develop future cardiovascular disease. At present, risk factors underlying this association are not well characterized. We investigated whether the risk of recurrent preeclampsia is associated with pre-pregnancy levels of common cardiovascular and inflammatory markers.

Methods: Reproductive follow-up and cardiovascular parameters were obtained for 150 primiparae with a history of early-onset preeclampsia 6 – 12 months after their first delivery. Simultaneously, fasting plasma samples were collected and tested for lipids, glucose, C-reactive protein and fibrinogen. The relative contribution of each marker to the recurrence risk of preeclampsia and preterm delivery was estimated by Cox proportional hazard models.

Results: Forty-two women (28%) developed preeclampsia in a next pregnancy. Recurrent preeclampsia was related to elevated pre-pregnancy levels of C-reactive protein and fibrinogen when compared to women who did not develop recurrent disease. We found no associations between recurrent preeclampsia and maternal age, pre-pregnancy BMI, smoking or fasting levels of total cholesterol, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, triglycerides and glucose.

Conclusion: These observations support a role for inflammation in recurrent hypertensive disorders of pregnancy similar to its contribution to later-life atherosclerosis and risk of cardiovascular disease.

INTRODUCTION

Preeclampsia is a common hypertensive disorder of pregnancy with an incidence of 1 – 5% defined as de-novo systemic hypertension and proteinuria in the second half of pregnancy. (1,2) In recent years, it has become clear that a history of preeclampsia predisposes a woman to premature cardiovascular disorders, such as hypertension, ischaemic heart disease, stroke and venous thromboembolism, and is associated with excess long-term cardiovascular mortality. (3 – 5) Mechanisms underlying the shared maternal predisposition to preeclampsia, atherosclerosis and cardiovascular disease (CVD) are poorly understood. (6,7) Previous studies have suggested a role for common CVD risk factors (obesity, dyslipidaemia, hyperinsulinaemia, chronic hypertension and family history of CVD) (8), and for markers of inflammation as markers of future CVD risk after preeclampsia (9). We and others previously reported an association between elevated postpartum levels of C-reactive protein (CRP) and fibrinogen (9), as well as a high prevalence of other common CVD risk factors, among women with a history of preeclampsia. (10 – 12) Future CVD-related morbidity and mortality appears to be highest in women who experienced early-onset preeclampsia, that is, preeclampsia that required delivery before 34 weeks. These women have a three-fold higher risk of ischaemic heart disease and stroke compared to women who experienced late-onset disease. (3,13) Also, recurrent preeclampsia occurring in two or more consecutive pregnancies is associated with higher cardiovascular mortality and a higher prevalence of CVD risk factors postpartum. (14,15) In this study, we tested the hypothesis that – apart from common CVD risk factors (total cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, triglycerides, blood pressure and smoking) – acute-phase inflammatory markers (CRP, fibrinogen) contribute to the increased risk of recurrent preeclampsia in a next pregnancy following a first pregnancy complicated by early-onset preeclampsia. To this end, we conducted a prospective cohort study of 150 women included after a first pregnancy complicated by early-onset preeclampsia and studied the relationship between pre-pregnancy cardiovascular and inflammatory markers and the risk of recurrent preeclampsia.

METHODS

Study population

We conducted a prospective cohort study to include 304 women after a first pregnancy complicated by early-onset preeclampsia, who had been admitted to our Tertiary Referral Center (UMC Utrecht, the Netherlands) between1995 and 2006. Details of inclusion criteria, recruitment and study protocol have been described elsewhere. (16) In brief,

women were asked to participate by their consultant perinatologist after delivery and provided written informed consent prior to enrolment. Between 6 and 12 months postpartum, we registered demographic, general medical, obstetric data and relevant information on family history, and collected fasting blood samples for the measurement of thrombophilia-related factors and metabolic, inflammatory and lipid markers. (16,17) Women were advised to discontinue breastfeeding and intake of any vitamin and folic acid supplements, or either of them, at least 6 weeks prior to inclusion. Preeclampsia was defined as de-novo development of hypertension, that is, a DBP rise to above 90 mmHg and a SBP rise to above 140 mmHg, or either of the two, measured twice with an interval of at least 4h, accompanied by new-onset proteinuria, dipstick 2+ or more than 300 mg/24 h, according to the criteria of the International Society for the Study of Hypertension in Pregnancy (ISSHP). (18) We defined the Haemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome according to previously published criteria: a platelet count below 100 10^9/l, lactate dehydrogenase in excess of 600 U/l or haptoglobin below 0.3 g/l, and if serum aspartate aminotransferase or serum alanine aminotransferase had increased to a value above 50 U/l. (19) We considered a newborn to be small for gestational age (SGA) when birth weight was below the 10th percentile for gestational age, based on the most recent Dutch birth weight charts. (20) We defined chronic hypertension as hypertension requiring treatment with antihypertensive medication before the tar- get pregnancy. In this study cohort, we included all women with a second pregnancy continuing until after the 16th week and completed before December 2007. Only data of singleton pregnancies were considered. At the time of analysis, the study cohort included 184 (65.8%) women who had completed a second pregnancy beyond 16 weeks gestational age. There was no loss to follow-up. For the purpose of this study, we further excluded 34 women because data on inflammatory markers were unavailable. Details about pregnancy course and outcome of the second pregnancy were collected prospectively. All women received low-dose aspirin (80 mg daily) between 12 and 36 weeks of the second pregnancy as part of the standard protocol for routine care. Women with hyperhomocysteinemia used daily supplements of 5 mg folic acid with 100 mg vitamin B6 daily, also according to our hospital protocol. All participants provided informed consent and the study was approved by the Medical Research Ethics Committee of the University Medical Center Utrecht (proposal number 12 – 641).

Assessment of cardiovascular risk factors cardiovascular risk factors determined in this study were derived from the Framingham Risk Score and the Reynolds Risk Score for women. (21,22) We added fibrinogen levels as an additional marker of the acute-phase inflammatory response related to cardiovascular risk. (23) Height, weight, BMI and the presence of diabetes or chronic hypertension were registered at inclusion. We measured

blood pressure sphygmomanometrically, as the mean value of two separate measurements using the Korotkoff V cut-off point for DBP. Blood samples were collected after an overnight fast, immediately centrifuged and analyzed for lipid markers, glucose and triglyceride levels by standard procedures at the routine Clinical Chemistry Laboratory of our hospital. A description of measurements and laboratory procedures has been detailed previously. (9,12,24) In brief, high-sensitivity CRP was determined using commercially available reagents for nephelometric analysis (Dade Behring, Marburg, Germany). Fibrinogen levels were measured by the Claus' clotting method using a StaR automated coagulation analyzer with STA Fibrinogen reagent (Diagnostic Stago, Taverny, France) according to routine and extensively validated standard operating procedures used within our tertiary ISO 9001:2008 accredited diagnostic facility. Similar, fasting levels of total cholesterol, HDL-cholesterol, triglycerides and glucose were determined using a Vitros950 dry-chemistry analyzer (Johnson & Johnson, Rochester, New York, USA). LDL-cholesterol was estimated using the Friedewald formula. Within-run variation coefficients were less than 4% for all variables. Technicians were blinded for the study group and unaware of the hypothesis to be tested.

Statistical analysis

Baseline data were independently tested for their association with recurrence risk of preeclampsia. Student's t-tests were used for continuous variables and chi-square tests for categorical variables. Hazard ratios were calculated using Cox proportional hazard models, with days of gestation in second pregnancy as timescale, as previously reported. (24) Hazard ratios were adjusted for chronic hypertension and other potential confounders by multivariable Cox proportional hazard ratio analysis. The relation between CRP, fibrinogen, BMI, smoking status, diabetes, age, total cholesterol level on the one hand and recurrent preeclampsia on the other, was further analyzed by comparing consecutive tertiles, with cut-off points based on the distribution of values within the study population; hazard ratios and 95% confidence intervals (CIs) were calculated for each tertile with the lowest tertile serving as reference group using multivariable Cox proportional hazard models. Trend was evaluated using the tertiles as one independent variable (0 – 2) in a multivariable logistic regression model. Goodness-of-fit tests for trends were performed using the Hosmer – Lemeshow test and were considered acceptable at P greater than 0.05. Statistics were performed with SPSS 20.0.0 (IBM Corp.). P-values less than 0.05 were considered to indicate statistically significant differences.

RESULTS

We obtained complete follow-up for 150 participants who had completed a second pregnancy after a first pregnancy complicated by early-onset preeclampsia at a mean inter- birth interval of 2.8 years. None of the included women were lost to follow-up. Table 1 lists the baseline characteristics of the study population. Women with a history of early-onset preeclampsia are known to have a high prevalence of CVD risk factors (12), which is in line with the relatively high mean values for all CVD markers observed in this study as compared to the distribution of the Dutch population. (12,25) Table 2 shows the clinical characteristics of the first and second pregnancy. Outcome of a second pregnancy in former patients affected by early-onset pre- eclampsia was generally more favorable compared to the first pregnancy. Consequently, pregnancies complicated by preterm delivery, HELLP syndrome, placental abruption or fetal growth restriction were less common and caesarean sections were performed less often, that is, in less than half of the second pregnancies. Forty-two women had developed recurrent preeclampsia (28%) in the second pregnancy, 6(4%) of whom before 34 weeks of gestation, 10 women (6.6%) between 34 and 37 weeks and 26 women (17%) after 37 completed weeks of gestation. Five (3.3%) second pregnancies were complicated by perinatal deaths. Two neonates died due to complications of prematurity after indicated preterm delivery in pregnancies complicated by severe recurrent early-onset preeclampsia. Two infants died secondary to placental abruption and one neonate died due to sepsis following an

Table 1. Baseline Characteristics

Variable	Women with a History of Early-Onset Preeclampsia (N=150)
Age, y	30.5 (29.8–31.1)
Inter-birth interval, y	2.8 (2.6–3.0)
Postpartum, mo	10.0 (7.6–12.3)
Body-mass index, $kg \cdot m^{-2}$	26.4 (25.6–27.2)
Smoking, %	31.3
Chronic hypertension, %	33.1
Diabetes, %	1.3
Total cholesterol, mmol/L	5.20 (5.0–5.4)
HDL-cholesterol, mmol/L	1.36 (1.3–1.4)
LDL-cholesterol, mmol/L	3.20 (3.0–3.3)
Triglycerides, mmol/L	1.41 (1.3–1.6)
Glucose, mmol/L	4.87 (4.7–5.1)
C-reactive protein, mg/L	3.33 (1.8–4.9)
Fibrinogen, mg/dL	297 (271–323)

Data are presented as mean and 95% confidence interval. HDL indicates high-density lipoprotein and LDL, low-density lipoprotein.

Table 2. Characteristics of First and Subsequent Pregnancy

Variable	First pregnancy (N=150)	Subsequent pregnancy (N=150)
Gestational age at delivery, weeks	29.5 (29.1–29.9)	38.1 (37.5–38.6)
PIH, %	-	26.9
Preeclampsia, %	100	28.0
Delivery < 34 weeks gestational age, %	100	6.0
HELLP-syndrome, %	45.3	2.0
Placental abruption, %	4.7	2.7
Infant birthweight, g	1029 (955–1103)	2972 (2838–3106)
SGA <10th percentile, %	52.7	16.8
Cesarean section, %	82.0	40.1
Perinatal deaths, %	32.2	2.0

Data are presented as mean and 95% confidence interval. PIH indicates pregnancy-induced hypertension; HELLP, hemolysis, elevated liver enzymes, low platelets; SGA, small-for-gestational-age.

intrauterine infection. Mean birth weight in the subgroup of women with recurrent preeclampsia was higher than in the preceding preeclamptic pregnancy (3142 ± 713 versus 2540 ± 937 g; P < 0.001). Similar differences between women with and without recurrent preeclampsia were observed for gestational age at delivery (38.7 3.3 versus 36.4 4.0

Table 3. Comparison of Clinical Characteristics and Cardiovascular Markers among Women with a History of First Pregnancy Early-Onset Preeclampsia, with or without Recurrent Preeclampsia in Subsequent Pregnancy

Variable	Recurrent Preeclampsia (N=42)	Non-Recurrent Preeclampsia (N=108)	*P*
Age, y	30.6 (29.3–31.9)	30.4 (29.6–31.2)	.811
Inter-birth interval, y	2.5 (2.1–2.9)	2.9 (2.6–3.2)	.125
Body-mass index, kg · m^{-2}	26.7 (25.2–28.3)	26.3 (25.3–27.3)	.637
Smoking, %	27.0	32.7	.520
Chronic hypertension, %	19.0	18.5	.940
Diabetes, %	2.4	0.9	.483
Total cholesterol, mmol/L	5.41 (5.1–5.7)	5.12 (4.9–5.3)	.115
HDL-cholesterol, mmol/L	1.36 (1.2–1.5)	1.36 (1.3–1.4)	.995
LDL-cholesterol, mmol/L	3.36 (3.1–3.6)	3.13 (2.9–3.3)	.180
Triglycerides, mmol/L	1.49 (1.2–1.8)	1.38 (1.2–1.6)	.518
Glucose, mmol/L	4.78 (4.5–5.1)	4.90 (4.6–5.1)	.622
C-reactive protein, mg/L	5.89 (0.9–10.9)	2.34 (1.3–3.4)	.041
Fibrinogen, mg/dL	347 (292–403)	276 (248–303)	.010

Data are presented as mean and 95% confidence interval. HDL indicates high-density lipoprotein and LDL, low-density lipoprotein.

weeks; P = 0.001) and the incidence of small-for-gestational-age newborns (26.2 versus 13.1%; P = 0.054). Further, we found a higher rate of caesarean sections in women who had developed recurrent disease (61.0 versus 32.1%; P = 0.001). Women with and without recurrent disease showed no differences in mean maternal age, inter-birth interval, BMI and levels of total cholesterol, LDL-cholesterol, triglycerides and glucose (Table 3). Both groups were also similar with respect to current smoking status and the presence of diabetes mellitus. Former patients who had developed recurrent preeclampsia in the second pregnancy had higher mean pre-pregnancy levels of fibrinogen (347 versus 276 mg/dl; P = 0.01) and CRP (5.89 versus 2.34 mg/l; P = 0.041), when compared to women who did not develop recurrent disease. Hazard ratios and corresponding 95% CIs for recurrent preeclampsia according to tertiles of CRP and fibrinogen levels revealed a rise in the mid and high biomarker categories in a dose-effect manner up to hazard ratio 4.84 (95% CI 1.3 – 18.0) for the highest compared to the lowest tertile of CRP and hazard ratio 2.72 (1.0 – 7.2) for fibrinogen, respectively. The strength of these associations was not attenuated after adjustment for BMI, smoking status, diabetes mellitus, age and total cholesterol levels (Table 4). As shown in Fig. 1, combined stratified analysis of baseline CRP and fibrinogen levels revealed further potential improvements to predict recurrent preeclampsia in the next pregnancy with hazard ratios up to 10.8 for the highest tertiles.

