Placental syndrome

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early pregnancy adaptation and placental development

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General introduction

Placental syndrome and its clinical implications

A healthy pregnancy is often taken for granted. Most pregnancies indeed progress without complications and result in the delivery of a healthy neonate. However, a substantial number of women experience complications. Many 'great obstetrical syndromes' have recently been shown to be associated with disorders of deep placentation (see further).[1] This spectrum of pregnancy complications includes gestational hypertension (GH), preeclampsia (PE)/HELLP syndrome (acronym for Hemolysis, Elevated Liver enzymes and Low Platelet count), fetal growth restriction (FGR), spontaneous preterm labor, late spontaneous abortion and placental abruption.[1] We denominate this group as "placental syndrome", derived from the term "maternal placental syndrome" as given by Ray *et al*. to GH, PE, placental abruption and placental infarction.[2-4] Together the different entities in placental syndrome account for roughly 15-25% of pregnancies.[5-9] In addition, placental syndrome is an essential cause of both maternal and perinatal morbidity and mortality.[10-12] Although there remains much to be clarified regarding its etiology, it is believed that a common denominator is defective deep placentation, starting early in pregnancy with absent or defective spiral artery remodeling. [1,13-15] This deficient vasculature is thought to result from 'occult' cardiovascular risk factors, identified in women with placental syndrome which already exist prior to pregnancy, as first hypothesized by Sattar.[16] These occult risk factors are exacerbated by the metabolic stress of pregnancy, and contribute to the occurrence of placental syndrome. In line with this hypothesis, our research group has previously demonstrated a high-risk cardiovascular profile in women with a previous pregnancy complicated by placental syndrome. These findings include a subnormal plasma volume,[17] microvascular reactivity,[18] hypertension,[19,20] increased vascular resistance[19] and electrocardiographic abnormalities.[21] Several other investigators also have shown that capillary rarefaction is declined in women with gestational hypertension[22] and preeclampsia,[23] even before clinical onset.[24]

Aging also exacerbates the occult cardiovascular risk factors. There is growing evidence that women with a history of placental syndrome are at increased risk of cardiovascular disease later in life, as recently reviewed by Rich-Edwards *et al*.[25] In this way, placental syndrome may reveal women at increased risk of metabolic and vascular diseases in later life. Furthermore, intra-uterine growth restriction and fetal programming may also play important roles in the development of occult cardiovascular risk factors. Barker demonstrated that intra-uterine growth restriction is associated with increased risk of cardiovascular disease.[26-28] In agreement, infants born to hypertensive pregnancy[29] and preterm born infants[30] show diminished capillary rarefaction. Successively, this will direct to a continual cycling of risk factors through generations.[16]

Clinical picture of placental syndrome

Placental syndrome consists of different entities, all with a different clinical picture. PE/HELLP is the most complicated syndrome and is associated with both maternal and fetal complications. PE is defined as gestational hypertension (≥140/90 mmHg) with proteinuria (≥300 mg/24 hr).[10] It is a multi-systemic progressive disorder, in which clinical symptoms appear after 20 weeks of gestation. Commonly involved organs include the liver, kidneys and brain. Fetal complications include FGR and intra-uterine fetal demise. The role of the placenta is crucial for both the development and progression of the disease. The symptoms usually disappear within a few days after delivery of the placenta. The syndrome has been postulated as a two-stage disease; the pathogenic process of PE begins during early deep placentation in the first trimester, long before the manifestation of clinical symptoms.[31] It is now recognized that the other clinical diseases in placental syndrome are also associated with disorders of deep placentation, starting with absent or defective spiral artery remodelling.[1]

Pathophysiology of placental syndrome

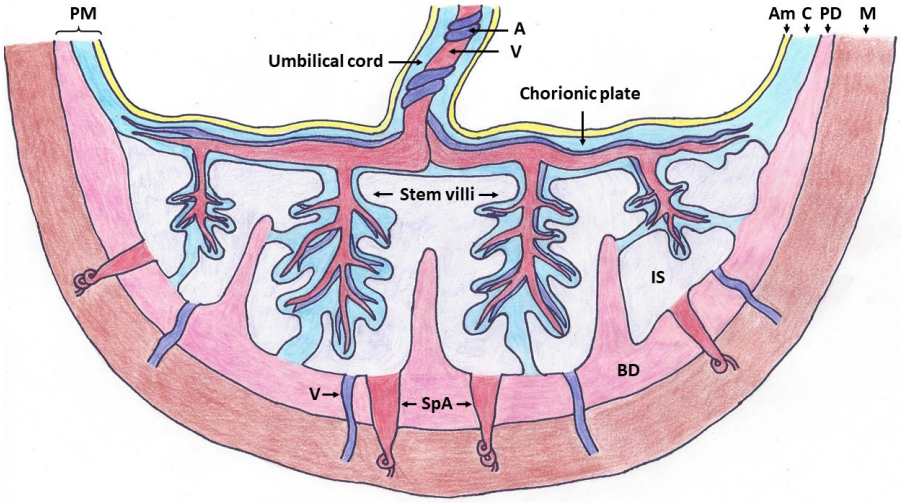
Normal placental anatomy

After fertilization, the zygote develops into a blastocyst surrounded by trophoblast cells, eventually giving rise to placental villi.[32] The placental villi carry the structure of a branching tree, originating from anchoring villi which are attached to the basal plate at the maternal side. Oxygen, nutrients and waste products are exchanged between the fetal and maternal circulation through the placenta. The maternal circulation provides oxygenated blood into the intervillous space by spiral arteries. The fetal circulation is located inside the villi and extracts the oxygen and nutrients from the intervillous space. Waste products are disposed in the opposite direction. The villous vessels drain in the umbilical cord and provide the fetus with blood (Figure 1).[32]

Spiral arteries in placental syndrome

Spiral artery remodeling is facilitated by invasive cytotrophoblast originating from the blastocyst-stage embryo.[33] The distal segments of the uterine spiral arteries are structurally modified (Figure 2). Endovascular cytotrophoblast cells replace the endothelium and intercalate within the smooth muscle cells of the tunica media. The initially high-resistance uterine arteries are now transformed into low-resistance vessels in which the muscle cells are broken down.[34] These changes effect in a high maternal blood flow into the intervillous space with a low blood pressure, thereby creating optimal circumstances for the fetus to extract nutrients and oxygen. In placental syndrome however, spiral artery adaptation is absent or defective.

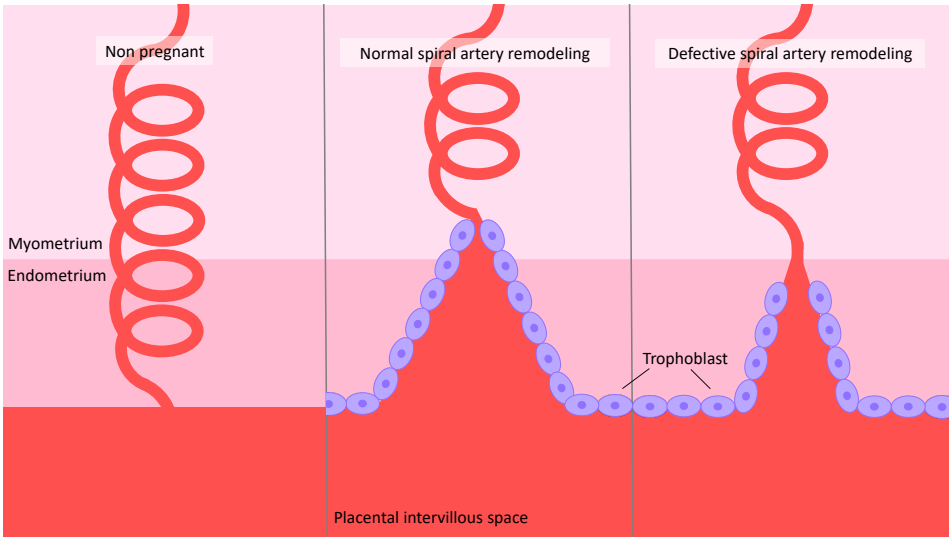
Figure 1. Schematic presentation of placental anatomy



Placental anatomy with the placental membranes (PM) composed of amnion (Am), chorion (C), and parietal decidua (PD); the umbilical cord with two arteries (A) and one vein (V); the chorionic plate; the villous parenchym composed of villi; the basal decidua (BD); and the myometrium from with spiral arteries (SpA) and veins (V) arise to supply the placenta with maternal blood into the intervillous space (IS).

In various phenotypes of placental syndrome, and predominantly in severe cases, a typical lesion, decidual vasculopathy, may occur. Generally, fibrinoid necrosis of the vessel wall is the most consistent feature of decidual vasculopathy and is sufficient for diagnosis.[35] Combined with the infiltration of foamy macrophages into the vessel wall the lesion is called acute atherosis. These changes are mostly found in the parietal decidua but can also be seen in the basal decidua.[35] Another type of decidual vasculopathy is mural hypertrophy,[36] which theoretically represents an early stage that possibly evolves into acute atherosis. Although the etiology of decidual vasculopathy is still unknown, these lesions are mostly seen in spiral arteries with deficient remodeling and in women with risk factors for cardiovascular diseases.[37] In addition, there are striking histological similarities with atherosclerotic lesions in larger arteries, hence the name "acute atherosis". Not only is the presence of decidual vasculopathy correlated with adverse perinatal outcome and placental pathology,[38] but lesion characteristics also correlate with increased placental pathology and adverse maternal and fetal outcome, most relevantly with perinatal mortality.[39] Women whose placentas exhibit decidual vasculopathy are at increased cardiovascular risk in the future.[40,41] It is obvious from the above that spiral artery maladaptation leads to a relative hypoxic state in the intervillous space (resulting in uteroplacental hypoxia). The presence of decidual vasculopathy has an additional negative effect on placental circulation. These changes embody large effects on placental functioning.

Figure 2. Schematic presenation of spiral artery remodeling



Placental development in placental syndrome

Placental development is largely controlled by the complex process of fetoplacental angiogenesis, which in turn is greatly influenced by placental oxygenation.[42] Hypoxia has a stimulating effect on certain angiogenic factors, namely vascular endothelial growth factor-A (VEGF-A) and its receptors. VEGF-A stimulates both VEGF-R1 (also known as flt-1, which results in endothelial tube formation) and VEGF-R2 (also known as KDR, which results in endothelial cell proliferation), resulting in a specific type of angiogenesis called branching angiogenesis. For this type of angiogenesis to take place, endothelial cells need to migrate and break down the surrounding matrix to form new branches of vessels. VEGF-R2 stimulation leads to upregulation of fibronectin, which stimulates cell migration.[43,44] It also leads to proteolytic activity by urokinase (uPA), which is part of the uPA system and inhibited by plasminogen activator inhibitors type 1 and 2 (PAI-1 and PAI-2).[45] The upregulation of VEGF-A by hypoxia is induced by HIF-1 (hypoxia induced factor 1).[46] HIF-1 is a transcription factor and a heterodimer consisting of an alpha and a beta subunit. HIF-1α accumulates under hypoxic conditions, dimerizes with HIF-1β and binds to the nucleus to act as a transcription factor. Its accumulation is enhanced by reactive oxygen species (ROS), including nitric oxide, superoxide and hydrogen peroxide,[47] while anti-oxidants are able to downregulate ROS. Indicating the complexity of angiogenesis, there are many other surprising factors able to influence VEGF-R1 and VEGFR-2. One example being low-density lipoprotein (LDL), which has been shown to dose-dependently reduce both receptors.[48] Furthermore, acute atherosis (presence of lipid laden foam cells in maternal spiral arterioles) as seen in placental syndrome

resembles the early stages of atherosclerosis,[49] which in turn has also been shown to be majorly influenced by hypoxia.[50] In addition, uteroplacental hypoxia is not the only type of hypoxia involved in placental syndrome.

Postplacental hypoxia is another type of hypoxia that has a role in placental syndrome. It occurs when there is an absent or reversed blood flow of the fetal umbilical artery, which is seen in fetal growth restriction (FGR). The fetoplacental circulation is compromised and causes reduced extraction of oxygen from the intervillous space, resulting in poor fetal oxygenation but higher than normal intervillous oxygen levels.[51] This relative hyperoxic state leads to an attenuation of the VEGF system and enhanced PlGF secretion. In normal pregnancies, branching angiogenesis takes place until mid-gestation. Hereafter non-branching angiogenesis predominates, stimulated by placental-like growth factor (PlGF) which only acts on VEGF-R1.[42,52] Overstimulation of VEGF-R1 in postplacental hypoxia subsequently results in more non-branching angiogenesis.[51] In contrast to VEGF-A, PlGF gene expression is not influenced by HIF.[53] The exact mechanism of gene expression regulation in postplacental hypoxia still remains to be elucidated. Complicating the understanding of the etiology of placental syndrome further, both types of hypoxia can also co-exist within the same woman. However, it is known that all these changes in placental angiogenesis already start to take place early in pregnancy, while the clinical outcomes of placental syndrome express in the second half of pregnancy. In addition, several factors involved in angiogenesis can be detected in the maternal circulation, thereby creating opportunities for early counselling.

Why study placental syndrome in early pregnancy?

Since changes in placental development in placental syndrome already occur previous to the expression of clinical symptoms, this would create opportunities to reveal women at high risk of developing the syndrome. Women at high risk are in particular those with a previous pregnancy complicated by the syndrome. All the different disorders in placental syndrome predispose to each other in a next pregnancy,[54] although there are still many women having a subsequent normal pregnancy. Preeclampsia for example has a recurrence rate of approximately 25%,[55] FGR 23%,[56] and preterm birth up to 52%.[57] It would be a great opportunity to evaluate recurrence risk of placental syndrome in early pregnancy, given the hypothesis of Sattar. Sattar explains that the maternal genotypes and phenotypes associated with increased risk of cardiovascular disease may also underlie fetal growth restriction and fetal programming, leading to a continual cycling of risk factors through generations. Although we cannot influence genotype, there is an opportunity in altering phenotype. Therefore, improving the maternal risk factor status early in pregnancy could benefit fetal development

and reduce cardiovascular risk of future generations.[16] Women with a previous pregnancy complicated by placental syndrome could be offered more intensive prenatal care in order to early detect signs and symptoms associated with the syndrome.[58] To detect women at high risk of developing recurrent placental syndrome, screening methods could be used.

Many investigators have studied screening methods in placental syndrome, but there are mixed results.[59] There are several defined WHO criteria for screening,[60] which could be translated to placental syndrome as follows: the screening should be performed before the onset of clinical disease creating room for therapeutic options (or more frequent follow up) and should be easy/minimally invasive and cheap to execute with a high sensitivity and specificity in a defined target population. Naturally, there should be scientific evidence of screening program effectiveness. By considering these criteria, an ideal screening method would be an easy and cheap biochemical marker that can be measured in maternal serum in the first or early second trimester of pregnancy. Although placental syndrome is a heterogeneous group of pregnancy complications, investigating markers associated with placental angiogenesis might offer opportunities to detect an accurate (set of) biomarker(s).

Concluding, placental development is greatly controlled by villous angiogenesis, which in turn is influenced by maternal cardiovascular status in placental syndrome. Therefore, in this thesis we study maternal circulating cardiovascular- and angiogenic factors and investigate villous vascular development both quantitatively and qualitatively in relation with placental syndrome.

Hypothesis

In this thesis we hypothesize that placental angiogenesis plays an important role in the pathophysiology of placental syndrome. We believe that in early pregnancy differences can be detected between those women who are destined to develop placental syndrome later on in that pregnancy and those who do not. We speculate that these differences are expressed in markers for cardiovascular risk and placental angiogenesis, in which cardiovascular risk markers show effects on angiogenesis. We further believe that by investigating placental angiogenesis we could gain more insight in the pathophysiology of placental syndrome.