Table 4. Hazard Ratios for Recurrent Preeclampsia in a Subsequent Pregnancy after Early-Onset Preeclampsia, According to Tertiles of C-Reactive Protein and Fibrinogen

		Unadjusted HR	**Adjusted HR**		
	CRP (mg/L)		Multivariable model 1*	Multivariable model 2†	Multivariable model 3‡
Low	< 0.48	1	1	1	1
Mid	0.48 – 1.68	2.3 (0.6–8.9)	2.5 (0.6–9.9)	2.7 (0.5–13.6)	2.7 (0.5–14.3)
High	> 1.68	4.8 (1.3–18.0)	4.6 (1.2–17.1)	6.8 (1.4–33.4)	5.5 (1.0–30.6)
	P trend	*0.013*	*0.019*	*0.009*	*0.012*

		Unadjusted HR	**Adjusted HR**		
	Fibrinogen (mg/dL)	Crude HR	Multivariable model 1*	Multivariable model 2†	Multivariable model 3‡
Low	< 240	1	1	1	1
Mid	240 – 330	1.6 (0.6–4.1)	1.7 (0.7–4.5)	2.4 (0.8–7.2)	2.3 (0.7–7.2)
High	> 330	2.7 (1.0–7.2)	2.8 (1.1–7.5)	4.1 (1.3–12.8)	4.2 (1.3–14.0)
	P trend	*0.031*	*0.021*	*0.008*	*0.011*

HR indicates hazard ratio. CRP, C-reactive protein.

* Adjusted for chronic hypertension.

† Additionally adjusted for body-mass index.

‡ Additionally adjusted for smoking status, diabetes, age and total cholesterol levels.

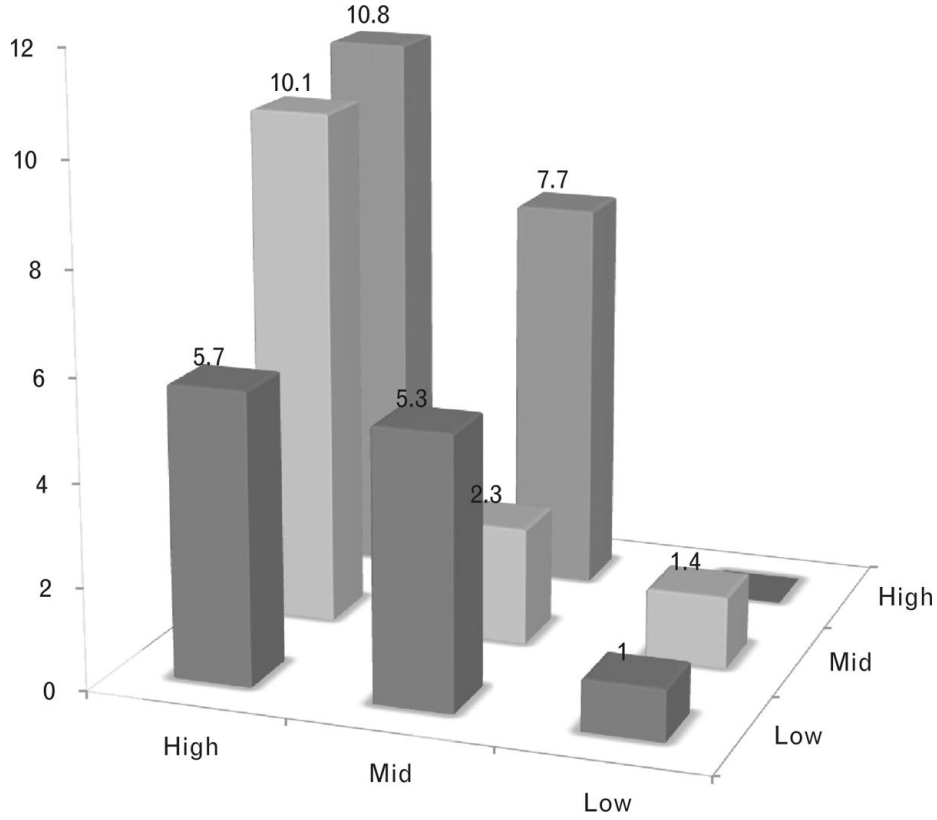

Figure 1. Hazard Ratios for Recurrent Preeclampsia in Women with First Pregnancy Early-Onset Preeclampsia According to Prepregnancy Levels of Fibrinogen (Z-axis) and C-Reactive Protein (X-axis).

DISCUSSION

In this prospective cohort study among primiparae with a history of early-onset preeclampsia, we found two markers of systemic acute-phase inflammation measured at 6 – 12 months postpartum (CRP and fibrinogen) that are associated with recurrent preeclampsia in a next pregnancy. Although women with a history of early-onset preeclampsia are more likely to have an abnormal lipid profile and altered glucose metabolism after delivery (12), we found no consistent differences in postpartum levels of total, HDL and LDL-cholesterol or triglycerides between women who did or did not develop recurrent disease. The observed associations between fibrinogen, CRP and recurrent preeclampsia could not be explained by differences in age, chronic hypertension, BMI, incident diabetes and smoking, or by interaction with lipid levels. Of interest and in line with previous studies (2,24), clinical parameters of first pregnancies complicated by early- onset preeclampsia such as gestational age at delivery, the presence of HELLP syn-

drome, placental abruption, fetal growth restriction, perinatal death or mode of delivery, were not related to recurrent preeclampsia in a next pregnancy. In contrast to previous findings in women with a history of late-onset preeclampsia (15), in our data, recurrence risk was not associated with BMI and markers of abnormal lipid or glucose metabolism. Also, as opposed to previous studies in late-onset or mild preeclampsia, the associations with raised levels of inflammatory markers observed in our study were not attenuated by adjustment for BMI (26,27). This may indicate that early-onset preeclampsia is related to an innate pro-inflammatory phenotype of the mother (9), rather than to inflammation as surrogate marker for acquired weight gain or excessive body fat.

Some limitations to this study merit consideration. First, data were only those of primiparae with a history of early-onset disease. Therefore, the findings of our study cannot be extrapolated to women with late-onset disease or multiparas. Second, we measured CRP and fibrinogen as robust markers of the acute-phase inflammatory response known to be predictive of CVD risk. Although many studies suggest a central role for the inflammatory system in the pathogenesis of early-onset preeclampsia, an exact mechanism for development of preeclampsia remains uncertain and may well involve multiple other components of the inflammatory system, such as interleukin-6, tumor necrosis factor- alpha, von Willebrand factor and components of the complement system. Third, we cannot rule out any temporary effects of pregnancy itself on CRP and fibrinogen levels measured at 6 – 12 months postpartum. However, this seems unlikely as baseline levels of CRP and fibrinogen have a strong hereditary component and are relatively stable over time. (28,29) Finally, although we adjusted for known confounders including BMI, chronic hypertension, smoking, diabetes and lipid levels, any effect of other unknown confounders (such as routine aspirin use, anti- hypertensive agents and other types of medication) on the associations of CRP and fibrinogen with recurrent preeclampsia cannot be fully excluded. Also, the a priori risk of CVD and indeed the prevalence of most cardiovascular risk factors in the Dutch population is relatively low compared to some other background populations (such as the UK and North America), hence caution should be applied when extrapolating our results to other populations (e.g. to populations with higher obesity rates) as the effect sizes may vary.

The results of this study have several implications. First, the findings reveal that markers of chronic low-grade inflammation are related to recurrent severe hypertensive complications of pregnancy. (9,26,30 – 32) From a pathophysiological perspective, our data support the view that an underlying maternal pro-inflammatory phenotype may be relevant to the development of early-onset preeclampsia and could be a potential target for risk assessment and intervention. (33,34) Second, our data are relevant to the previous observation that women who experience pre-eclampsia in more than one pregnancy are more likely to develop CVD, than women who experience preeclampsia in the first pregnancy only. (3) Although follow-up of women in this study was not

continued beyond the second pregnancy, it is likely that the underlying maternal pro- inflammatory phenotype associated with recurrent hypertensive complications of pregnancy will remain present after the second delivery as a potential long-term risk factor for premature cardiovascular morbidity later in life. In this respect, recent strategies aimed at lowering cardiovascular risk in women with mildly raised levels of baseline inflammatory markers (CRP >2 mg/l), but without any other known cardiovascular or lipid risk factors (35), may prove to be particularly relevant for women with a history of recurrent preeclampsia.

In summary, in this follow-up study of cardiovascular risk markers in non-pregnant women with a first pregnancy complicated by early-onset preeclampsia, we demonstrated that CRP and fibrinogen levels measured 6 – 12 months postpartum are closely related to recurrent preeclampsia in the next pregnancy. Increased levels of inflammatory markers may partly explain the shared predisposition to recurrent preeclampsia and the development of atherosclerosis later in life.

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Chapter 8

Maternal Cardiovascular Risk Profile After Placental Abruption

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ABSTRACT

The prevalence of premature cardiovascular diseases (CVD) is increased in women with a history of maternal placental syndromes, including pregnancy-associated hypertensive disorders (eg, preeclampsia), fetal growth restriction, and placental abruption. Whereas previous studies have shown a high prevalence of CVD risk factors after pregnancies complicated by preeclampsia, this has not been studied for women with a history of placental abruption. To explore the association of placental abruption with CVD risk factors after delivery, we compared 75 women with a history of placental abruption with a control group of 79 women with uneventful pregnancies at 6 to 9 months postpartum for the presence of common CVD risk factors. In a subanalysis, data were stratified according to the presence or absence of concomitant hypertensive disease and further adjusted for potential confounders. Women with previous placental abruption had significantly higher mean systolic blood pressure, body-mass index, fasting blood glucose, C-reactive protein, total cholesterol, high-density lipoprotein-cholesterol, and low-density lipoprotein-cholesterol as compared with controls with only uneventful pregnancies. In the subanalysis, all differences remained significant for women with a history of placental abruption only (ie, without concomitant gestational hypertension), except for the associations with low-density lipoprotein-cholesterol and diastolic and systolic blood pressure. Most likely, the identified CVD risk factors predispose to placental abruption and development of premature CVD later in life.

INTRODUCTION

Several large-scale population studies have found a strong link between pregnancy-specific disorders linked to abnormal development of the placental bed, such as preeclampsia Hemolysis, Elevated Liver enzymes and Low Platelet count syndrome (HELLP), gestational hypertension, placental abruption, placental infarction, and fetal growth restriction, also known as maternal placental syndromes (MPS), and subsequent maternal risk of premature cardiovascular disease (CVD). (1–4) MPS may be considered as a cluster of pregnancy-related disorders that appear when the cardiovascular system fails to adapt to the increased metabolic, inflammatory, and hemodynamic demands during pregnancy and represent the first manifestation of compromised cardiovascular health of the mother. (5,6)

Placental abruption, the separation of the placenta before delivery, is a serious complication of pregnancy, associated with a high morbidity and mortality for both mother and child. Placental abruption complicates ≈1% of pregnancies. (7) The precise pathogenesis of placental abruption is unclear, although recent studies suggest an important role for defective development remodeling of uterine spiral arteries. This may lead to inadequate blood and nutrient supply to the placenta in the first and second trimesters of pregnancy preceding subsequent placental abruption. Data from placental bed biopsy studies obtained in women with placental abruption show a higher prevalence of abnormal spiral artery remodeling, decidual thrombosis, inflammation, and intimal and subintimal thickening (so-called acute atherosis lesions) than that observed in normal pregnancy. (7–11) Although the pathophysiology of these characteristic vascular abnormalities is not well understood, intriguing similarities exist in the vascular biology of early-stage atherosclerosis preceding most CVD. (12)

For preeclampsia and fetal growth restriction, previous studies on postpartum CVD risk factors revealed a higher prevalence of multiple modifiable risk factors for CVD within the first year after delivery. (5,13,14) However, to date this has not been separately studied for women with previous placental abruption. In this study, we assessed common CVD risk factors in women with a history of placental abruption at 6 to 12 months after delivery, in comparison with a control group of women with a history of only uneventful pregnancies.

METHODS

Study Population

All women with a pregnancy complicated by placental abruption who delivered at the University Medical Center Utrecht, The Netherlands, between November 1994

and December 2009 were considered to be eligible for inclusion. Placental abruption was diagnosed as the following: retroplacental bleeding or clots at cesarean section, sonographic visualization of abruption, or a combination of abdominal pain or vaginal bleeding accompanied by a nonreassuring fetal heart rate trace, uterine hypertonicity, or as evident signs of placental abruption on histopathologic examination. (11) Patients with chronic hypertension, that is, using antihypertensive treatment for known chronic hypertension before pregnancy and patients with traumatic injury before hospital admission were excluded. Preeclampsia was defined as the presence of gestational hypertension and concomitant proteinuria in the second half of pregnancy. Gestational hypertension was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy as diastolic blood pressure >90 mm Hg and systolic blood pressure >140 mm Hg, measured on ≥2 separate occasions ≥4 hours apart. Proteinuria was diagnosed with urinary protein ≥300 mg per 24 hour or >2+ at dipstick urinalysis. (15) Infants were considered small-for-gestational age if the birth weight was below the fifth centile based on standardized Dutch population charts. (16) Women with abruption were divided into 2 groups: abruption with or without concomitant MPS in the index pregnancy or obstetric history. The control group was recruited from the same background population as cases and consisted of healthy women who had experienced only uncomplicated pregnancies. Control women were randomly selected and asked to participate by the research team in collaboration with the low-risk primary care antenatal clinic of the University Medical Center Utrecht and at a local midwifery practice nearby, within the same referral population as the cases to prevent selection bias. Control subjects were recruited according to the same inclusion protocol, were enrolled by the same research team, and were subject to identical sample handling and laboratory procedures as the cases. None of the women had a subsequent pregnancy at screening, and all stopped breastfeeding ≥6 weeks before screening. The study was approved by the Institutional Review Board of the University Medical Center Utrecht, and all participants provided written informed consent.

Assessment of Classic CVD Risk Factors

CVD risk factors were assessed 6 to 9 months after delivery. Breast-feeding and vitamin or folic acid supplements were discontinued ≥6 weeks before the risk assessment. The presence of diabetes mellitus and smoking were recorded, and body-mass index (BMI) was calculated, using self-reported height and measured weight at inclusion. A trained research nurse measured blood pressure by auscultatory sphygmomanometer, using an aneroid sphygmomanometer with normal cuff size, in sitting position. Diastolic blood pressure values were determined using the fifth Korotkoff sound. Where appropriate, cuff sizes were adjusted to arm circumference. The mean value of 2 separate measurements 30 minutes apart was used for analysis. All fasting venous blood samples were im-

mediately centrifuged and analyzed directly for lipid markers, glucose, and triglyceride levels by standard operating procedures at the routine Clinical Chemistry Laboratory of our hospital. A detailed description of measurements and laboratory procedures was previously published elsewhere. (5,17) Briefly, fasting total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, and glucose were determined using a Vitros950 dry-chemistry analyzer (Johnson & Johnson, Rochester, NY). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula. The Homeostatic Model Assessment (HOMA) score was used to calculate the level of insulin resistance with the following equation: (fasting glucose×fasting insulin)/22.5. Within-run variation coefficients were 1.7% for total cholesterol, 2.3% for HDL-cholesterol, 1.9% for triglycerides, and 4.3% for fasting glucose levels. Technicians were blinded for outcome.