Outline of this thesis

Aim 1 – Investigate possible biomarkers in the circulation of pregnant women in placental syndrome

This part investigates early-to-mid pregnancy adaptation in women at high risk of developing recurrent placental syndrome. **Chapter 1** explores the adaptation of the lipid profile to pregnancy women with recurrent PE and compares this to their counterparts who do not develop recurrent disease. Low density lipoprotein (LDL) is not only a marker for cardiovascular risk but is also involved in angiogenesis by reducing both VEGF receptors. In **Chapter 2** serum levels of asymmetric dimethylarginine (ADMA) and related metabolites are compared between women who developed recurrent placental syndrome (either GH or PE) and those who remained normotensive. ADMA impedes the synthesis of nitric oxide by inhibiting nitric oxide synthase (NOS) activity. NO, as well as other reactive oxygen species, in turn influence placental angiogenesis. In **Chapter 3** we investigate circulating fibronectin and plasminogen activator inhibitor-2 (PAI-2) levels in these women. Both biomarkers are also involved in branching angiogenesis. **Chapter 4** investigates anti-oxidant capacity, which is the primary defense mechanism against reactive oxygen species.

Aim 2 – Investigate placental microvascular development in placental syndrome

This part aims to provide more insight into the pathophysiology of placental syndrome and focuses on placental angiogenesis. **Chapter 5** summarizes normal placental angiogenesis and villous development and depicts the deviating angiogenesis in placental syndrome. The involved molecular mechanisms are discussed in uteroplacental and postplacental hypoxia, which are both observed in placental syndrome. We hypothesize that villous vascularization in placental syndrome is influenced by the type of hypoxic state. In **Chapter 6** we present a pilot study to test our hypothesis. The placental microvasculature is investigated in histology slides by stereological measurements. We subdivide our data according to 1) gestational age (<34 and ≥34 weeks of gestational age); and 2) clinical picture (early and late placental syndrome versus early and late normotensive controls). Finally, we perform a small sub-analysis in which we take umbilical artery blood flow into account as a measure for the type of hypoxic state.

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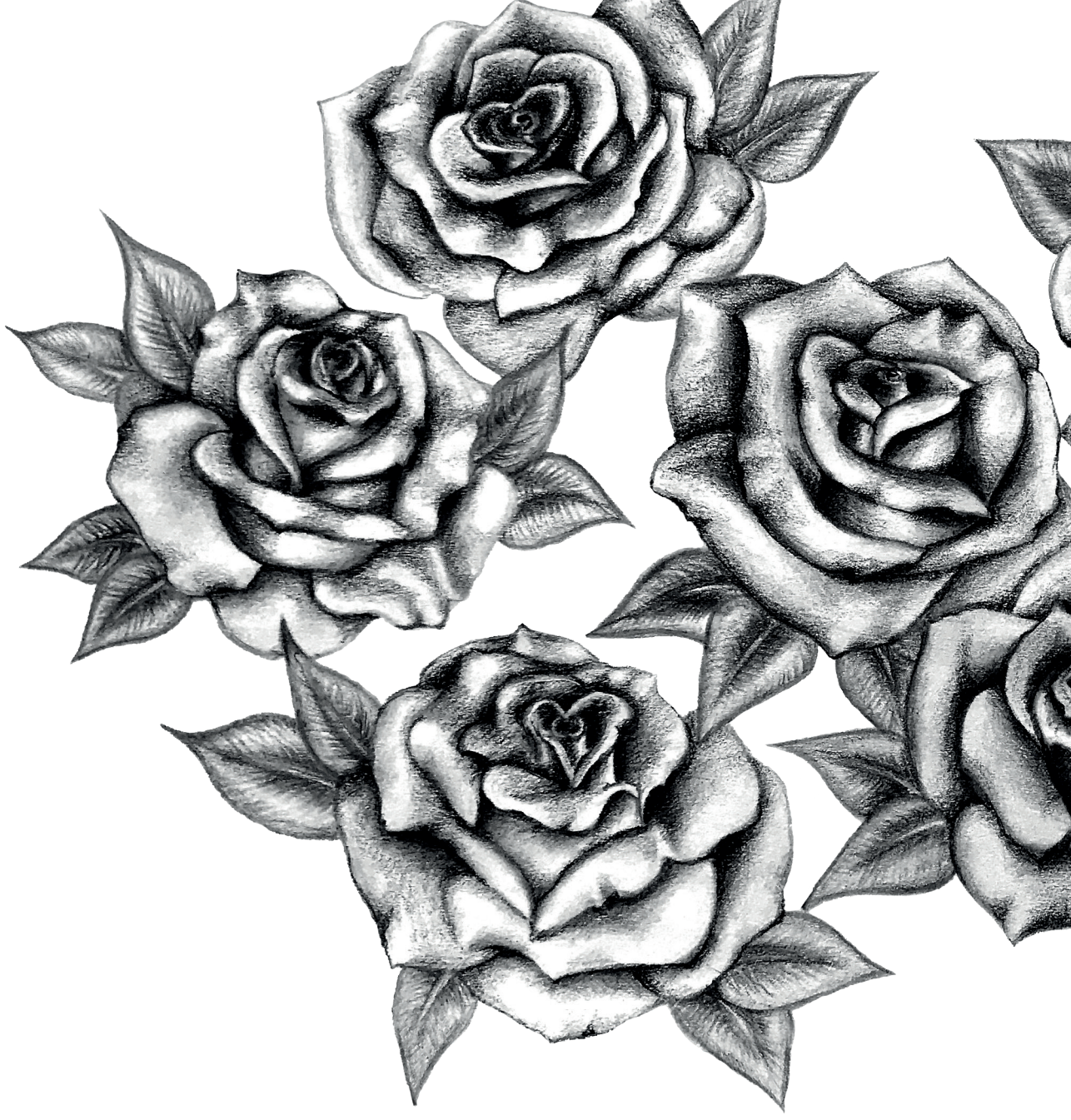
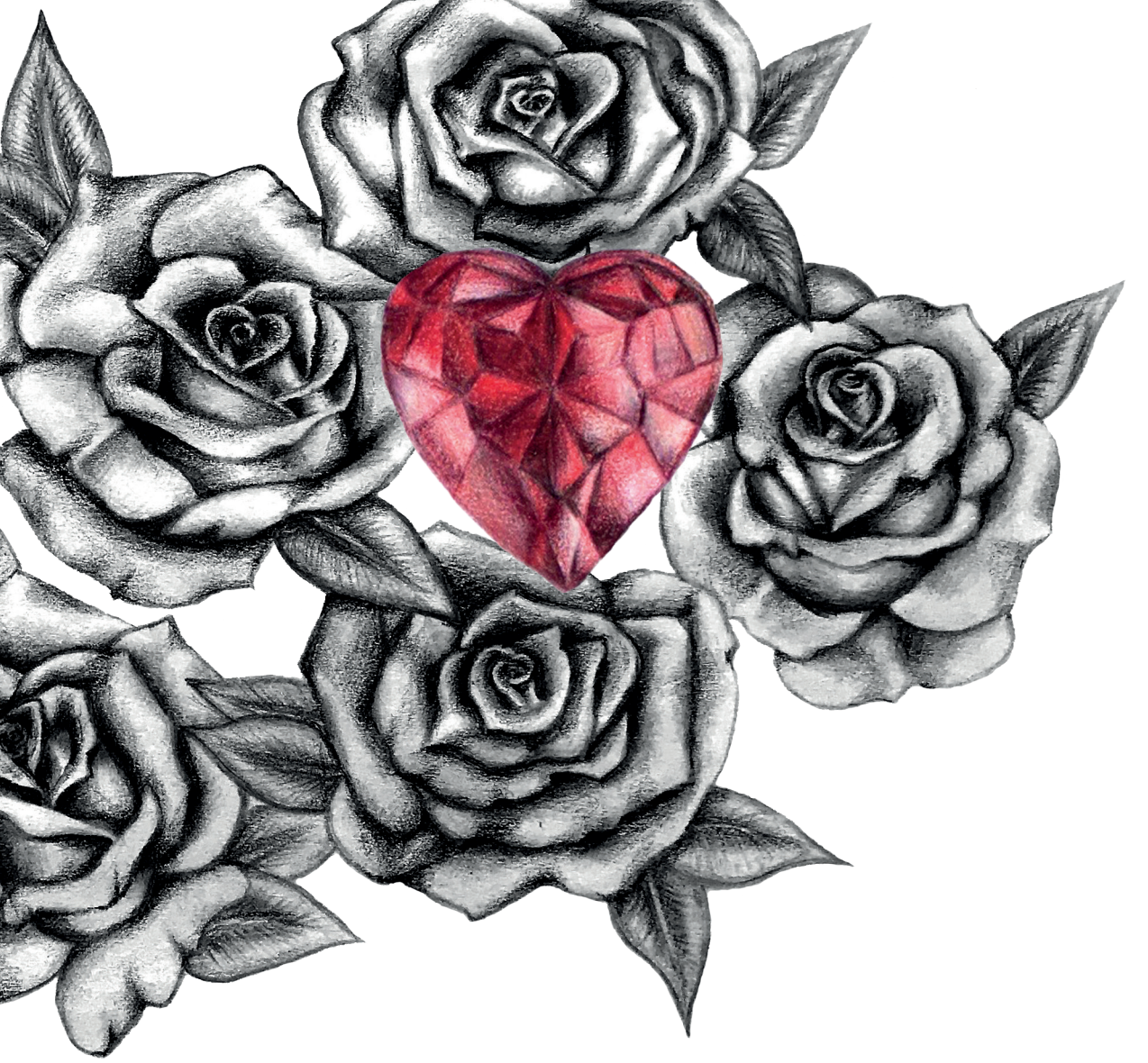