Statistical Methods

Statistical analyses were performed using Statistical Package for the Social Sciences (version 17.0 SPSS Inc, Chicago, IL). Baseline variables in the group with and without a previous abruption were expressed as means with 95% confidence intervals, or number and percentage. Statistical comparison was performed using generalized linear models. In the original data set, several women had missing data, and for some variables selective missing may have occurred. Average missing rate per variable was 17% in controls (confidence interval, 10–25) and 20% in cases (confidence interval, 10–35). To avoid any potential bias that may occur in complete-case analysis, multiple imputations (20×) were applied using observed patient characteristics. (18,19) Missing data were imputed using a logistic regression model that included the following variables: maternal age, BMI, nulliparity, blood pressure, glucose, insulin, high sensitive C-reactive protein, triglycerides, and cholesterols. Generalized linear models were used to analyze the data in each imputation set separately, before pooling the data using Rubin's rules. (20)

For subgroup analyses, the patient population was stratified into abruption without additional MPS (ie, preeclampsia/HELLP, gestational hypertension, or small-for-gestational age) and abruption with additional MPS. Where appropriate, variables were adjusted for potential confounders that were identified in the baseline comparison. $P<0.05$ were considered statistically significant. In addition, several parameters were dichotomized using the common cutoff values for metabolic syndrome, or those described in the JUPITER trial, and were subsequently compared between the groups by $\chi 2$ test. (21)

Results

Seventy-five women with placental abruption and 79 population-based controls were included in the analysis. Baseline characteristics are summarized in Table 1. Women in the control group were slightly older compared with the cases, with a mean difference in age of 2.2 years. Placental abruption was associated with a 63% rate of concomitant

gestational hypertension, preeclampsia, and intrauterine growth restriction (47 cases). Of the 28 women without MPS in the index pregnancy, 8 women were multiparous. None had MPS in their obstetric history. Women with previous placental abruption had significantly higher mean BMI, systolic blood pressure, fasting blood glucose, total cholesterol, HDL-cholesterol, and LDL-cholesterol than women in the control group (Table 1). No significant differences were found for diastolic blood pressure, cholesterol/HDL ratio, triglycerides, high sensitive C-reactive protein, and HOMA score.

Subgroup analysis between multiple determinants of CVD risk and a history of placental abruption is shown in Table 2. After adjustment for age, BMI, and nulliparity, the associations between increased systolic blood pressure, fasting blood glucose, total cho-

Table 1. Baseline Patient Characteristics of Controls and Placental Abruption Cases

Parameter	Control Group n=79	Placental Abruption n=75
Age	33,1 (32.9-33.4)	30.9 (30.7-31.1)*
BMI (kg/m^2)	22.8 (21.8-23.7)	25.7 (24.8-26.7)*
White (%)	79 (100)	73 (97.3)
Nulliparity (%)	47 (59.5)	58 (77.3)*
Smoking (%)	14 (17.7)	18 (24)
Pregnancy outcome Gestational age at delivery (days)	282 (7.5)	207 (37.8)*
Infant's birth weight	3592 (467)	1024 (619)*
Small-for-gestational age (%)	1 (1.3)	22 (29,3)*
Gestational hypertension (%)	-	11 (14,7)
Pre-eclampsia (%)	-	31 (41,3)
Systolic blood pressure (mmHg)	113 (110-116)	122 (117-126)*
Diastolic blood pressure (mmHg)	75 (72-77)	80 (76-83)
Fasting glucose (mmol/L)	4.11 (3.9-4.3)	5.09 (4.9-5.3)*
Fasting Insulin (ulU/L)	12.35 (9.2-15.4)	10.75 (9.4-12.1)
HOMA score	1.97 (1.5-2.5)	2.43 (2.2-2.7)
hsCRP (mg/L)	1.59 (0.6-2.6)	4.36 (1.6-7.1)
Triglycerides (mmol/L)	1.08 (0.9-1.3)	1.2 (1.1-1.4)
Cholesterol (mmol/L)		
Total cholesterol	3.76 (3.5-4.0)	5.01 (4.7-5.3)*
HDL	1.22 (1.1-1.3)	1.44 (1.1-1.3)*
LDL	2.06 (1.8-2.3)	3.00 (2.8-3.2)*
Cholesterol/HDL ratio	3.40 (3.0-3.8)	3.71 (3.4-4.1)

Data are presented as mean and 95% confidence interval (CI) hsCRP: high sensitive C-reactive protein; HDL: high-density lipoprotein; LDL: low-density lipoprotein; HOMA: Homeostatic Model Assessment. * p<0.05

lesterol, HDL-cholesterol and LDL-cholesterol levels, and a history of placental abruption remained significant for both the subgroup of women with a placental abruption and concomitant MPS, as well as for women with placental abruption without concomitant MPS, except for blood pressure and LDL cholesterol

Table 3 shows stratified results according to clinical cutoff values used in the JUPITER trial and according to established criteria for components of the metabolic syndrome. Significant differences were observed for all CVD risk factors between women who experienced placental abruption with other concomitant MPS as compared with the control group, Similar, for placental abruption without MPS differences remained significant except for the difference in glucose levels.

Table 2. Determinants of Cardiovascular Risk in Placental Abruption Cases with or without concomitant MPS and Controls

Parameter	Control Group n=79	Placental abruption with concomitant MPS n=47	Placental Abruption n=28
Systolic blood pressure (mmHg)	115 (111-118)	122 (117-127)*	118 (110-125)
Diastolic blood pressure (mmHg)	75 (72-78)	80 (77-84)	78 (72-83)
Fasting glucose (mmol/L)	4.13 (3.9-4.3)	5.10 (4.8-5.4)*	5.02 (4.7-5.4)*
Fasting Insulin (uIU/L)	12.96 (10.2-15.8)	9.79 (8.1-11.5)	8.14 (6.0-10.3)
HOMA score	2.23 (1.8-2.7)	2.32 (2.0-2.6)	1.90 (1.5-2.3)
hsCRP (mg/L)	1.95 (0.9-3.0)	3.33 (1.3-5.4)	5.07 (0.4-9.8)
Triglycerides (mmol/L)	1.18 (1.0-1.4)	1.26 (1.0-1.5)	0.98 (0.7-1.3)
Cholesterol (mmol/L)			
Total cholesterol	3.87 (3.6-4.2)	5.06 (4.7-5.4)*	4.77 (4.3-5.3)*
HDL	1.16 (1.1-1.3)	1.44 (1.3-1.6)*	1.60 (1.4-1.8)*
LDL	2.19 (1.9-2.4)	3.05 (2.7-3.4)*	2.73 (2.3-3.1)
Cholesterol/HDL ratio	3.64 (3.2-4.1)	3.64 (3.1-4.2)	3.15 (2.4-3.9)

Data are presented as mean and 95% confidence interval (CI), adjusted for age, BMI and nulliparity.
hsCRP: high sensitive C-reactive protein; HDL: high-density lipoprotein; LDL: low-density lipoprotein; HOMA: Homeostatic Model Assessment. MPS: Maternal Placenta Syndrome i.e. PE/HELLP, gestational hypertension or SGA. * $p<0.05$ vs. control

DISCUSSION

This study demonstrates an association between placental abruption and increased prevalence of CVD risk factors several months after delivery. Women with a history of placental abruption seem to have a different CVD risk profile compared with women with a history of only uncomplicated pregnancies. Blood pressure, BMI, fasting blood

Table 3. Cut off values used in JUPITER trail and Metabolic Syndrome in Placental Abruption Cases with or without concomitant MPS and Controls

Cut off values	Control Group n=79	Placental abruption with concomitant MPS n=47	Placental Abruption n=28
hsCRP >2mg/ml	15 (19%)	21 (45%)*	10 (36%)*
LDL cholesterol >1.8 mmol/L	47 (59%)	45 (96%)*	26 (93%)*
HDL cholesterol <1.29 mmol/L	53 (67%)	19 (40%)*	12 (43%)*
BMI>30 kg/m^2	3(4%)	6 (13%)*	7 (25%)*
Triglycerides >1.7 mmol/L	10 (13%)	8 (17%)*	5 (18%)*
Glucose >5.6 mmol/L	5 (6%)	5 (11%)*	3 (11%)

Data are presented as number and percentage.
hsCRP: high sensitive C-reactive protein; HDL: high-density lipoprotein; LDL: low-density lipoprotein; MPS: Maternal Placenta Syndrome i.e. PE/HELLP, gestational hypertension or SGA. * p<0.001 vs. control

glucose, total cholesterol, and LDL-cholesterol are significantly higher in women after placental abruption compared with population-based controls.

Previous studies have shown that preeclampsia and small-for-gestational age are associated with CVD risk factors early in life and an increased risk of future CVD. (2,3,13,14,22) Even the 10-year CVD risk in women with a history preeclampsia is significantly higher with an odds ratio of 1.31 according to the Framingham risk score. (23) Therefore, in our study, the presence of concomitant MPS may be considered as a potential confounder for the association between placental abruption and subsequent CVD risk factor levels. However, with the exception of blood pressure and LDL cholesterol, subgroup analysis of cases in our study without concomitant MPS demonstrated a virtually unaltered significant difference in CVD risk profiles compared with healthy controls. This is also reflected in Table 3, where several cutoff points were used. Next to the metabolic syndrome cutoff values, we chose to use the JUPITER trial criteria to estimate clinically relevant cutoff points for this population of young apparently healthy women. Hence, the fact that this apparently healthy group of men and women with only mildly elevated high sensitive C-reactive protein (>2.0 mmol/L) and <1.8 mmol/L LDL values after rosuvastatin treatment seemed to have improved event free survival. Placental abruption seems to be independently related to the presence of CVD risk factors ≥6 months after delivery, irrespective of concomitant MPS in the index pregnancy.

Several studies have shown a strong correlation between placental lesions and placental abruption. (9,10) It has been hypothesized that placental abruption results from poor vessel quality of placental spiral arteries in women who are already predisposed to CVD. (1–4) Defective spiral artery remodeling is assumed to cause underperfusion of the placental bed with subsequent infarction and increased resistance of the placental vessels. (11,24,25) Specific decidual vasculopathy like muscular thickening, thrombosis,

and acute atherosis lesions possibly arise as response to increased sheer stress and are indeed seen more often in cases of placental abruption and other MPS. (10,11) In this concept, pregnancy acts as a metabolic stress test revealing poor cardiovascular health in women with a pregnancy complicated by an MPS, such as placental abruption. (6,26)

Of note, HDL-cholesterol levels were mildly lower in the control group. Because HDL-cholesterol is shown to be inversely related to the CVD risk in several epidemiological studies, we expected levels to be lower in women with a history of placental abruption. (27–30) It is difficult to explain this finding. HDL levels are known to be higher in women and show a temporal decline with increasing age; thus, one may speculate that increased HDL-cholesterol levels observed in women with previous placental abruption may (temporarily) protect them against early atherogenesis, despite alterations in other lipid parameters and metabolic disturbances. (31)

Some limitations of this study need to be addressed. First, controls were significantly older than women with previous placental abruption. However, this attenuates rather than explains the differences in CVD risk factors between cases and controls because advanced age is associated with an increment in CVD risk factor levels. We estimate this effect to be rather small because age-adjusted logistic regression models had virtually no effect on the observed associations. Second, although to date our study represents the first data set on CVD risk factors after placental abruption, for some outcomes in the stratified subanalysis, our sample size had limited power to draw any definitive conclusions. Third, our data were collected several months after delivery. It is not certain that abnormal risk profiles were already present before pregnancy in women who experienced placenta abruption. However, because the minimum delivery-to-assessment interval was 6 months, we assume that the levels of blood pressure and all biochemical risk factors had returned to prepregnancy values. Furthermore, there is no evidence that maternal weight decreases more or faster after normal pregnancy as compared with pregnancy complicated by placental abruption.

In spite of the higher prevalence of CVD risk factors in women with previous placental abruption, the estimated absolute CVD risk is low for most women during the first years after delivery. Nevertheless, this is mainly attributable to the young age of the study population masking the long-term impact of a positive history of placental abruption on CVD risk. Because the CVD risk profile is already significantly different in these young and apparently healthy women without known CVD shortly after delivery, the observed alterations in CVD risk profiles are likely to precede the appearance of clinically relevant metabolic abnormalities and signs of accelerated development of atherosclerosis in some of these women, leading to premature development of CVD later in life. (1–4)

Clinical Perspective

Evidence exist that CVD is largely preventable by early modification of CVD risk factors. (32) However, the first presentation of CVD usually does not occur before menopause, making it difficult to identify women at risk for future CVD. The presence of modifiable risk factors in women with a history of multiple placental syndromes, including placental abruption, may therefore be of potential use for primary prevention programs. Currently, CVD follow-up of women with a history of placental abruption or other MPS is not routine practice in The Netherlands and is largely clinic dependent. At present, very few clinics worldwide have started such cardiovascular risk assessment programs. (33,34) The American Heart Association updated the guideline for the prevention of CVD in women in 2011 in which they recognized preeclampsia, gestational diabetes mellitus, and pregnancy-induced hypertension as independent risk factors for CVD. (35) The update emphasizes referring these women to a primary care physician or cardiologist in the years after pregnancy. Recently, Spaan et al (33) suggested a structured cardiovascular screening program for these women by multidisciplinary teams, including an obstetrician. Our findings suggest that such multidisciplinary routine assessment and reduction of CVD risk factors may also be offered to women with placental abruption in the future. However, as for the hypertensive disorders of pregnancy, the feasibility and clinical and cost-effectiveness of such a strategy of screening and preventive interventions in women who experienced placenta abruption must be evaluated before wide implementation in clinical practice.