Part 1

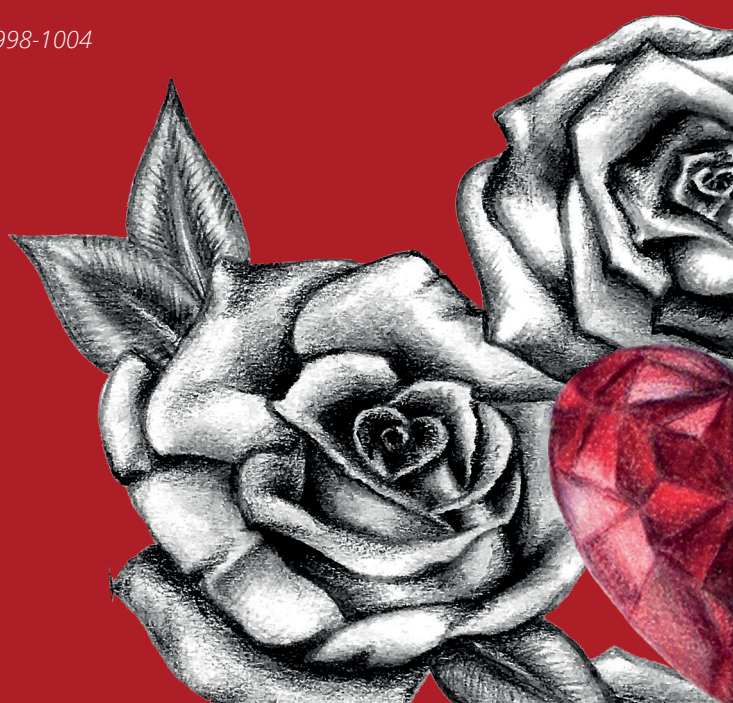
Circulating biomarkers during early pregnancy
and the recurrence of placental syndrome


2

Early-pregnancy changes in maternal lipid profile in women with recurrent preeclampsia and previously preeclamptic women with normal next pregnancy

S. Sep, C. Rijvers, L. Smits, M. van Bilsen, O. Bekers, L. Peeters

Reproductive Sciences 2011;18(10):998-1004



Abstract

Objective: To evaluate early-pregnancy changes in lipid profile in recurrent preeclampsia.

Methods: In this retrospective observational study, blood samples were obtained from 41 normotensive women with a history of early-onset preeclampsia preconceptionally and at 12 and 16 weeks in the next pregnancy. We assessed triglycerides (TGs), total cholesterol (TC), and high- and low-density lipoprotein cholesterol (HDL-C and LDL-C, respectively). We analyzed differences in longitudinal patterns between normal and recurrent preeclamptic next pregnancy using mixed-design repeated measurements analysis of covariance (ANCOVA).

Results: Eleven (28%) women developed recurrent preeclampsia. Eighteen (45%) women had a normal pregnancy. In normal pregnancy, LDL-C declines transiently in the first trimester ($p<0.01$). In women who develop recurrent preeclampsia later on this decline was absent. Moreover, from 12 weeks onward the elevating levels of HDL-C stagnates in women who subsequently develop recurrent preeclampsia ($p=0.02$).

Conclusion: These observations point to an abnormal early adaptation of lipid metabolism to pregnancy preceding clinical manifestation of preeclampsia.

Introduction

Pregnancy-induced changes in lipid metabolism promote the accumulation of maternal fat stores in the first half of normal pregnancy and enhance fat mobilization in late gestation. After an initial decrease during the first trimester, plasma lipids increase steadily throughout gestation.[1,2] Cholesterol is used in the placenta for the synthesis of steroid hormones, while fatty acids are oxidized and used for cellular membrane synthesis.[3] The changes in the concentrations of total cholesterol reflect changes in lipoprotein fractions. High-density lipoprotein (HDL)-cholesterol increases from the 12th week in response to the estrogen raise and remains high during gestation.[1,2] Total and low-density lipoprotein (LDL)-cholesterol progressively increases from the second trimester. Reduction of insulin sensitivity may contribute to an increase in triglyceride (TG) concentration from the first trimester onward.

In preeclampsia, plasma lipids have been reported to reach levels substantially above those seen in normal pregnancy.[4-10] However, in most studies, blood samples were collected cross-sectional after diagnosis.[5,6,11,12] The pathogenesis of preeclampsia originates in early pregnancy, presumably even during the first stages of placentation.[13] Unlike normal early pregnancy, when apoptotic syncytiotrophoblast nuclei are packed into syncytial knots and released in the maternal circulation,[14-16] in preeclampsia, impaired trophoblast differentiation is proposed to lead to mechanisms as necrosis and aponecrosis.[17] Eventually, the latter may cause systemic alterations of the maternal endothelium and inflammatory system. These effects subsequently lead to clinical manifestation of the disease.

Whether abnormal lipid metabolism or maladaptation in preeclampsia can be detected early in pregnancy is unclear, but this knowledge could improve our understanding of the pathophysiology of preeclampsia and potentially offer opportunities for prediction and/or counseling strategies. The objective of this study was to evaluate early pregnancy changes in lipid profile over time in formerly preeclamptic women and to compare longitudinal patterns between women who do and do not develop recurrent disease subsequently.

Methods

This is an observational cohort study performed at Maastricht University Medical Center in the Netherlands, in which we observed 41 women with a history of early-onset preeclampsia (diagnosis ≤34 weeks and delivery ≤37 weeks of gestation). The adaptation of the lipid profile to early pregnancy was compared between women who subsequently did and did not develop recurrent preeclampsia.

All measurements originate from high-risk obstetric care provided to women with a history of early-onset preeclampsia in our tertiary referral center. This high-risk care consists of a preconceptional check-up (at least 6 months after first delivery) combined with medical check-ups at 12 and 16 weeks pregnancy and aims to detect underlying abnormalities and abnormal adaptation to pregnancy.

The study protocol was approved by the institutional medical ethical committee (MEC 10-4-049). Although observations had been made prospectively, data were collected retrospectively. This study includes women with a history of early-onset preeclampsia (index pregnancy) and an ongoing (>20 weeks of gestational age) next pregnancy (target pregnancy) resulting in delivery between March 2002 and December 2009. These women had singleton pregnancies only.

Figure 1. Selection process of the study population and outcome groups.

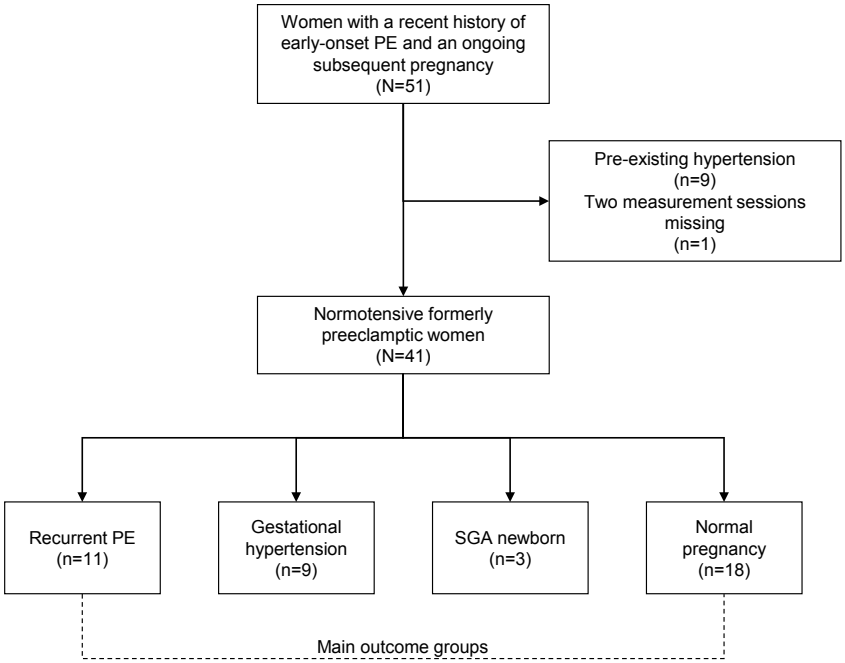


Figure 1 illustrates the selection procedure of the women included in this study. Fifty-one women with a history of early-onset preeclampsia were screened for eligibility. Nine women were excluded because of preexisting hypertension (elevated blood pressure [≥140/90 mm Hg] or use of antihypertensive medication), one because she participated

in the 12 weeks measurement session only. Eventually, the study population consisted of 41 normotensive formerly preeclamptic women.

We defined preeclampsia according to the guidelines of the American National working group on High Blood Pressure in Pregnancy, as de novo hypertension (blood pressure >140/90 mm Hg, occurring after 20 weeks of pregnancy) accompanied by proteinuria (>300 mg/24 h or in the absence of a 24-hour urine sample >30 mg/mmol creatinine).[18] We defined the HELLP syndrome as the concomitant occurrence of hemolysis (evidence on peripheral blood smear, serum lactate dehydrogenase [LDH] level >600 IU/L), elevated serum aspartate aminotransferase (ASAT, ≥70 IU/L) and/or alanine aminotransferase (ALAT, ≥70 IU/L), and a low platelet count (<100 × 10^9/L), as proposed by Sibai *et al*.[19] Newborns were considered small-for-gestational-age (SGA) when the birth weight was below the 10th percentile, based on the most recent Dutch birth weight reference curves.[20]

Before conception, and again at 12 and 16 weeks pregnancy, venous blood was sampled using a vacutainer after an overnight fast. After centrifugation, the blood was stored at −30°C. Centrifugation was performed at 4000 RPM for 10 minutes at 4°C. Serum concentrations of TG, total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were determined on a Beckman Coulter LX20 PRO Clinical Chemistry analyzer (Beckman Coulter, Fullerton, California). We calculated LDL-C by the Friedewald equation.[21] Glucose was determined on the Beckman Coulter LX20 as well. Insulin has been measured on an Auto-Delfia system (PerkinElmer Life and Analytical Sciences, Turku, Finland) with a fluoroimmunometric test based on a direct sandwich technique. Prepregnant body length and weight were determined in a standardized fashion, while wearing light clothing and using calibrated scales.

Statistical analysis was performed using SPSS version 15.0 for Windows. Missing values (15%) were imputed using a single imputation regression procedure.[22] Non-normal data were transformed logarithmically. We used mixed design analysis of covariance (ANCOVA) with a multivariate approach to test differences between longitudinal patterns during pregnancy. Within-group changes were tested by dependent *t*-test. We adjusted for body mass index (BMI) and circulating levels of fasting glucose and insulin to correct for the influence of estrogen and insulin resistance on lipid metabolism in early pregnancy. Data are presented as mean ± standard error (SE), unless otherwise noted. The means and 95%-confidence intervals presented in the figures were corrected for between-participant variability according to the methods of Loftus and Masson.[23,24] A result was considered statistically significant when $p<0.05$.

Results

Pregnancy outcomes are presented in Table 1. Preeclampsia recurred in 11 (27%) women, of which 3 had early-onset preeclampsia. Nine (22%) women had gestational hypertension without proteinuria. Maternal hypertensive complications were absent in 21 (51%) women, although 3 of them gave birth to an SGA infant. It follows that 18 (44%) women had a completely normal next pregnancy with healthy maternal and fetal outcome.

Table 1. Maternal and fetal pregnancy outcome of 41 normotensive women with a history of early-onset preeclampsia. Data are presented as n (%).

Maternal outcome	
Early-onset preeclampsia/HELLP	3 (7%)
Late-onset preeclampsia/HELLP	8 (20%)
Gestational hypertension without proteinuria	9 (22%)
No maternal hypertensive disease	21 (51%)
Fetal outcome	
Intra-uterine demise	1 (2%)
Extremely preterm birth (<32 wks)	1 (2%)
Preterm birth (32-37 wks)	4 (10%)
birth weight <1500 g	2 (5%)
SGA (<p10)	11 (27%)

Early-onset preeclampsia: diagnosis <34 weeks of pregnancy and delivery <37 weeks. SGA = small-for-gestational-age. Intra-uterine demise occurred at pregnancy duration <32 weeks with fetal weight <1500 g (<p2.3) in preeclamptic mother. Other fetal outcomes concern liveborns. The extremely preterm birth resulted from early-onset preeclampsia. Seven mothers with an SGA newborn had preeclampsia/HELLP.

Table 2 summarizes obstetric history, next pregnancy outcome, clinical characteristics, and lipid profile in the overall study population and in the 2 main outcome groups: recurrent preeclampsia (n=11) and normal pregnancy (n=18). Overall, mean gestational age of the previous pregnancy was 31 weeks, with a tendency towards shorter previous pregnancy duration of on average 1 week in women with recurrent preeclampsia. Infant's birth weight tended to be 155 g lower and was more often SGA in the latter. Mean maternal age tended to be 2 years higher in women with a normal next pregnancy. Hyperinsulinemia was more frequent in women with recurrent preeclampsia, while the proportion of women being overweight or obese was equal in the 2 groups. Except for a higher mean circulating level of TGs, the lipid profile tended to be more favorable in women with recurrent preeclampsia as compared to their counterparts with a normal next pregnancy.

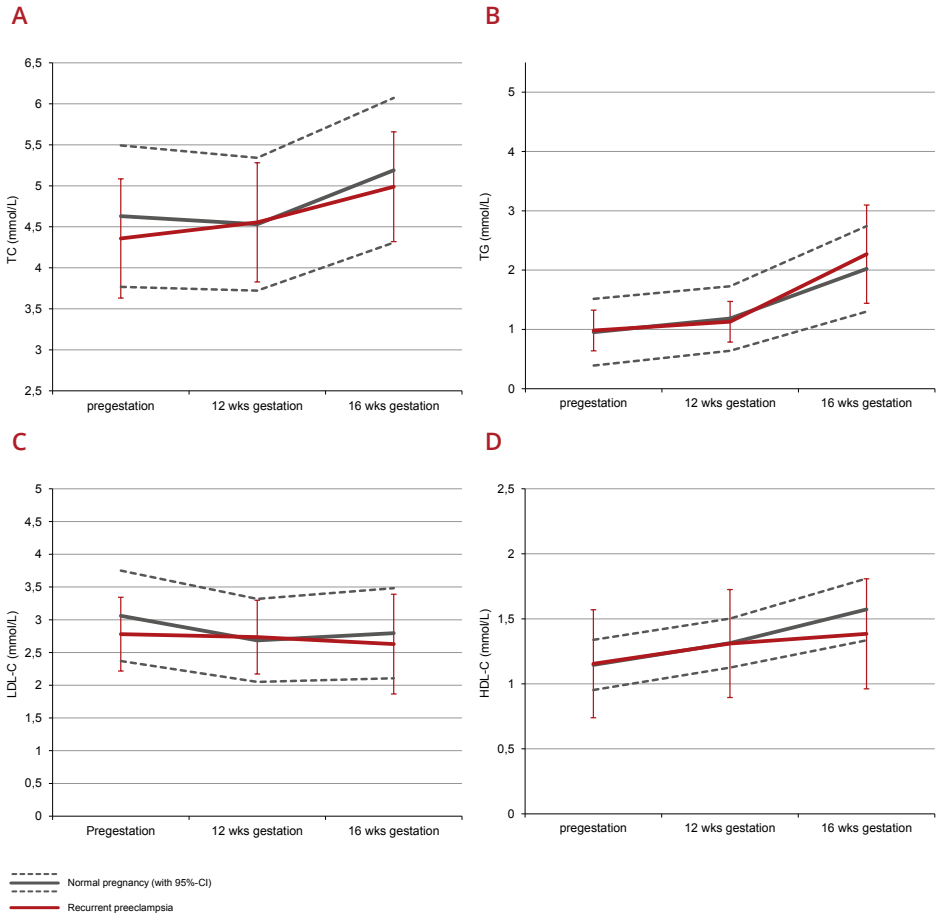
Table 2. Obstetric history, clinical characteristics and lipid profile in the overall study population of normotensive formerly preeclamptic women and the main outcome groups 'recurrent preeclampsia (PE)' and 'normal pregnancy'. Data are presented as mean ± SE or n (%), unless otherwise noted.