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Chapter 9

Cardiovascular disease risk factors after early-onset preeclampsia, late-onset preeclampsia, and pregnancy-induced hypertension

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ABSTRACT

Observational studies have shown an increased lifetime risk of cardiovascular disease (CVD) in women who experienced a hypertensive disorder in pregnancy. This risk is related to the severity of the pregnancy-related hypertensive disease and gestational age at onset. However, it has not been investigated whether these differences in CVD risk factors are already present at postpartum cardiovascular screening. We evaluated postpartum differences in CVD risk factors in 3 subgroups of patients with a history of hypertensive pregnancy. We compared the prevalence of common CVD risk factors postpartum among 448 women with previous early-onset preeclampsia, 76 women with previous late-onset preeclampsia, and 224 women with previous pregnancy-induced hypertension. Women with previous early-onset preeclampsia were compared with women with late-onset preeclampsia and pregnancy-induced hypertension and had significantly higher fasting blood glucose (5.29 versus 4.80 and 4.83 mmol/L), insulin (9.12 versus 6.31 and 6.7 uIU/L), triglycerides (1.32 versus 1.02 and 0.97 mmol/L), and total cholesterol (5.14 versus 4.73 and 4.73 mmol/L). Almost half of the early-onset preeclampsia women had developed hypertension, as opposed to 39% and 25% of women in the pregnancy-induced hypertension and late-onset preeclampsia groups, respectively. Our data show differences in the prevalence of common modifiable CVD risk factors postpartum and suggest that prevention strategies should be stratified according to severity and gestational age of onset for the hypertensive disorders of pregnancy.

INTRODUCTION

Cardiovascular disease (CVD) has gained interest in obstetrics in recent years because large observational studies revealed a remarkable increase in the long-term risk of CVD in women who experienced different types of gestational hypertensive disorders. (1–3) These include pregnancy-induced hypertension (PIH) and preeclampsia, which affect 2% to 7% of all pregnancies worldwide. (4)

A review by Bellamy et al (1) showed an increase of the postpartum risk of CVD events according to the severity of the hypertensive pregnancy disorder, with the highest risk in women who experienced early-onset preeclampsia. Women with a normal pregnancy have an advantage according to these results, but still develop CVD later in life. Currently, it is not possible to identify individual women who have the highest risk in developing CVD. In recent years, other studies did reveal that common modifiable risk factors such as fasting blood glucose and lipid levels are significantly elevated 6 months after a pregnancy complicated by early-onset preeclampsia. (5–8) The dose–response relationship with the severity of a hypertensive pregnancy disorder and future CVD suggest that the differences in long-term CVD risk between women with a history of a hypertensive pregnancy may be dependent on variation in the underlying maternal CVD risk profiles. However, studies that compare cardiovascular risk factors between women with a previous pregnancy complicated by early-onset preeclampsia, late-onset preeclampsia, or PIH within the same population are lacking.

In this study, we compare CVD risk profiles >=3 months postpartum between women with previous early-onset preeclampsia, term preeclampsia and term gestational hypertension. We hypothesize that there is a difference in the prevalence of modifiable CVD risk factors postpartum between women with a history of a hypertensive disorder of pregnancy. Identification of women at high risk of CVD at a relatively young age may provide an opportunity for early-personalized follow-up and prevention.

METHODS

Study Population

The study population consists of data from 2 cohorts: the Utrecht cohort and patients who participated in the Hypitat Risk Assessment Study (HyRAS). Both are discussed separately below.

Utrecht Cohort

All women delivered at the University Medical Center Utrecht between November 1994 and January 2012, with a pregnancy complicated by early-onset preeclampsia were

asked to participate in a follow-up program starting >=3 months postpartum. Patients with chronic hypertension, defined as known hypertension who required antihypertensive treatment before pregnancy, as well as chronic kidney disease and diabetes mellitus, were excluded. Characteristics of the study population and inclusion criteria have been published in detail elsewhere. (8,9) For this analysis, women with an interval of >5 years between delivery and follow-up were excluded to ensure comparability with the HyRAS cohort.

HyRAS Cohort

Between June 2008 and November 2010, 306 women who had participated in the Hypertension and Preeclampsia Intervention Trial at Term (HYPITAT) study were enrolled in the HyRAS study between 2 and 5 years postpartum. Details on inclusion criteria of this cohort study have been published elsewhere. (10) In short, women who have participated in the HYPITAT trial (11) were consented for the HyRAS study, a cardiovascular follow-up 2 to 5 years after their pregnancy. The HYPITAT study evaluated if induction of labor between 36+0 and 41+0 weeks of gestation improved maternal outcome in women with late-onset preeclampsia and PIH. Exclusion criteria of the HYPITAT study included: diabetes mellitus, gestational diabetes mellitus needing insulin treatment, renal disease, heart disease, previous caesarean section, hemolysis elevated liver enzymes and low platelets syndrome, oliguria of <500 mL/24 h, pulmonary edema or cyanosis, HIV seropositivity, use of antihypertensive drugs before pregnancy, fetal anomalies, suspected intrauterine growth restriction, abnormalities detected during fetal heart rate monitoring, and postpartum preeclampsia. Seventy-six of these women had late-onset preeclampsia and 230 women had PIH; all women were >=18 and delivered between 36+6 and 41+0 weeks of gestation. Women with a history of preeclampsia were excluded from the PIH group. The patient selection of this study is represented in Supplemental Figure 1.

Definitions

Preeclampsia was defined as the presence of PIH and concomitant proteinuria in the second half of the pregnancy. PIH was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy as diastolic blood pressure >90 mm Hg and/or systolic blood pressure >140 mm Hg, measured on >=2 separate occasions >=4 hours apart. Proteinuria was diagnosed with urinary protein was >300 mg/24 h or >=2 at dipstick urinalysis. (12)

Homeostasis Model Assessment score (fasting plasma glucose [mmol/L]×fasting insulin levels [mU/L]/22.5) was used as a measurement for insulin sensitivity, where higher values correspond with higher insulin resistance. (13)

Small for gestational age offspring was defined as birth weight below the 10th percentile according to the most recent growth charts used in the Netherlands. (14) Hypertension at inclusion was defined as systolic blood pressure >=140 mm Hg or diastolic blood pressure >=90 mm Hg or taking antihypertensive drugs as described in The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. (15)

Metabolic syndrome was defined according to the International Diabetes Foundation as body mass index (BMI) >30 kg/m2 and >=2 of the following: triglycerides >=1.7 mmol/L, high-density lipoprotein cholesterol <1.3 mmol/L, systolic blood pressure >=130 mm Hg or diastolic blood pressure >=85 mm Hg, and fasting plasma glucose levels >=5.6 mmol/L. (16) The cutoff points total cholesterol >6.2 mmol/L, low-density lipoprotein cholesterol >1.8, and high-sensitive C-reactive protein (hsCRP) >2.0 mg/mL were based on the Adult Treatment Panel III guidelines and Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) trial. (17,18)

Assessment of Classic CVD Risk Factors

The 3 study groups were compared for baseline characteristics, pregnancy outcome, and CVD risk factors postpartum, including BMI, blood pressure, plasma lipids, glucose, insulin, Homeostasis Model Assessment score, hsCRP, smoking status, and current hypertension or diabetes mellitus. In all groups, breastfeeding was stopped >=6 weeks before inclusion.

Both the assessment of risk factors in the early-onset preeclampsia group as well as in the late-onset preeclampsia and PIH group have been described in detail elsewhere. (8–10) Study protocols on assessment of risk factors were the same in both hospitals. In summary, BMI was calculated using measured height and weight at inclusion. A trained research nurse measured blood pressure by auscultatory sphygmomanometry, using an aneroid sphygmomanometer, in sitting position. Diastolic blood pressure values were determined using the fifth Korotkoff sound. Where appropriate, cuff sizes were adjusted to arm circumference. The mean value of 2 separate measurements 30 minutes apart was used for analysis.

Interassay Variations

After centrifuging the blood samples at the local center, samples were sent to the routine clinical chemistry laboratory of the Medical Center Haaglanden (late-onset preeclampsia/PIH groups) or University Medical Center Utrecht (early-onset preeclampsia group). Protocols for blood analysis used by the laboratories were comparable and all technicians were blinded for outcome. Details about the assays used and their mutual comparability are presented in Supplemental Table 1. Results from the different assays were harmonized with the use of annual data from the Dutch Foundation for Quality

Assessment in Clinical Laboratories (SKML) from 1994 to 2013. This independent foundation uses assays from different laboratories to measure the same samples during 6 annual measuring rounds. As a result, accordance between centers is assured and the quality of laboratory performance is monitored.

Outcome Measures

The primary outcome measure was the difference in modifiable classic cardiovascular risk factors, that is, blood glucose, insulin, cholesterol, triglycerides, and hsCRP. Secondary outcome measures were the presence of hypertension and use of antihypertensive drugs.

Statistical Methods

All statistical analyses were performed using PASW statistics 21.0, SPSS Inc.

Average missing rates per variable were 15% in women with early-onset preeclampsia (95% confidence interval, 1%–28%), 5% (95% confidence interval, 2%–11%) in women with late-onset preeclampsia, and 5% (95% confidence interval, 2%–12%) in PIH, respectively. To avoid any potential bias that may occur in complete-case analysis, we used a previously described multiple imputation method (10×) using the observed patient characteristics. (19,20) Missing data were imputed using a logistic regression model that included the following variables: follow-up time, group, smoking, maternal age, BMI, nulliparity, blood pressure, glucose, insulin, hsCRP, triglycerides, and cholesterol.

For all parameters, mean and SE or numbers and percentages were calculated. Baseline characteristics were compared using ANOVA for continuous variables and chi^2 tests for categorical variables. Generalized linear models were used to compare the cardiovascular risk factors between early-onset preeclampsia, late-onset preeclampsia, and PIH. Variables were adjusted for age, BMI, and nulliparity. $P<0.017$ were considered to indicate statistical significance and were adjusted with the Bonferroni correction for multiple testing.

Ethical Approval

All women gave written informed consent for participation in the studies. The Institutional Review Boards of the participating hospitals and the local ethics committees approved both the original studies. HyRAS is a follow-up of the HYPITAT study (trial registration: ISRCTN08132825). The Utrecht cohort is registered under Institutional Review Board number 12–641/C. The study adhered to the principles of the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001.

RESULTS

Baseline characteristics are shown in Table 1. Maternal age, percentage of non-whites, gestational age, and birth weight were significantly lower in women who had early-onset preeclampsia when compared with women with late-onset preeclampsia and PIH. Furthermore, nulliparity and percentage of small for gestational age offspring were lower when women with early-onset preeclampsia were compared with PIH. BMI was higher in women with a history of PIH compared with women who experienced early-onset preeclampsia during pregnancy. Follow-up time was significantly different between the Utrecht cohort (mean, 213 days [SE, 10.4]) and HyRAS cohort (late-onset preeclampsia: mean, 919 days [SE, 19.7] and PIH: mean, 921 days [SE, 11.1]).

The percentage of women with hypertension at time of screening varied from 25% in the late-onset preeclampsia group to 39% in PIH and 45% in the early-onset preeclampsia group, respectively. The use of antihypertensive drugs varied from 9% to 16% and 21% in women with late-onset preeclampsia, PIH, and early-onset preeclampsia, respectively. However, the difference between late-onset preeclampsia and PIH was nonsignificant for the use of hypertensive drugs.

Table 1. Baseline characteristics of the study population

Parameter	Early-onset PE	Late-onset PE	PIH	Early vs. Late	Early vs. PIH	Late vs. PIH
	n=448	n=76	n=224			
Characteristics at postpartum screening						
Age (years)	31.5 (0.23)	34.2 (0.63)	33.5 (0.34)	**<0.001**	**<0.001**	0.271
BMI (kg/m^2)	26.2 (0.26)	26.6 (0.72)	28.0 (0.35)	0.565	**<0.001**	0.045
Caucasian	425 (94.9%)	69 (90.7%)	202 (90.2%)	**0.014**	**<0.001**	1.000
Smoking	72 (16.1%)	11 (14.5%)	30 (13.4%)	0.865	0.355	0.841
Hypertension	201 (44.9%)	19 (25.0%)	88 (39.3%)	**0.002**	**0.017**	**0.016**
Antihypertensive medication	94 (20.9%)	7 (9.2%)	35 (15.6%)	**0.017**	0.023	0.085
Time interval pregnancy-screening (days)	213 (10.4)	919 (19.7)	921 (11.1)	**<0.001**	**<0.001**	0,935
Number of women with pregnancies between index and screening	6 (1.3%)	24 (31%)	63 (28%)	**<0.001**	**<0.001**	0.661
Pregnancy (index)						
Nulliparity	374 (83.5%)	59 (77.6%)	164 (73.2%)	0.251	**0.002**	0.544
Gestational age (days)	206.5 (0.84)	272.8 (1.11)	276.4 (0.55)	**<0.001**	**<0.001**	**0.002**
Birth weight (kg)	1042 (19.5)	3146 (54.7)	3482 (34)	**<0.001**	**<0.001**	**<0.001**
SGA	123 (27.5%)	12 (15.8%)	16 (7.1%)	0.033	**<0.001**	0.038

Data are presented as means (SE) unless otherwise indicated
BMI: Body mass Index, SGA: Small for Gestational Age

CVD Risk Factors

A generalized linear model was used to compare mean and SDs in the 3 study groups after adjustment for maternal age, BMI, and nulliparity (Table 2). Blood pressure, glucose, Homeostasis Model Assessment scores, triglycerides, and total cholesterol were significantly different between the early-onset preeclampsia and late-onset preeclampsia groups (P<0.017). All parameters were significantly different between women with early-onset preeclampsia and PIH, except for diastolic blood pressure and hsCRP. Significant differences in the late-onset preeclampsia and PIH groups were only seen in blood pressures and high-density lipoprotein levels.

Table 2. Determinants of Cardiovascular Risk in early-onset PE, late-onset PE and PIH

Parameter	Early-onset PE	Late-onset PE	PIH	Early vs. Late	Early vs. PIH	Late vs. PIH
	n=448	n=76	n=224			
Systolic blood pressure (mmHg)	128 (0.80)	119 (1.49)	124 (0.94)	**<0.001**	**0.006**	**0.004**
Diastolic blood pressure (mmHg)	82 (0.61)	79 (1.26)	82 (0.67)	**0.007**	0.952	**0.003**
Fasting glucose (mmol/L)	5.29 (0.04)	4.80 (0.09)	4.83 (0.05)	**<0.001**	**<0.001**	0.754
Fasting Insulin (ulU/L)	9.12 (0.32)	6.31 (0.08)	6.17 (0.43)	**<0.001**	**<0.001**	0.786
HOMA score	2.19 (0.08)	1.37 (0.18)	1.37 (0.11)	**<0.001**	**<0.001**	0.93
hsCRP (mg/L)	4.83 (0.52)	3.85 (0.69)	4.46 (0.42)	0.197	0.546	0.414
Triglycerides (mmol/L)	1.32 (0.04)	1.02 (0.08)	0.97 (0.05)	**0.004**	**<0.001**	0.629
Cholesterol (mmol/L)						
Total cholesterol	5.14 (0.05)	4.73 (0.11)	4.73 (0.06)	**0.002**	**<0.001**	0.937
HDL	1.36 (0.02)	1.34 (0.04)	1.44 (0.02)	0.627	**0.002**	**0.012**
LDL	3.19 (0.04)	2.93 (0.1)	2.85 (0.06)	0.048	**<0.001**	0.432

Data are presented as means (SE), adjusted for age, BMI and nulliparity.
hsCRP: high sensitive C-reactive protein; HDL: high-density lipoprotein; LDL: low-density lipoprotein; HOMA: Homeostatic Model Assessment

Metabolic Syndrome and CVD Cutoff Points

The prevalence of metabolic syndrome was the same in all the 3 groups (Table 3). Several individual components of the metabolic syndrome, however, were significantly different between the groups. Glucose levels >5.6 mmol/L occurred in 16% of women with previous early-onset preeclampsia, compared with 5% and 6% in women with late-onset preeclampsia and PIH, respectively (P<0.001). A BMI>30 was present in 21% to 29% of the women, but was only significantly different between early-onset preeclampsia and PIH (21% versus 29%, P<0.001). Blood pressures measured >=3 months postpartum did not differ between women with early-onset preeclampsia and PIH.

Besides the cutoff points defined in the metabolic syndrome, we also chose to compare other clinically relevant cutoff points for total cholesterol and hsCRP (Table 2). Again the

results showed significantly altered lipid levels in women with early-onset preeclampsia versus PIH. However, total cholesterol >6.21 mmol/L was nonsignificant between women with early-onset preeclampsia and late-onset preeclampsia. Significantly higher levels of hsCRP were found in women with early-onset preeclampsia and PIH compared with late-onset preeclampsia. The main findings are summarized in Supplemental Figure 2.

Table 3. Cut-off values used in Metabolic Syndrome, ATP III guidelines and JUPITER trial in early-onset PE, late-onset PE and PIH

Cut off values	Early-onset PE	Late-onset PE	PIH	Early vs. Late	Early vs. PIH	Late vs. PIH
	n=448	n=76	n=224			
Metabolic syndrome	69 (15%)	11 (14%)	36 (16%)	0.873	0.846	0.755
BMI>30 kg/m^2	93 (21%)	16 (21%)	66 (29%)	0.957	**<0.001**	0.151
Systolic BP >130	209 (47%)	22 (29%)	95 (42%)	**0.006**	0.395	0.042
Diastolic BP> 85	202 (45%)	23 (30%)	108 (48%)	**0.009**	0.406	**0.008**
Glucose >5.6 mmol/L	70 (16%)	4 (5%)	11 (5%)	0.024	**0.004**	0.858
Triglycerides >1.7 mmol/L	88 (20%)	8 (11%)	18 (8%)	0.065	**<0.001**	0.540
LDL cholesterol >1.8 mmol/L	430 (96%)	74 (97%)	213 (95%)	0.629	0.580	0.458
ATP III guidelines and JUPITER trial						
HDL cholesterol <1.29 mmol/L	200 (45%)	36 (47%)	79 (35%)	0.748	0.020	0.081
Total cholesterol >6.21 mmol/L	62 (14%)	4 (5%)	14 (6%)	0.050	**0.005**	0.840
hsCRP >2mg/ml	249 (56%)	27 (36%)	126 (56%)	**0.006**	0.873	**0.002**

Data are presented as number and percentage.
hsCRP: high sensitive C-reactive protein; HDL: high-density lipoprotein; LDL: low-density lipoprotein; BMI: Body mass Index

DISCUSSION

This study provides evidence that the number and levels of postpartum modifiable CVD risk factors differ between the different hypertensive disorders of pregnancy; early-onset preeclampsia, late-onset preeclampsia, or PIH. Women with a previous early-onset preeclampsia showed an overall less favorable CVD risk profile, compared with women with late-onset preeclampsia and PIH, particularly reflected in glucose and lipid levels. Hypertension postpartum is observed in almost half of the patients with a history of early-onset preeclampsia and PIH. Furthermore, we showed that blood pressure was significantly higher in women who experienced early-onset preeclampsia and PIH compared with late-onset preeclampsia. These results support the hypothesis that the CVD risk profile after pregnancy may reflect the risk of CVD later in life, in particular, the risk of chronic hypertension.

Large observational studies have shown that there is an increased risk of future CVD for women who experienced hypertensive pregnancy complications, such as early-onset preeclampsia, late-onset preeclampsia, and PIH. For instance, women who experienced preeclampsia have a 2.28 higher risk of developing CVD according to a recent meta-analysis on this subject. (2) Only few articles have addressed the changes in risk for developing CVD between different hypertensive pregnancy complications. In the study of Wikström et al, (21) the risk for developing ischemic heart disease was significantly higher in women with severe preeclampsia compared with PIH and mild preeclampsia similar to the study of Lykke et al. (22) Women with mild preeclampsia had an [almost equal to] 4-fold increased risk of subsequent hypertension later in life. However, the risk increased to 5- and 6-fold in women with a history of PIH and severe preeclampsia, respectively. (21) Although we acknowledge that stratification in early versus late preeclampsia is not the same as mild and severe preeclampsia, we observed similar patterns in mean blood pressure, usage of antihypertensive medication and the presence of hypertension with the highest incidence in early-onset preeclampsia followed by PIH and late-onset preeclampsia. It is striking that 25% to 45% of women in this relatively young population has hypertension compared with 8% in the Dutch female population aged 30 to 39 years. Even more, the use of antihypertensive drugs was only 2% in this age category compared with the 9% to 21% in our cohort. (23)

Patterns of maternal vascular remodeling and responsiveness show a distinct vascular adaptation between early and late preeclampsia. (24) It has also been shown that women with early preeclampsia show high total vascular resistance and women with late preeclampsia have low total vascular resistance 1 year postpartum. (25) Increased vascular resistance might lead to systolic and diastolic dysfunction and could be a possible mechanism in the development of chronic hypertension. This also supports the theory that pregnancy is a stress test for cardiovascular health and that hypertensive disorders unmask the patients more prone to develop CVD later in life. However, we cannot exclude the reverse: more severe endothelial damage and inflammatory stress in early-onset preeclampsia cause (more) permanent vascular damage apposed to the milder late-onset preeclampsia and PIH. (26) These differences in permanent vascular damage may contribute to the pathogenesis of CVD later in life and explain the seemingly gliding scale in differences in CVD prevalence between the hypertensive pregnancy disease early-onset preeclampsia and late-onset preeclampsia, PIH.

Up to now, PIH has not been included in postpartum research as a separate clinical entity. Our data show that the postpartum biochemical CVD risk factors do not differ between late-onset preeclampsia and PIH. Nonetheless, significant differences were found between early-onset preeclampsia on the one hand and late-onset preeclampsia and PIH on the other hand. Fasting blood glucose, insulin, Homeostasis Model Assessment score, triglycerides, and total cholesterol were significantly higher than in women with

a history of late-onset preeclampsia and PIH. BMI seemed not to relate to this difference hence the fact that BMI was only significantly higher in the PIH group when compared with the early-onset preeclampsia group.

Interestingly, mean hsCRP levels did not differ between the groups. However, when the cutoff value of 2 mg/mL was used, there was a significant difference in prevalence when early-onset preeclampsia and PIH were compared with the late-onset preeclampsia group. The role of hsCRP as a predictor for CVD is controversially and several reports have questioned its usefulness. (27,28) Nonetheless, recent data from a large cohort reconfirmed the associations found in the JUPITER trial. (29) Our results show that women with early-onset preeclampsia or PIH in their medical history are more prone to hypertension than late-onset preeclampsia. This difference is also reflected in hsCRP cutoff levels. Recent data show that elevated prepregnancy CRP predisposes to recurrent preeclampsia in women with a history of early-onset preeclampsia, (30) which might explain the higher incidence of hypertension as well. Further research and follow-up are needed to confirm the relationship between hsCRP and subsequent hypertension after complicated pregnancy.

Some limitations of this study need to be addressed. First, women in the early-onset preeclampsia were significantly younger than women with previous late-onset preeclampsia or PIH. Advanced age is associated with an increased risk of CVD. However, in this relatively young population of women, the effect of a few years difference is estimated to be small. Besides, data were corrected for age and showed no effect on the associations found. Second, data were obtained from 2 different cohorts. However, population differences between both cohorts are highly unlikely due the fact that the HyRAS cohort was based on a national study, including the Utrecht region. Although almost identical in design, some inclusion criteria were different with follow-up time being the most notable. The follow-up time within the groups was not correlated with any of the other variables tested. Besides, several articles have shown that as early as 6 to 7 weeks serum lipid levels rapidly decrease to normal values. (31,32) Therefore, it is unlikely that the differences between groups have confounded importantly by a difference in follow-up time. The exception would be the number of intervening pregnancies that was lower in the early-onset preeclampsia group. However, there is no proper data available that indicate that multiple pregnancies would further increase the risk on CVD later in life. Finally one of the limitations of this study is the lack of prepregnancy values of the measured CVD risk factors. It is possible that women with early-onset preeclampsia exhibit more risk factors simply because there is a difference in prepregnancy values. Nonetheless, this does not detract from our main conclusion that these pregnancy-related diseases present an opportunity for early detection of CVD risk factors in young women.

In conclusion, our study showed that early-onset preeclampsia, late-onset preeclampsia, and PIH differ significantly on the levels of postpartum biochemical CVD risk factors and the presence of hypertension. Women with a previous early-onset preeclampsia show an overall less favorable risk profile compared with late-onset preeclampsia and PIH, in particular, for glucose and lipid levels. The high prevalence of hypertension in both early-onset preeclampsia and PIH compared with late-onset preeclampsia may be of benefit for the development of targeted prevention programs. With high rates of hypertension found in our study, it seems justified to assess the CVD risk profile within 1 year after pregnancy routinely. Specific follow-up strategies may be required for the different hypertensive disorders of pregnancy.

PERSPECTIVES

Hypertension increases the risk for a variety of CVDs, including stroke, coronary artery disease, heart failure, and peripheral vascular disease. (33) Of great interest is the gliding scale of prevalence of hypertension postpartum. In early-onset preeclampsia, the prevalence is the highest followed by PIH and late-onset preeclampsia. Furthermore, the women with previous early-onset preeclampsia have the most deviant risk profile compared with late-onset preeclampsia and PIH in terms of glucose levels and lipid profile. Our results not only emphasize on early prevention programs for these high-risk women but also suggest that physicians should stratify for the different hypertensive pregnancy complications to further personalize preventive strategies.

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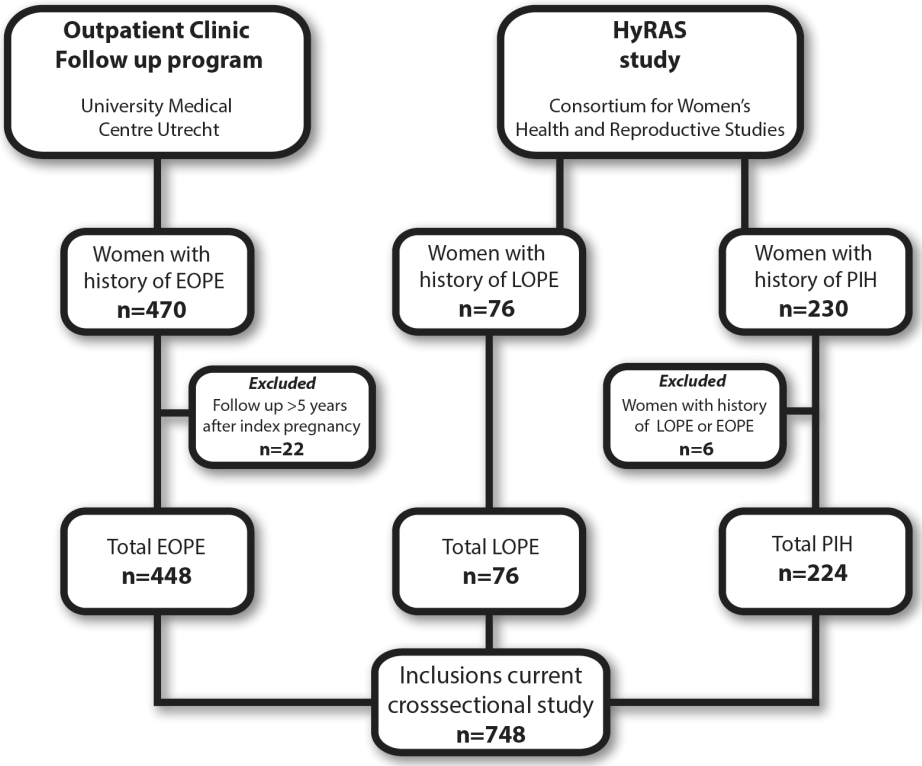
Supplemental data chapter 9

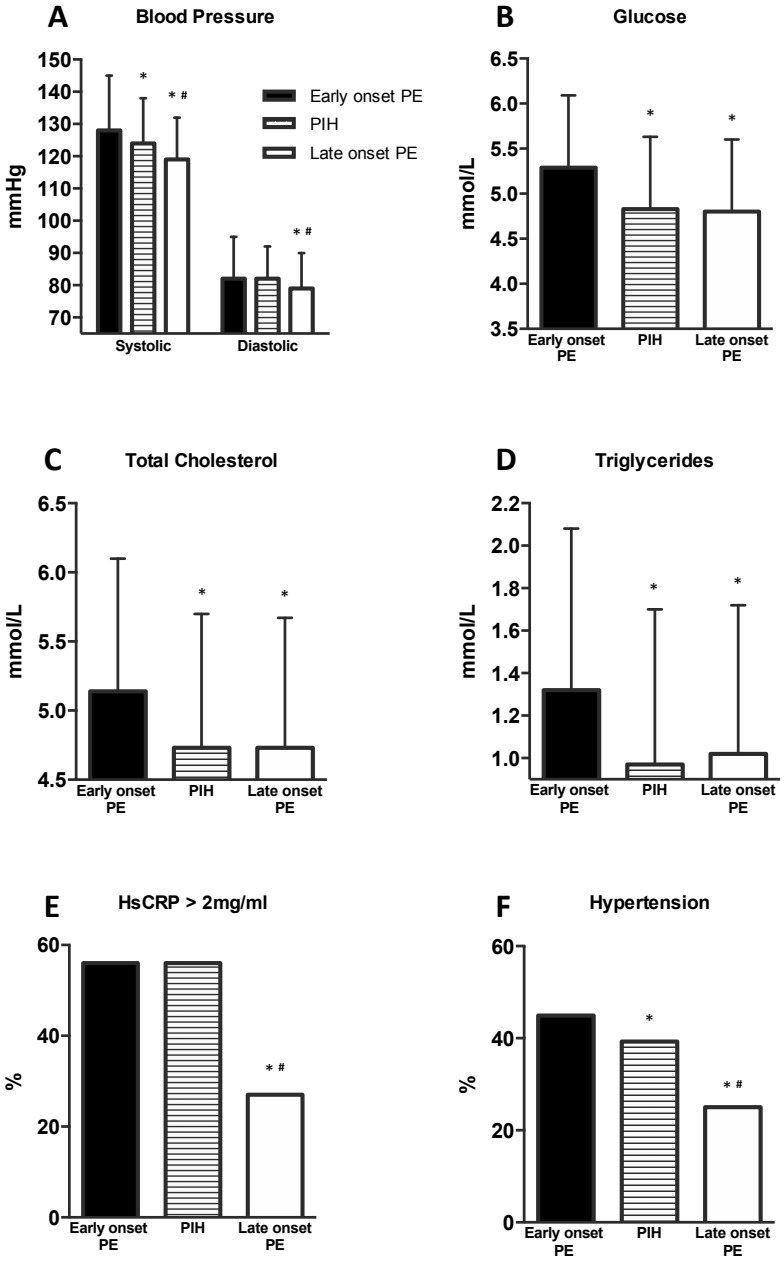
Supplemental table 1. Assays used in assessment of endocrine and cardiometabolic profile per center