	Overall population	Recurrent PE	Normal next pregnancy	p
n	41	11	18	
Obstetric history				
HELLP syndrome	n=28 (68%)	n=8 (72%)	n=13 (72%)	0.98
Gestational age at birth (weeks)	31.0 ± 0.5	30.5 ± 1.0	31.7 ± 0.7	0.33
Birth weight newborn (g)	1120 (705-1840)	1100 (750-1700)	1255 (743-1953)	0.67
SGA newborn (<p10)	n=14 (34%)	n=4 (36%)	n=5 (28%)	0.70
Intra-uterine demise	n=6 (15%)	n=2 (18%)	n=2 (11%)	0.59
Clinical characteristics				
Time between pregnancy and first (pregravid) measurements (months)	10 (6-22)	10 (6-17)	13 (6-36)	0.46
Time between pregravid and 12 weeks measurements (months)	7 (5-14)	10 (5-20)	7 (5-11)	0.20
Age (y)	28.7 ± 0.6	27.3 ± 1.1	29.3 ± 1.2	0.25
BMI (kg/m^2)	25.6 ± 0.7	24.2 ± 1.2	26.7 ± 1.4	0.22
Obesity	n=6 (15%)	n=1 (9%)	n=4 (22%)	0.36
Hyperglycemia	n=2 (5%)	n=1 (9%)	n=1 (6%)	0.72
Hyperinsulinemia	n=10 (24%)	n=4 (36%)	n=3 (17%)	0.23
Lipid profile				
Total cholesterol (mmol/L)	4.5 ± 0.1	4.4 ± 0.2	4.6 ± 0.2	0.44
Triglycerides (mmol/L)	0.73 (0.56-1.30)	0.87 (0.56-1.44)	0.66 (0.50-1.40)	0.41
HDL-cholesterol (mmol/L)	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.95
LDL-cholesterol (mmol)	2.9 ± 1.1	2.8 ± 0.2	3.1 ± 0.2	0.33

p-value refers to comparison recurrent PE versus normal pregnancy. Hyperglycemia: fasting glucose level >5.6 mmol/L. Hyperinsulinemia: fasting insulin level >15.0 mmol/L. Maternal age at 12 weeks gestation. Time intervals, birth weight and circulating levels of triglycerides are presented as median (inter-quartile range).

Figure 2 displays the changes in lipid parameters with pregnancy in women with recurrent preeclampsia and those with a normal next pregnancy. In women with a normal pregnancy, LDL-C declined temporarily in the first 12 weeks of pregnancy (p<0.01). This decline was absent in women with recurrent preeclampsia (p=0.78). The difference between groups in the patterns of change in LDL-C in the first 12 weeks of pregnancy reached borderline statistical significance (p=0.08). High-density lipoprotein cholesterol increased significantly in both groups in the first 12 weeks of pregnancy (p<0.01 and p=0.04 in normal and preeclamptic pregnancy, respectively), but after 12 weeks, the increase in HDL-C was less strong in women who developed recurrent preeclampsia later on (p=0.02 for between-groups difference in patterns).

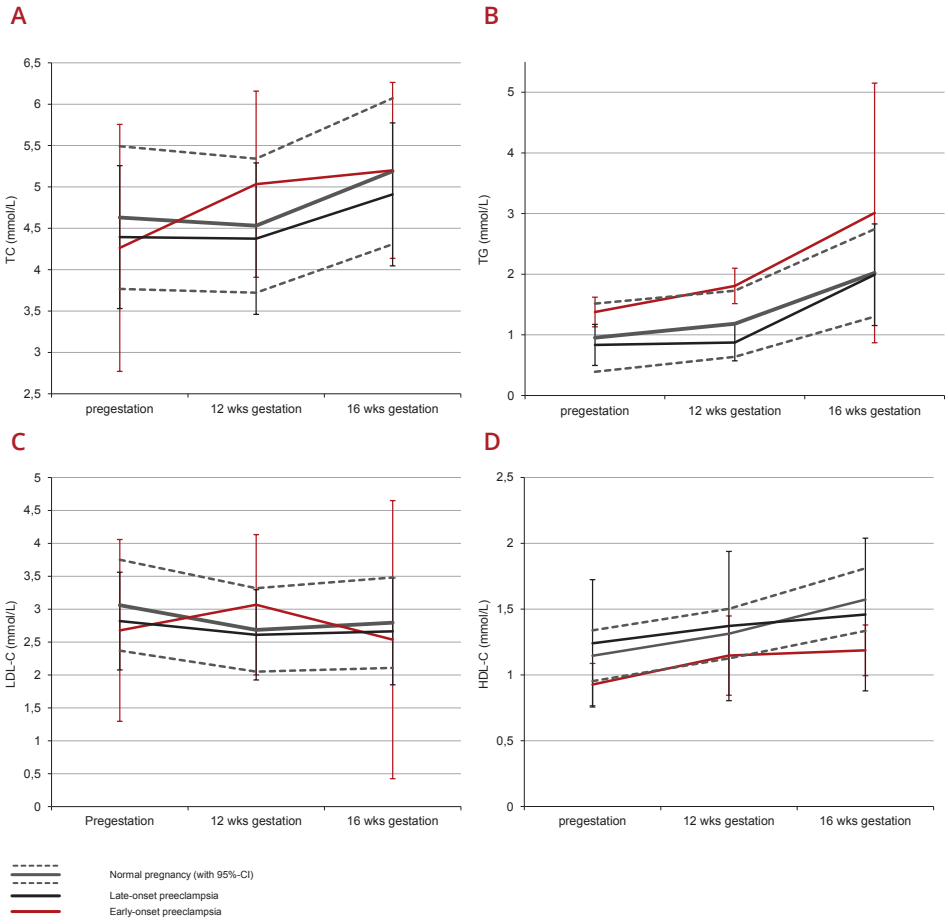
Figure 2. Circulating levels of total cholesterol (TC), triglycerides (TG), LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C) before and at 12 and 16 weeks pregnancy in 11 women with recurrent preeclampsia and 18 formerly preeclamptic women with a normal next pregnancy.



Data are presented as mean and corresponding 95%-confidence interval, adjusted for between-subject variability.

No statistically significant differences in altering circulating levels of TC were observed between groups (p=0.29). However, the tendency (p=0.50) toward a temporary decline in women with normal pregnancy seemed to be absent in women with recurrent preeclampsia. Changes in TGs did not differ between groups (p=0.74) and increased significantly between 12 and 16 weeks pregnancy (p<0.01 within both groups).

Figure 3. Circulating levels of total cholesterol (TC), triglycerides (TG), LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C) before and at 12 and 16 weeks pregnancy in 3 women with recurrent early-onset preeclampsia, 8 with late-onset preeclampsia and 18 formerly preeclamptic women with a normal next pregnancy.



Data are presented as mean and corresponding 95%-confidence interval, adjusted for between-subject variability.