Parameter	**Medical Center Haaglanden**				**University Medical Center Utrecht**			
	Date	***Assay***	***Interassay variation***	***Conversion formulas***	***Date***	***Assay***	***Interassay variation***	***Conversion formulas***
Insulin (mIU/L)	*	Immulite platform	4.1-6.4%	#	start-01/07/2003	In house competitive radioimmunoassay (RAI) **(X)**	7.9; 5.4; and 7.8% at 10.6; 43 and 106 mE/L respectively	*Z=0,62X*
					start – 15/12/2010	Immulite platform **(Z)**	9% at 6 mE/L and <5,5% at 20 - 120 mE/L	#
					16/12/2010 – end	Roche Modular **(Y)** E170	<3.3%	*Z=0,77Y*
Glucose (mmol/L)	*	Roche Modular P800	0.9-1.0%	#	start – 11/30/2006	VITROS	2.4%	#
					12/1/2006 – end	Unicell DxC 800		
HsCRP mg/L	*	Roche Modular P800	1.5-3.2%	#	start – 11/30/2006	VITROS	2.5%	#
					12/1/2006 – end	Unicell DxC 800		
Lipids (mmol/L) Total-C, TG, LDL-C, HDL-C	*	Roche Modular P800	0.8-0.9% for total cholesterol 2.1-3.0% for HDL cholesterol 1.0% for triglycerides	#	start – 11/29/2006	VITROS	1.5% for total cholesterol 2.0% for HDL-cholesterol 2.0% for triglycerides	#
					11/30/2006 – 5/6/2013	Unicell DxC 800		

For insulin the results of assays were internally adjusted according to the standards of the last used assay. This internal correction was derived from the quality controls performed by the Dutch Foundation for Quality Assessment in Clinical Laboratories (SKML) using a reference sample used in all laboratories in the Netherlands to ensure comparability nation wide. This way no "between center conversion factor" needed to be applied to ensure between center homogeneity.
Glucose, HsCRP and lipids did not differ between the platforms tested so no conversion formula was needed. These were again meticulously tested 6 times a year by the SKML as part of routine care in the Netherlands.* assay used during entire study period, # conversion factor not applicable.
Beckman Dxi system, Unicell DxC 800, AU 5811: *Beckman Coulter, Woerden, Netherlands;* Roche Modular E170, Roche Modular P800: *Roche Diagnostics Almere, Netherlands*; Immulite platform, Immulite 1000, Immulite 2000, RIA DPC: *Diagnostics Products Corporation Breda, Netherlands;* VITROS Chemistry System*: Ortho-Clinical Diagnostics, Strasbourg, France;* In house RIA: in house developed extraction RIA.



Supplemental figure 1. Flowchart of patient selection. A total of 748 women enrolled after excluding >5 year follow-up time and women with a history of PE in the PIH group. EOPE: early-onset preeclampsia; LOPE: late-onset preeclampsia; PIH: pregnancy induced hypertension.



Supplemental figure 2. Summary of main findings between the three groups studied. A. Systolic and diastolic blood pressures (mean and SD); B. Fasting glucose levels (mean and SD); C. Total cholesterol (mean and SD); D. Triglycerides (mean and SD); E. Percentage of women with HsCRP >2mg/ml (%); F. Percentage of women with hypertension at screening (%). PE: preeclampsia; PIH: pregnancy induced hypertension. * Significant versus early onset PE, # Significant versus PIH

Chapter 10

Summary and General discussion

Preeclampsia, intra uterine growth restriction (IUGR), placental abruption and preterm birth are major contributors to maternal and perinatal morbidity and mortality. (1,2) In these disorders the placenta is a key aetiological factor and therefore preeclampsia, IUGR and placental abruption are also referred to as maternal placental syndromes (MPS). In order to understand these syndromes one has to study placental development. Over the last century, research has provided us with knowledge on how the functional human haemochorial placenta develops. Especially the invasive growth into the uterine wall and the underlying maternal blood vessels has been the focus of attention. Since the vascular morphology within the placental bed was first characterised (3) and subsequent identification of the extravillous trophoblast some years later, the number of publications on this subject has risen exponentially. (4) It is now widely accepted that impaired spiral artery remodelling in the placental bed is a key factor in the development of early-onset, severe preeclampsia and other pregnancy complications, such as placental abruption, IUGR and preterm birth. (5-7) Extravillous trophoblasts fail to remodel maternal spiral arteries, into high capacity and low resistance vessels, in complicated pregnancies. Despite extensive research, the mechanism of this failure remains unclear. It is probable that several maternal factors (genetic, environmental and behavioural), many of which are also associated with the development of atherosclerosis and cardiovascular risk, interact with the development of the placenta. Failure of this process results in "shallow" placentation and, ultimately, the preeclamptic syndrome, which is clinically defined as a multisystem syndrome by its key signs hypertension and concomitant proteinuria after 20 weeks of gestation. Interestingly, the risk of cardiovascular disease is increased after a hypertensive pregnancy and common risk factors suggest a shared pathogenesis. (8) Given that pregnancy is considered as a stress test for future cardiovascular health, it has been proposed that women with MPS fail this stress test and are at higher risk for developing cardiovascular disease later in life. (9)

This thesis focuses on spiral artery remodelling during pregnancy, the role of the maternal immune system and endoplasmic reticulum (ER) stress as contributing factors when remodelling failure occurs. In addition, we investigated the possible shared pathogenesis of failed spiral artery remodelling with cardiovascular disease. Finally, we show differences in cardiovascular risk factors in several MPS. The conclusions, implications and questions that arise from our findings are summarised and discussed in this final chapter.

SAMPLING THE PLACENTAL BED

The 'physiologic change' of spiral arteries in the placental bed is crucial to the establishment of a healthy pregnancy. (4) The study of the absence or failure of this morpho-

logical change requires placental and placental bed tissues. In **chapter 2,** we summarize all previous studies that have used placental bed biopsies to study various aspects of placentation. Ninety-four papers were identified including the first placental bed biopsy study in 1958. (3) Few groups have collected bed biopsies, most of them with only a small number of patients. By far the largest collection of placental bed samples was taken through punch biopsy. This cohort comprised 417 patients undergoing pregnancy terminations or evacuation of retained products of conception. (10) Although these samples are important for elucidating the physiology of spiral artery remodelling in early pregnancy, they cannot be correlated with pregnancy outcome and, as such, are less relevant when studying preeclampsia. Although prediction models for preeclampsia still suffer from low sensitivity and specificity, recent studies indicate that early preeclampsia can be accurately predicted in the first trimester of pregnancy using maternal characteristics, biomarkers and Doppler flow measurements of the uterine artery. (11,12) Obtaining these parameters before termination of pregnancy might enable us to distinguish women at high risk of developing (early) preeclampsia if their pregnancy would continue. Furthermore, one could stratify the samples according to the degree of spiral artery remodelling. However, caution must be taken when this approach is followed due to variations in gestational age at the start of spiral artery remodelling. (13) Changes in the myometrial region of the spiral arteries progressively increase from 10-12 weeks of gestation onwards, whereas in 6-8 weeks samples, changes are only seen in the decidual region. (13,14) Due to the speculation of pregnancy outcome with these early pregnancy placental bed specimens, there is still need for placental bed biopsies sampled in (near) term pregnancies. Therefore, we proposed a sample protocol in **chapter 2** for the uniform collection of placental bed biopsies. Caution should be taken when the placental bed has been subjected to labour, which induces changes in the normal, *in vivo* molecular profile of the tissue. (15) In **chapter 5** we showed that labour also induces ER stress in the placenta. Although labour presents the opportunity to study placental cellular changes to oxidative and ER stress in the absence of maternal factors, it might be less useful for other purposes. These data, together with a recently published paper on sample handling by Burton *et al* (16), have been incorporated in the suggested protocol in **chapter 2.** This protocol was used in our spiral artery remodelling (SPAR) study that investigated the distribution of maternal immune cells in the placental bed and how this correlated with cardiovascular risk factors, which is presented in **chapter 3**. The success rate of sampling the placental bed was 57%, depending on the criteria used to determine success. "Successful" sampling of the placental bed was achieved when biopsies contained trophoblasts and both decidua and myometrium. However, in order to study acute atherosis, these biopsies should contain at least one spiral artery. (10) The success rates shown in this chapter are comparable to the success rates presented in

chapter 2. The feasibility and safety of the procedure were also evaluated and found to be sufficient to continue biobanking placental bed biopsies at our institution.

MATERNAL IMMUNE SYSTEM IN PREGNANCY DISORDERS

Immune cells are generally thought to play a role in spiral artery remodelling, as discussed in **chapter 1**. Previous work, aiming to identify the involvement of specific immune cell types in spiral artery remodelling, has been conflicting due to the use of different markers for identification. Nonetheless, we found that the number of CD3 positive cells was significantly lower in both the myometrium and decidua of preeclampsia patients in the third trimester of pregnancy **(chapter 3)**. Sasaki *et al*, however, did not detect a change in myometrial or decidual CD3 abundance between preeclampsia and control pregnancies. (17) The small group of patients used by this group may have accounted for this negative result. The scoring system proposed in **chapter 3** of this thesis involves visualizing the whole placental bed biopsy slide, in order to categorise the presence of immune cells. This way, sampling error is less likely, due to assessment of larger areas. Sasaki *et al* found that the population of T-regulatory cells was significantly lower in preeclamptic cases. (17) Based on the results presented in **chapter 3**, it is tempting to speculate that the number of T-regulatory cells is also reduced in the placental bed of preeclamptic pregnancies, resulting in insufficient control of the maternal immune system. This, in turn, could account for the excessive maternal immune response in preeclampsia, as has been described previously. (18) To further substantiate this theory, it is known that there is a shift from the T-helper (Th) 2 type immunity in normal pregnancy to an abnormal Th1 type state in preeclampsia, with increased proinflammatory circulating factors, such as CRP, IL-6, TNFα, IFN-γ, IL-12p70 and IL-18. (19,20) Also, experiments in animal models of preeclampsia suggest a causative role of inflammatory stimulation. (21). Future work should therefore focus on investigating subsets of immune cells in the placental bed in greater detail. A larger sample size is needed to make definite conclusions about the role of specific cell types. However, we acknowledge the limitation that placental bed biopsies were collected at term/preterm and, thus, changes in these biopsies are unlikely to reflect early firt trimester pregnancy. Furthermore, researchers should incorporate cases of preterm birth, IUGR and placental abruption into investigations on immune regulation of the placental bed, given the shared morphological placental phenotype of these disorders.

POSSIBLE ROLE FOR ENDOPLASMIC RETICULUM (ER) STRESS IN FAILURE OF SPIRAL ARTERY REMODELLING

The effect of the maternal immune system on trophoblast invasion has been the subject of many papers. (18,22,23) A growing body of evidence suggests that immune cells at the feto-maternal interface play an important role in controlling trophoblast invasion. (18,24,25) Inflammatory factors like Interferon γ (INFγ) can, in turn, cause ER stress by downregulating the sarcoendoplasmic reticulum Ca2+ pump and depleting ER Ca2+. (26) A potential relationship between trophoblast invasion and ER stress has not been studied previously. In **chapter 6,** we have shown that ER stress could be successfully induced *in vitro* in an HTR8/SVneo trophoblast cell line. Subsequently, we showed that ER stressed HTR8/SVneo cells display a diminished invasive capacity, most likely due to reduced MMP activity. This suggests that ER stress may negatively impact trophoblast invasiveness *in vivo* and, thus, may be implicated in the pathophysiology of MPS. In order to test this hypothesis, first trimester trophoblasts from pregnancies, that in the future will be complicated by maternal placental syndromes should be analysed for ER stress. Unfortunately, as reviewed in **chapter 2**, it is very challenging, if not impossible, to obtain first trimester tissues from on-going pregnancies. In further elucidating the role for ER stress, future research could focus on trying to alleviate ER stress with orally active chemical chaperones such as 4-Phenyl butyric acid and taurine-conjugated ursodeoxycholic acid. This may provide us with potential future treatment options. The effectiveness of chaperones that relieve the cell from ER stress has been shown previously in a mouse model of type 2 diabetes where ER stress was reduced and glucose homeostasis restored. (27) Although proven to be clinically safe, teratogenicity of such drugs remain to be investigated.

PLACENTAL CHANGES

As a result of defective spiral artery remodelling, characteristic placental pathology associated with hypoxia and reperfusion can be identified. Although the direct relationship has never been properly identified. Pathological lesions can be classified into two major categories; [1] those related to reduced vascular supply of nutrients and oxygen due to ineffective spiral artery remodeling *(e.g. infarction, villous hypoplasia, increased syncytial knotting and elevated foetal nucleated red blood cells (NRBCs))* and [2] those related to an altered inflammatory response (*e.g. chorioamnionitis, villitis and acute funisitis*). (28-32) In **chapter 4** we found that chronic chorioamnionitis was more common in IUGR pregnancies with concomitant preeclampsia possibly reflecting the excessive systemic inflammation in preeclampsia as discussed earlier. Interestingly, the severity of IUGR,

expressed in absence of end diastolic flow in the umbilical artery, seemed to correlate with signs of chronic ischemia and poor nutrient supply (i.e. severely elevated NRBCs and the presence of distal villous hypoplasia) and not with (excessive) inflammation. Overall, both groups showed significant placental changes, although there were subtle immunological changes when IUGR was further complicated by preeclampsia. Paradoxically, not all these women developed preeclampsia, despite the largely shared placental phenotype. This suggests a different pathophysiologic pathway even though poor placentation is thought to underlie both conditions. Possibly, women with concomitant preeclampsia are more susceptible to oxidative, ER and inflammatory stress from the placenta, causing the global maternal inflammatory response.