Small sample size hampered the separate analysis of early- and late-onset recurrent preeclampsia. However, Figure 3 shows that the observations described above (primarily the lack of a temporary decline in TC and LDL-C) are particularly valid for recurrent early-onset preeclampsia. Circulating levels of HDL-C were lowest in women with early-onset preeclampsia, although the patterns of change were similar between the groups. Furthermore, the TG levels in women with early-onset preeclampsia reached values considerably above those seen in normal next pregnancies.

Discussion

Our results indicate that an initial transient decline in LDL-C is absent in women who developed recurrent preeclampsia. Moreover, from 12 weeks onward, the elevating levels of HDL-C stagnate in women who subsequently develop recurrent preeclampsia.

We believe that our observations are important, since they address the initial adaptation of the lipid profile during the subclinical stage of preeclampsia. To the best of our knowledge, early-pregnancy alterations of serum lipid levels in preeclampsia—with a preconception level as a starting point—have not been reported previously.

The initial fall in LDL-C observed in formerly preeclamptic women with a normal next pregnancy is in line with previous observations and is believed to result from placental uptake of cholesterol for the synthesis of steroid hormones.[1-3,25] Although results are contradictory, estrogen concentrations have shown repeatedly to be reduced relative to uncomplicated pregnancies.[26,27] Since estrogen is produced by syncytiotrophoblast, the latter could be the result of increased aponecrosis of syncytiotrophoblast cells in preeclampsia. As we did not measure circulating levels of estrogen in these women, we can only further speculate that the absence of a decline in LDL-C in women with recurrent preeclampsia is due to a lower placental steroid production. In a sub-analysis including the subgroup (n=5) of women without preeclampsia who gave birth to an SGA infant, the initial fall in LDL-C was absent as well, providing additional support for reduced placental steroid production to be involved. The absent decline in LDL-C could therefore be considered a marker for recurrent preeclampsia rather than a predisposing factor in its pathological pathway.

Reduced circulating levels of HDL-C in (recurrent) preeclampsia have been reported before.[28-30] High-density lipoprotein cholesterol is characterized by anti-inflammatory and endothelium-protective properties.[31] Reduced circulating levels of HDL-C may facilitate an overkill of the mechanisms that dispose released apoptotic trophoblast fragments. In turn, this can induce systemic activation and damage to endothelial cells, which may eventually result in preeclampsia.

Whether our results can be generalized to nulliparous pregnant women is unknown. To test the hypotheses generated, future research on this topic including the assessment of steroid hormones in nulliparous women is essential.

Conclusion

In conclusion, the characteristic transient decline in LDL-C in early gestation was absent in women who developed recurrent preeclampsia later on. Moreover, after the 12th week, the circulating level of HDL-C stopped rising in women who subsequently developed recurrent preeclampsia, indicating that the reduced levels of HDL-C reported in preeclamptic pregnancy result from maladaptation in the early (preclinical) stages. The insight that an abnormal early adaptation of the lipid metabolism to pregnancy precedes clinical manifestation of (recurrent) preeclampsia can be of pathophysiologic importance and could contribute to the development of prediction strategies in the future.

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3

Early-pregnancy asymmetric dimethylarginine levels in women prone to develop recurrent hypertension

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Abstract

Objective: To evaluate early-pregnancy levels of ADMA (asymmetric dimethylarginine) in recurrent hypertensive pregnancy.

Methods: In this retrospective observational study, blood samples from 35 normotensive women with a previous hypertensive pregnancy were obtained preconceptionally and at 12, 16 and 20 weeks in their next pregnancy. We assessed ADMA, symmetric dimethylarginine (SDMA), l -arginine and l -citrulline. We analyzed differences in longitudinal patterns between normotensive (NT, n=18) and recurrent hypertensive (HT, n=17) pregnancies by linear mixed models, with a sub-analysis for preeclampsia (PE, n=6).

Results: Pre-pregnant SDMA and l -citrulline were lower in HT. At 12 weeks, ADMA and ADMA/SDMA ratio correlated inversely with PAPP-A and β-hCG, respectively. In both groups, ADMA-related compounds changed inconsistently with advancing (mid-trimester) pregnancy, although in HT, l -arginine tended to decrease between 16 and 20 weeks, a decline consistent in PE.

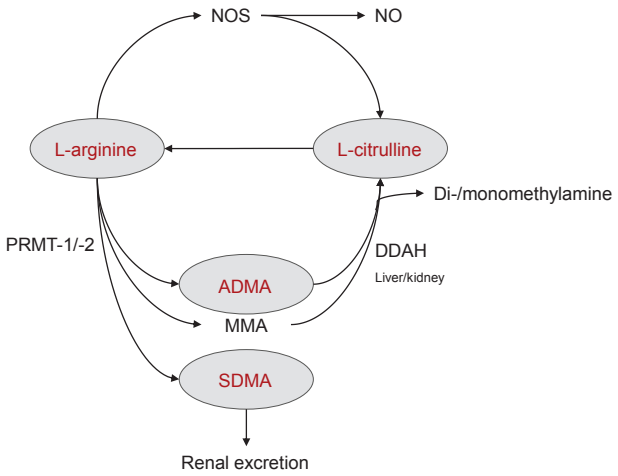
Conclusion: These data support a modest role for ADMA and related metabolites in the pathogenesis of hypertensive pregnancy.

Introduction

Preeclampsia (PE) affects 5-8% of first pregnancies and is a major cause of maternal and perinatal mortality and morbidity.[1,2] PE is a hypertensive pregnancy disorder that, along with gestational hypertension, recurs in either form in about 25% of next pregnancies.[3,4]

Although the etiology of PE is only partly understood, there is general agreement about a central role for endothelial dysfunction. The synthesis by the endothelium of nitric oxide (NO), an important regulator of vascular tone, is impeded by asymmetric dimethylarginine (ADMA) (Figure 1).[5]

Figure 1. ADMA metabolism and function.



Methylarginines are formed from L-arginine residues in proteins by enzymes called protein arginine methyltransferases (PRMTs). Asymmetric dimethylarginine (ADMA) is formed by PRMT type 1 and symmetric dimethylarginine (SDMA) is formed by PRMT type 2, while both PRMT types are able to form mono-methylarginine (MMA). ADMA as well as MMA are mainly metabolized by dimethylarginine dimethylaminohydrolase (DDAH) into L-citrulline and di- or monomethylamine, respectively. SDMA is mainly eliminated by urinary excretion, however, small amounts of ADMA and MMA are also excreted by the kidneys. Although ADMA and MMA both inhibit nitric oxide synthase (NOS) activity, ADMA can be regarded as the predominant endogenous inhibitor of NOS since physiological levels of MMA are approximately ten times lower. In addition, SDMA has been demonstrated to inhibit NO synthesis via competition with L-arginine uptake by endothelial cells. NOS uses L-arginine as a substrate to produce nitric oxide (NO) and L-citrulline. L-citrulline is reused to synthesize L-arginine.

As reviewed by Sibal *et al*,[6] there is evidence that elevated ADMA levels may either cause or aggravate endothelial dysfunction. Furthermore, raised circulating levels of ADMA coincide more often in cardiovascular disorders, such as hypertension, hypercholesterolemia and myocardial infarction. Therefore, chronically raised circulating levels of ADMA are gener-

ally regarded as a risk factor for cardiovascular disease.[7] Based on these inferences it is conceivable that raised circulating ADMA levels contribute to the endothelial dysfunction observed in PE, a concept supported by the observation in PE pregnancies that elevated ADMA levels precede the onset of clinical symptoms by several weeks.[8,9]

In this retrospective, observational study, we tested the hypothesis that circulating ADMA levels are already elevated in the mid-trimester of recurrent hypertensive pregnancy in parous women at high risk of PE because of their history. To this end, we measured circulating levels of ADMA, symmetric dimethylarginine (SDMA), L-arginine and L-citrulline before pregnancy and again at 12, 16 and 20 weeks amenorrhea.