FROM INFLAMMATION IN PREGNANCY TO FUTURE CARDIOVASCULAR DISEASE

Systemic inflammation observed in normal pregnancy generates physiological hyperlipidaemia. Due to exaggerated systemic inflammation in preeclampsia and other maternal placental syndromes, hyperlipidaemia and dyslipidaemia (hypertriglyceridemia and increased circulating low-density lipoproteins) becomes excessive. (33) However, since dyslipidaemia itself contributes to excessive inflammation cause and effect are difficult to distinguish. Of interest, dyslipidaemia seen in preeclampsia patients resembles that seen in atherosclerosis, including higher levels of oxidised low-density lipoprotein (LDL) cholesterol. (34) In line with this, preeclampsia and atherosclerosis are both considered inflammatory diseases. (18,35,36) Both are characterised by increased concentrations of circulating pro-inflammatory cytokines, such as TNFα and IL-6, which, in turn, play an important role in trophoblast migration (**chapter 6**) and in the development of coronary heart disease. (37) Acute phase proteins, such as C-reactive protein (CRP) and markers of leukocyte activation, are augmented in both conditions. (38,39) In **chapter 7,** we showed that elevated pre-pregnancy CRP and fibrinogen also predispose to recurrent preeclampsia in women with a history of early-onset preeclampsia. This supports the hypothesis that an underlying pro-inflammatory phenotype in the mother may be a predisposition for developing early onset preeclampsia. We were not able to show any correlation between serum levels of cardiovascular risk markers and recurrent preeclampsia. Magnussen *et al*., however, did find positive associations between pre-pregnancy serum levels of triglycerides, cholesterol, LDL cholesterol, non-high density lipoprotein (HDL) cholesterol and blood pressure and the risk of developing preeclampsia. (40) Differences in sample size and patient population (only women with a history of early onset preeclampsia were included in the present study) may explain the discordance between the present data and those of Magnussen *et al*. Nonetheless,

our findings suggest that routine assessment of inflammatory markers postpartum may improve stratification of former patients into risk categories for recurrent preeclampsia and facilitate the development of effective preventative strategies tailored to the individual's recurrence risk. Interestingly, elevated CRP levels have also been associated with higher risk of cardiovascular disease (41,42), suggesting that the underlying maternal pro-inflammatory phenotype may also predispose women to cardiovascular disease later in life.

From the previous findings we hypothesised that excessive systemic inflammation seen in preeclampsia might be predictive in the future development of cardiovascular disease. In **chapter 3** we compared the findings of the scoring system with cardiovascular disease risk factors directly post partum. We found small differences in HDL triglycerides, creatinine and homocysteine. It is known that, among others, medication use, severity of disease, interval between caesarean section and sampling can influence lipid profiles, glucose and blood pressures. (43) As the blood samples were taken on the morning after the caesarean section, this could potentially have interfered with the measurements. Therefore, a definitive conclusion on differences between cardiovascular disease risk factors could not be drawn from the data at present. Despite the small sample size, some correlations between placental bed characteristics and cardiovascular disease risk factors were observed. Women had higher levels of triglycerides and cholesterol when the placental bed biopsies contained acute atherosis. This preliminary finding will be further investigated in our on-going study, in which included patients will be enrolled into a follow-up programme for cardiovascular risk evaluation ~ 6-12 months after pregnancy, hereby diminishing the effects of pregnancy on cardiovascular disease risk factors.

Two theories have been postulated that link preeclampsia and cardiovascular disease: [1] preeclampsia and future cardiovascular disease share risks factors for augmented inflammation and [2] preeclampsia, or even pregnancy in general, causes (permanent) vascular alterations implicated in the development of future cardiovascular disease. (44-46) The theories are not mutually exclusive and may even co-exist. In **chapters 8 and 9** we investigated if such shared risk factors could already be identified several months post partum.

The long-term risk for cardiovascular disease is highest in early onset preeclampsia. (8) In **chapter 9**, we hypothesized that differences in long-term cardiovascular disease risk of women with a history of a hypertensive disorder of pregnancy is reflected in the prevalence of modifiable cardiovascular disease risk factors postpartum. Accordingly, our data suggest that the prevalence of post-partum hypertension increases with the severity of preeclampsia. We showed that early onset preeclampsia is associated with an overall less favourable cardiovascular disease risk profile, reflected in glucose and lipid levels. Nonetheless, these values were all within clinical normal ranges in these relatively young women.

Although the highest CVD risk is found in early onset preeclampsia, Ray *et al* reported a hazard ratio of 1.7 for developing cardiovascular disease after placental abruption. (47) In **chapter 8,** we showed that, comparable to preeclampsia and IUGR (48-51), women with a history of placental abruption had a higher prevalence of multiple modifiable risk factors for cardiovascular disease within the first year after delivery. This difference remained in a subanalysis that excluded women with concomitant hypertensive disease. Although the pathophysiology of placental abruption remains to be characterised completely, one study reported an involvement of failed spiral artery transformation. (52) Moreover, there seems to be a relationship between (prolonged) inflammation and placental abruption, suggesting placental abruption may be a consequence of defects present in the first trimester, before placental abruption even presents. (53,54) If failed spiral artery remodelling is implicated in placental abruption, it is tempting to speculate that the pathogenesis might be similar to preeclampsia. However, placental abruption also occurs without preeclampsia. Possibly, as discussed as well in **chapter 4**, women with concomitant preeclampsia are more susceptible to oxidative, ER and inflammatory stress from the placenta. Due to a small sample size in the study by Dommisse *et al* (52) researchers were not able to stratify between abruption with and without concomitant disease in studying the placental bed. Thus, future work should focus on this caveat in knowledge and examine placental bed biopsies following placental abruption.

CLINICAL IMPLICATIONS

It is striking that as many as 25-45% of the relatively young women investigated in **chapter 9** had hypertension, compared to 8% in the Dutch female population aged 30-39 years. Moreover, antihypertensive drugs were used by only 2% in this age category compared to the 9-21% in our cohort. (55) Hypertension increases the risk for a variety of cardiovascular diseases, including stroke, coronary artery disease, heart failure and peripheral vascular disease. (56) The differences in prevalence of postpartum hypertension between early onset preeclampsia, late onset preeclampsia, and pregnancy-induced hypertension are interesting. In early-onset preeclampsia, the prevalence is the highest, followed by pregnancy induced hypertension and late-onset preeclampsia. Furthermore, women with previous early-onset preeclampsia have the most deviant risk profile, compared to late-onset preeclampsia and PIH, in terms of glucose levels and lipid profile. Collectively, this suggests that likelihood of developing cardiovascular disease later in life may be reflected in post-partum (biochemical) risk factors.

Existing evidence suggests that cardiovascular disease is largely preventable by early modification of cardiovascular disease risk factors. (57) However, the first presentation

of cardiovascular disease does not usually occur before menopause, making it difficult to identify women at risk for future cardiovascular disease. The presence of modifiable risk factors in women with a history of multiple placental syndromes, including placental abruption, preeclampsia and PIH, may therefore be of potential use for prevention programmes. Recently, the American Heart Association (AHA) recognised preeclampsia, gestational diabetes and pregnancy-induced hypertension as independent risk factors for cardiovascular disease. (58) This update also emphasised referring these women to a primary care physician or cardiologist in the years after their complicated pregnancy. In the Netherlands a national guideline for cardiovascular risk management after reproductive disorders has been developed recently. (59) Our results support development of early prevention programmes for high-risk women but also indicate that physicians should stratify for the different hypertensive pregnancy complications in order to further personalize preventive strategies.

FUTURE DIRECTIONS FOR RESEARCH AND CLINICAL PRACTICE

Although this thesis has shed new light on several aspects of maternal placental syndromes, we have by no means solved the mysteries that still underlie these pregnancy complications. Investigations remain hampered by the lack of sample material from first trimester on-going pregnancies. Although theories postulate why spiral artery remodelling does not occur adequately in maternal placental syndromes, they cannot be properly tested. We should therefore explore new techniques to overcome this problem.

In order to confirm the hypothesis that placental bed pathology, composition of the immune cell population and circulating risk factors correlate to the risks of developing cardiovascular disease later in life, future research should focus on structured, longitudinal follow-up of women with a history of maternal placental syndromes; studies that our research group are recently carrying out. Collections of tissues from the placenta and placental bed are indispensable in such studies. Studying these samples in correlation to data on cardiovascular health before, during and (long) after pregnancy may provide more knowledge on the pathogenesis of both maternal placental syndromes and cardiovascular disease. Therefore, we should also explore the common ground of vascular remodelling in obstetrics and cardiovascular biology to further elucidate the possible shared pathogenesis.

Women with maternal placental syndromes have an increased risk of developing cardiovascular disease later in life, and the risk is most pronounced in cases of early preeclampsia. One could argue the relevance of screening all women who have a history of a hypertensive pregnancy, as is being proposed in the recent national guideline. (59) Future research should focus on the feasibility, clinical relevance and cost-effectiveness of such screening and preventive interventions before wide implementation in clinical practice may take place.

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Chapter 11

Dutch Summary

(Nederlandse samenvatting)

Pre-eclampsie , intra-uteriene groeirestrictie (IUGR), loslating van de moederkoek (placenta) en vroeggeboorte zijn belangrijke oorzaken van ziekte en sterfte van moeders en kinderen rondom zwangerschap en geboorte. De placenta speelt een belangrijke rol bij het ontstaan van deze aandoeningen en daarom worden pre-eclampsie, IUGR en loslating van de placenta ook wel maternale placentasyndromen (MPS) genoemd. De kennis over de oorzaken van deze syndromen is nog altijd beperkt. Om die oorzaken beter te begrijpen moet de ontwikkeling van de placenta worden bestudeerd. In de loop van de vorige eeuw heeft onderzoek ons veel geleerd over hoe de menselijke placenta zich ontwikkelt. Vooral het binnendringen van cellen van het kind (trofoblastcellen) in de baarmoederwand en de onderliggende moederlijke bloedvaten (spiraalarteriën) vroeg in de zwangerschap is een belangrijk punt van aandacht geweest. Sinds ontdekt is dat de vaten in de baarmoeder omgevormd (geremodelleerd) worden tijdens de zwangerschap en dat de trofoblast hierbij een belangrijke rol speelt, is er veel gepubliceerd over dit onderwerp. De veranderingen vinden vooral plaats in het placentavaatbed, dit is het gedeelte van de baarmoeder direct onder de placenta en bevat de spiraalarteriën. Het is nu algemeen geaccepteerd dat onvolledige spiraalarterieremodellering in het placentaire bed een belangrijke factor is in de ontwikkeling van ernstige pre-eclampsie en andere zwangerschapscomplicaties, zoals loslating van de placenta, IUGR en vroeggeboorte. Trofoblastcellen slagen er dan niet in de spiraalarteriën om te vormen tot bloedvaten met een hoge capaciteit en lage weerstand. Ondanks het vele onderzoek dat is gedaan, blijft het mechanisme achter dit falen onduidelijk. Waarschijnlijk zijn een aantal moederlijke factoren (genetisch, milieu en gedrag) betrokken, die ook betrokken zijn bij de ontwikkeling van slagaderverkalking en hart- en vaatziekten. De slechte placenta-ontwikkeling resulteert later in de zwangerschap in een te sterke bloedstroom in de placenta. Dit veroorzaakt een overmatige productie van zuurstofradicalen en stress in de fabriek van de cel: het endoplasmatisch reticulum (ER). Dit alles resulteert in een algehele ontstekingsreactie bij de moeder en vermoedelijk resulteert deze overmatige ontstekingsreactie in de uitingen van pre-eclampsie. De belangrijkste verschijnselen van pre-eclampsie zijn een hoge bloeddruk met eiwitverlies in de urine in de tweede helft van de zwangerschap. Interessant is dat het lange termijn risico van de moeder op hart- en vaatziekten verhoogd is als zij in de zwangerschap een hoge bloeddruk heeft gehad. De zwangerschap kan worden beschouwd als een stresstest voor het vaatsysteem van de vrouw. Vrouwen die falen voor deze stresstest ontwikkelen MPS en gedacht wordt dat MPS kunnen worden beschouwd als een eerste vorm van hart- en vaatziekten. We denken dat het mogelijk afwijkende vaatstelsel (al voor de zwangerschap) bij sommige van deze vrouwen er voor zorgt dat zij al op relatief jonge leeftijd hart- en vaatziekten ontwikkelen. Welke vrouwen nu het hoogste risico op hart- en vaatziekten hebben is nog niet helemaal duidelijk, maar gedacht wordt dat MPS en hart- en vaatziekten ge-

meenschappelijke risicofactoren en daarmee vermoedelijk ook een gemeenschappelijk ontstaanswijze hebben.

Dit proefschrift gaat over spiraalarterieremodellering tijdens de zwangerschap, de rol van het afweersysteem van de moeder en ER stress als oorzaak van het falen van de remodellering. Daarnaast onderzochten wij overeenkomsten in de ontstaanswijze van MPS en hart- en vaatziekten.

In **hoofdstuk 2** worden alle gepubliceerde onderzoeken die placentabedweefsel bestudeerden door biopten af te nemen geanalyseerd. We vonden aanvankelijk 94 publicaties waarvan er slechts 25 het percentage gevallen beschreven waarbij er op de juiste plek van de baarmoederwand werd gebiopteerd. In de studie met verreweg de meeste biopten werd een biopsietang gebruikt waarmee in 47% van de patiënten succesvolle biopten werden afgenomen. Wij stellen in dit hoofdstuk een uniform biopsieprotocol voor en doen we aanbevelingen voor verwerking van het weefsel.

Dit protocol is in **hoofdstuk 3** gebruikt om bij 29 vrouwen met pre-eclampsie en 29 gezonde vrouwen met een normaal verlopen zwangerschap waarbij een keizersnede heeft plaatsgevonden biopten af te nemen van het placenta vaatbed. Het doel van deze studie was het bestuderen van de afwijkingen van het vaatstelsel en het afweersysteem in het placentavaatbed met behulp van een nieuw, door ons ontwikkeld systeem om deze afwijkingen te beoordelen. De biopten werden beoordeeld op remodellering en aanwezigheid van verschillende afwijkingen; [1] vaat afwijkingen zoals acute atherose (een aderverkalking-achtige afwijking) en [2] de hoeveelheid immuuncellen (T-cellen, NK-cellen en macrofagen). Daarnaast werden bij deze vrouwen direct na de bevalling risicofactoren voor hart- en vaatziekten in het bloed gemeten en gecorreleerd aan de mate van remodellering van de spiraalarteriën. In vrouwen met pre-eclampsie vonden we dat de spiraalarteriën inderdaad minder goed geremodelleerd waren. Verder bleken er minder T-cellen bij vrouwen met een pre-eclampsie aanwezig te zijn in zowel de spierlaag (myometrium) als het baarmoederslijmvlies (decidua). Bij slechts 3 patiënten was sprake van acute atherose. Deze vrouwen hadden een minder gunstig profiel van de risicofactoren voor hart- en vaatziekten in het bloed. We gaan dit onderzoek herhalen in een grotere groep zwangeren. Uiteindelijk hopen we door dit onderzoek van het placentavaatbed meer inzicht te krijgen in de gemeenschappelijke ontstaanswijze van hart- en vaatziekten en pre-eclampsie.

In **hoofdstuk 4** vergeleken we de pathologische kenmerken van de placenta in zwangerschappen die gecompliceerd werden door een vroege IUGR met of zonder pre-eclampsie en met of zonder terugstroom van bloed in de navelstreng. Dit laatste duidt op een zeer hoge weerstand in de placenta en komt meestal voor bij de ernstigste vormen van IUGR. In verreweg de meeste placenta's werden karakteristieke afwijkingen

gevonden zoals infarcten, ontstekingsverschijnselen, tekenen van zuurstoftekort en weefselschade en trombose. Vrouwen met IUGR met een afwijkend bloedstroomprofiel van de navelstreng hadden vaker verhoogde aantallen kernhoudende rode bloedcellen en onderontwikkelde placentavlokken, iets dat kan duiden op zuurstof tekort. Chronische ontsteking van de vliezen en toegenomen "syncytial knotting" (kenmerk van de placenta vlokken die met de duur van de zwangerschap meer wordt en de rijpheid weergeeft) werden vaker gezien als de moeder naast de IUGR ook een hoge bloeddruk had. Wij speculeren dat een chronische ontsteking van de vliezen vaker wordt gezien bij IUGR in combinatie met hoge bloeddruk omdat het afweersysteem van deze vrouwen wellicht anders reageert op de slecht ontwikkelde placenta. Mogelijk ontstaat dit verschil door een verhoogde afweerreactie van de moeder.

In het tweede deel van dit proefschrift hebben we gekeken naar de mogelijke rol van endoplasmatisch reticulum (ER) stress bij het verminderd binnendringen van de trofoblast en remodellering van de spiraal arteriën. Het ER is de fabriek van de cel waar alle eiwitten in het lichaam worden gemaakt, aangepast en opgevouwen voordat ze op transport gaan naar de locatie waar ze nodig zijn. Het ER communiceert met een eigen netwerk van signaaleiwitten met de cel. Veranderingen in de balans binnen de cel zorgen voor de "Unfolded Protein Response" (UPR) die als doel heeft deze balans weer te herstellen. Drie signaalcascades worden geactiveerd in de UPR waarin verschillende eiwitten een rol spelen. Deze drie signaalcascades worden gewoonlijk tegelijk geactiveerd afhankelijk van de ernst van de ER stress. Om een goed model te vinden om ER stress te onderzoeken zonder de invloed van factoren in de moeder is in **hoofdstuk 5** onderzocht of placenta's die aan weeën onderhevig zijn geweest ook ER stress hebben. Al eerder werd gevonden dat dit het geval was voor oxidatieve stress. In acht placenta's die blootgesteld zijn aan weeënactiviteit en acht placenta's die bij een keizersnede verkregen zijn is gekeken naar de bovenstaande ER stress markers. Verschillende eiwitten in de UPR waren verhoogd in de placenta's die aan weeën blootgesteld waren. We zagen dat één van de eiwitten, die we een kleur gaven en onder de microscoop bekeken, zich met name in de buitenste laag van de placentavlokken bevond en niet in de cellen in het midden van de vlok. Blootstelling aan weeën lijkt een nuttig model om ER stress in de placenta *in vivo* (in het lichaam) te bestuderen. Verder blijkt uit onze resultaten dat we voorzichtig moeten zijn met onderzoek in placenta's die blootgesteld zijn aan weeën, omdat zij door ER stress remming van eiwitproductie en functie kunnen laten zien. Het is daardoor moeilijk om oorzaak en gevolg te scheiden, omdat de processen die je wilt onderzoeken mogelijk veranderd zijn door de weeënactiviteit.

In **hoofdstuk 6** hebben we aangetoond dat ER stress opgewekt kan worden in een celkweek met trofoblastcellen die we onsterfelijk hebben gemaakt zodat ze oneindig

kunnen doorgroeien. Deze worden HTR8/SVneo cellen genoemd. Vervolgens hebben we aangetoond dat ER stress in deze HTR8/SVneo cellen minder goed een gel kunnen binnendringen, waarschijnlijk door verminderde activiteit van enzymen die de ruimte tussen de cellen kunnen afbreken: metalloproteinases (MMPs). Dit suggereert dat ER stress een negatieve invloed heeft op het binnendringen van de trofoblast en dus mogelijk betrokken is bij de ontstaanswijze van MPS. Om deze hypothese te testen hebben we trofoblastcellen (en dus weefsel) nodig uit de eerste drie maanden van de zwangerschap. Hier vind een belangrijk deel van de remodellering plaats. Helaas, zoals beschreven in **hoofdstuk 2**, is het zeer moeilijk, zo niet onmogelijk, om in die eerste maanden weefsel van een doorgaande zwangerschap te verkrijgen, waarbij we uiteindelijk ook de uitkomst (wel of niet pre-eclampsie) kunnen vaststellen danwel voorspellen. Om de rol van ER stress verder te ontrafelen zou toekomstig onderzoek gericht moeten zijn op het opheffen van ER stress met medicatie tegen ER stress. Dit zou in de verre toekomst een mogelijke behandeling kunnen zijn.

In het laatste deel van dit proefschrift hebben we gekeken naar risicofactoren voor hart- en vaatziekten bij de moeder na een MPS. Uit grote epidemiologische onderzoeken is gebleken dat vrouwen met een voorgeschiedenis van MPS een (sterk) verhoogd risico hebben op hart- en vaatziekten op latere leeftijd. Zoals in **hoofdstuk 3** al werd besproken zou dit mogelijk het gevolg kunnen zijn van een gezamenlijk onderliggend mechanisme. Van beide aandoeningen wordt gedacht dat het afweersysteem een belangrijke rol speelt bij de ontstaanswijze.

In **hoofdstuk 7** zijn de resultaten gepubliceerd van een studie waarin de rol van hart- en vaatziekten en immuunsysteem gerelateerde markers in herhaalde vroege pre-eclampsie wordt bepaald. Bij 150 vrouwen met een voorgeschiedenis van vroege pre-eclampsie (vóór 34 weken zwangerschap), werden 6-12 maanden na de bevalling factoren van het afweersysteem en risicofactoren voor hart- en vaatziekten gemeten. Naast cholesterol, glucose, HDL (goede cholesterol), LDL (slechte cholesterol), triglyceriden (vetten in het bloed) is ook gekeken naar factoren die verhoogd zijn bij ontsteking: CRP en fibrinogeen. De relatieve bijdrage van de markers aan het risico op herhaalde pre-eclampsie werd met een statistisch model bepaald. Van de 150 vrouwen ontwikkelde 42 (28%) van hen wederom een pre-eclampsie in de volgende zwangerschap. Ondanks dat vrouwen met een voorgeschiedenis van vroege pre-eclampsie vaker afwijkende risicofactoren voor hart- en vaatziekten hebben na de bevalling, vonden we geen relatie met een herhaling van pre-eclampsie. CRP en fibrinogeen waren wel gecorreleerd met een herhaalde pre-eclampsie. Deze uitkomsten waren niet te verklaren door bijvoorbeeld een verschil in gewicht. Het suggereert dat vroege pre-eclampsie inderdaad gerelateerd is aan een actiever immuunsysteem van de moeder. Het lijkt er op dat een overactief immuunsysteem van de moeder betrokken is bij de ontwikkeling

van vroege pre-eclampsie en daarmee kunnen we deze informatie wellicht gebruiken voor preventie strategieën.

Het risico op hart- en vaatziekten is niet alleen verhoogd in vrouwen met een vroege pre-eclampsie, maar ook bij een loslating van de placenta, zwangerschapshypertensie (Pregnancy Induced Hypertension, PIH) en late pre-eclampsie. In **hoofdstuk 8 en 9** zijn risicofactoren voor hart- en vaatziekten gemeten, 6-12 maanden na een zwangerschap met deze aandoeningen. Voor loslating van de placenta geldt dat deze vaak voorkomt samen met een pre-eclampsie, maar ook zonder. Daarom is in **hoofdstuk 8** gekeken in 75 vrouwen met een loslating van de placenta en werd de groep verder onderverdeeld in loslating in combinatie met en zonder pre-eclampsie of PIH en vergeleken met een normale gezonde groep zwanger vrouwen. Vrouwen met deze aandoening hadden significant hogere bloeddruk, gewicht, glucose, CRP, cholesterol, HDL en LDL, allen gecorrigeerd voor leeftijd, gewicht en nulliparitеit. Interessant is dat dit verschil blijft bestaan in de vrouwen met een loslating van de placenta zonder pre-eclampsie of PIH. Voor beide groepen geldt dat deze vrouwen mogelijk gebaat zijn bij een preventieprogramma. Tot op heden zijn er slechts enkele klinieken in Nederland die vrouwen met MPS controleren op risicofactoren voor hart- en vaatziekten. Onze data laten zien dat ook vrouwen met een loslating, al dan niet gecompliceerd door een pre-eclampsie of PIH, mogelijk ook baat kunnen hebben bij een dergelijke controle. Echter zijn de risico's op het ontwikkelen van hart- en vaatziekten op latere leeftijd een stuk kleiner dan bij vroege pre-eclampsie.

Voor PIH en late pre-eclampsie geldt ook dat het risico op hart en vaatziekten weliswaar verhoogd is maar niet zo hoog als bij vroege pre-eclampsie. Daarom vergeleken we in **hoofdstuk 9** risicofactoren voor hart- en vaatziekten tussen vroege pre-eclampsie, late pre-eclampsie en PIH om te kijken of dit verschil in risico ook al zichtbaar is in het verschil in risicofactoren in het bloed na de zwangerschap. In totaal werden 448 vroege pre-eclampsie patiënten, 76 late pre-eclampsie patiënten en 224 patiënten met PIH vergeleken met elkaar. Uit de resultaten blijkt dat inderdaad bij vroege pre-eclampsie glucose, insuline, triglyceriden en cholesterol significant hoger zijn dan bij PIH en late pre-eclampsie. Het voorkomen van hoge bloeddruk na de bevalling gaat van 45% in de vroege pre-eclampsie groep naar 39% van PIH en 25% van de late pre-eclampsie patiënten. Dit laat zien dat, ondanks het feit dat al deze vrouwen een verhoogd risico hebben op hart- en vaatziekten, de risicofactoren voor hart- en vaatziekten bij vroege pre-eclampsie patiënten het sterkst afwijken. Het is dus belangrijk om onderscheid te maken tussen deze verschillende zwangerschapscomplicaties en eventuele preventieprogramma's hierop aan te passen.

Hoewel dit proefschrift nieuwe inzichten belicht rondom de verschillende aspecten van MPS, zijn de mechanismen die ten grondslag liggen aan deze zwangerschapscomplicaties nog niet geheel ontrafeld. De verschillende theorieën betreffende de afwijkende spiraalarterieremodellering in MPS kunnen niet goed worden getest. Onderzoeken worden beperkt door het gebrek aan weefsel uit de eerste maanden van doorlopende zwangerschappen. We zullen daarom nieuwe methoden moeten vinden om dit probleem op te lossen en deze theorieën te toetsen.

Om onze hypothese te bevestigen dat het voorkomen van placenta-afwijkingen en risicofactoren voor hart- en vaatziekten zijn gerelateerd aan hart- en vaatziekten op latere leeftijd, moeten we in de toekomst gestructureerd en voor langere tijd deze vrouwen volgen. Het verzamelen van placenta en placentavaatbed biopten is hierbij essentieel. Het bestuderen van deze weefsels en de relatie met gezondheid van hart en vaten voor, tijdens en (lang) na de zwangerschap kan ons veel informatie geven over de ontwikkeling van MPS, maar ook over hart- en vaatziekten.

Vrouwen met MPS hebben een verhoogd risico op het ontwikkelen van hart- en vaatziekten op latere leeftijd en dit risico is het meest uitgesproken in vroege pre-eclampsie patiënten. Men kan zich afvragen of het zinvol is om al deze vrouwen te screenen voor risicofactoren. De recente richtlijn van de Nederlandse Vereniging Obstetrie en Gynaecologie heeft dit kritisch onderzocht. Voorlopig is hieruit gebleken en overeengekomen dat vooral de vroege pre-eclampsie patiënt baat heeft bij deze screening. Toekomstig onderzoek zal zich moeten richten op de haalbaarheid, de klinische relevantie en de kosteneffectiviteit van dergelijke screening en preventieve interventies voordat we het kunnen toepassen in de dagelijkse praktijk.

Chapter 12

List of Publications

Curriculum Vitae

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LIST OF PUBLICATIONS

(1) Veerbeek JH, Brouwers L, Koster MP, Koenen SV, van Vliet EO, Nikkels PG, van Rijn BB, Franx A. Spiral artery remodelling and maternal cardiovascular risk: the SPAR study. Under review

(2) Veerbeek JH, Post Uiterweer ED, Nikkels PG, Koenen SV, van der Zalm M, Koster MP, Burton GJ, van Rijn BB, Franx A. Biopsy techniques to study the human placental bed. Accepted for publication in Placenta

(3) Veerbeek JH, Hermes W, Breimer AY, van Rijn BB, Koenen SV, Mol BW, Franx A, de Groot CJ, Koster, MP. Cardiovascular disease risk factors after early-onset preeclampsia, late-onset preeclampsia, and pregnancy-induced hypertension. Hypertension 2015 Mar;65(3):600-606.

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CURRICULUM VITAE

Jan Veerbeek was born on August 24th 1981 in Zwolle. He attended high school at the Vechtdal College in Hardenberg, where he graduated in 2000. Following this, he studied Biology at the University of Groningen, specialising in Research and Medical Biology for the final two years. In his final year, he decided to pursue his dream of becoming a medical doctor and applied for the Selective Utrecht Medical Master (SUMMA), a post-graduate medical school aimed at training students to become medical doctors while providing a background in clinical research. He started his training in 2005 and completed clinical rotations at the 'Gelre Ziekenhuis Apeldoorn' in 2007, immediately after graduating as Medical Biologist at the University of Groningen in the same year. After obtaining his medical degree in the winter of 2009, he started as an Obstetrics and Gynaecology resident at the 'Diakonessenhuis' in Utrecht. Scientific research remained of great interest to him, so he decided to start a PhD in 2011 under the supervision of Arie Franx. For the last part of his PhD, Jan received the Termeulen stipend, which allowed him to continue his research at the University of Cambridge, with supervision from Graham Burton. Since April 2015 Jan has returned to the 'Diakonessenhuis' to work as a specialty registrar (AIOS) as part of the specialty-training programme at the University Medical Centre in Utrecht.

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