Methods

This retrospective longitudinal, observational cohort study was performed at the Maastricht University Medical Center, Maastricht, the Netherlands between January 2002 and December 2010. Women at increased risk of PE (defined as having either gestational hypertension or preeclampsia in the previous pregnancy, n=42) were followed prospectively with repeated measurements before and during the first half of their next pregnancy. Biomarker assays were performed single-batch-wise after completion of the study. The study protocol was approved by the institutional medical ethical reviewing committee (MEC 10-4-049). We only included a total of 35 normotensive women in this study (7 women with pre-existing hypertension were excluded), subdivided into subgroups of women with eventually a normotensive pregnancy (NT, n=18) and a hypertensive pregnancy disorder in their next pregnancy (HT, n=17). Hypertensive pregnancy was defined as developing gestational hypertension or PE after the 20th week of pregnancy, for which the criteria are described below. In addition, we performed a sub-analysis on the 6 women in the HT group, who had developed PE. Finally, we compared the 8 women who gave birth to a growth-restricted infant (SGA) with the 27 other women who delivered from an infant with a normal birth weight (NoSGA). All participants were Caucasian.

We defined PE as gestational hypertension (blood pressure > 140/90 mmHg, developing after the 20th week of pregnancy), accompanied by new-onset proteinuria (≥ 300 mg in a 24-hour sample), using the criteria described in detail previously.[1] The HELLP syndrome was defined according to the criteria proposed previously,[10] i.e. the concomitant occurrence of hemolysis (evidence on peripheral blood smear, serum LDH level > 600 IU/L), elevated (≥ 70 IU/L) serum aspartate aminotransferase (ASAT) and/or alanine aminotransferase (ALAT) and a low platelet count (platelets below 100 x 10^9/L). A newborn

was considered small-for-gestational age (SGA) when his or her birth weight was below the 10th percentile, based on the Dutch birth weight reference curves.[11]

After an overnight fast, maternal venous blood samples were collected in the pre-pregnant state and again at a gestational age (GA) of 12, 16 and 20 weeks. The median time elapsed between the pre-pregnant measurement and conception was 10 weeks (IQR 0.5-28 weeks). All blood samples were immediately centrifuged and plasma samples stored at -30 °C for later analysis. The following metabolites were measured (Figure 1): 1) L-arginine, a precursor of NO, as well as of ADMA, MMA and SDMA; 2) L-citrulline, a byproduct in the NO synthesis and a metabolite of ADMA and MMA metabolism; 3) ADMA, an endogenous inhibitor of NO synthase (NOS); and 4) SDMA, an inert ADMA isomer. These metabolites were determined in plasma using an Acquity UPLC (ultra-performance liquid chromatography) separation module coupled to a Quattro Premier ESI-MSMS (Waters, Etten-Leur, the Netherlands). Separation of the components of interest was performed as described for the determination of amino acids [12]. Briefly, plasma samples were mixed with stable isotope labeled arginine and ADMA and deproteinized with SSA (5-sulfosalicylic acid). The supernatant was diluted with separation buffer prior to analysis. L-arginine, L-citrulline, MMA, ADMA and SDMA were detected in the multiple reaction mode (MRM) in electrospray ionization (ESI) positive mode.[13] Intra- and inter-run variations at physiological concentrations were less than 5% for arginine, ADMA and SDMA. We also calculated ADMA/SDMA ratio, as well as L-arginine/ADMA ratio. The ADMA/SDMA ratio is relevant as it is considered a marker for ADMA catabolism providing a crude indicator for dimethylarginine dimethylaminohydrolase (DDAH) activity. The latter is important as ADMA is mainly metabolized by DDAH, as opposed to SDMA, which is almost completely cleared by the kidneys. The L-arginine/ADMA ratio is considered to provide an indirect estimate for the endothelial capacity to produce NO.

Placental associated plasma protein-A (PAPP-A) and β-human chorionic gonadotropin (β-hCG) concentrations at 12 weeks pregnancy were available from the first-trimester prenatal screening for Down-syndrome. They were determined using the AutoDELFIA method (Perkin-Elmer, Finland).

Data are presented as mean and standard deviation (SD) for continuous variables and as number (%) for categorical variables. Demographic features were compared between groups using independent-samples t-test or Mann-Whitney U-test for continuous data, where data distributions were evaluated using histograms, and Chi-square or Fisher's exact test for categorical data. We compared the longitudinal trends of plasma concentrations of ADMA and the other metabolites between the subgroups (NT and HT) using a linear mixed model with a random intercept to account for dependency of repeated mea-

surements within the same individual. Associations between renal clearance, inflammation and placental function with metabolites at 12 weeks gestational age were tested with Spearman's correlation because of non-normal distribution of these variables along with modest sample sizes. A p-value ≤0.05 was considered to indicate statistical significance. All statistical analyses were performed using SPSS version 17.0 for Windows.

Results

Table 1 lists baseline demographics of the study population. None of these variables differed statistically significant between the normotensive and the hypertensive groups. Table 2 lists pregnancy outcome in the two subgroups. From the 17 hypertensive women, 6 had developed PE. The HT group differed significantly from the NT group by a less advanced gestational age at birth and a lower birth weight.

Table 1. Baseline demographic characteristics of the subgroups. Data are presented as mean (SD) for continuous variables and n (%) for categorical variables.

	NT	HT	p
n	18	17	
Prepregnant BMI	26.1 (4.3)	26.6 (5.5)	0.776
Age	32.3 (8.1)	30.4 (3.5)	0.374
Primiparous	n=15 (88%)	n=15 (94%)	0.324
Obstetric history:			
GH/PE	n=18 (100%)	n=17 (100%)	1.000
SGA	n=3 (18%)	n=7 (44%)	0.103
GA at birth (weeks)	32.2 (3.0)	30.8 (3.7)	0.230
Birth weight (grams)	1522 (761)	1244 (661)	0.273

NT = normotensive pregnancy, HT = hypertensive pregnancy, BMI = body mass index, GH = gestational hypertension, PE = preeclampsia, SGA = small-for-gestational age, GA = gestational age

Table 3 shows the rate of change with advancing pregnancy in mean arterial pressure (MAP) and the circulating levels of ADMA, SDMA, L-arginine, L-citrulline and their ratios within- and between the groups NT and HT. Prepregnant SDMA and L-citrulline levels were significantly lower in HT than in NT (p=0.019 and p<0.001, respectively). Unfortunately, pre-pregnant data on estimated glomerular filtration rate in HT were too scant (n=2) to compare with the NT group. Both groups acted in response to pregnancy with a similar 10-30% fall in circulating levels of ADMA, SDMA, and L-citrulline (NT: p=0.001, p<0.001 and p<0.001, respectively, and HT: p=0.112, p=0.085 and p=0.047, respectively), with negligible change between 12 and 20 weeks. Conversely, HT differed significantly from NT by a later fall in MAP occurring at 16 (p=0.022) instead of 12 weeks of pregnancy in the

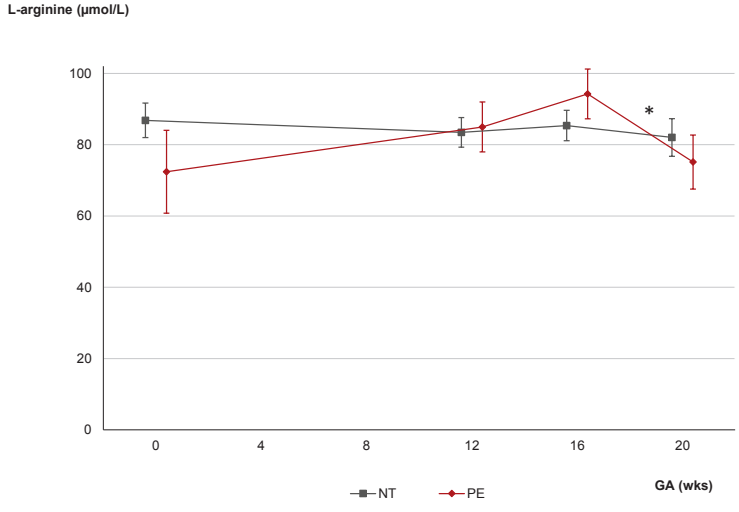
NT-group (p<0.001). L-arginine/ADMA ratio in HT also differed significantly from that in NT by an increase at 12 weeks (p=0.006) and a decrease at 20 weeks pregnancy (p=0.040). In addition, in the HT group L-arginine showed a marginally significant decrease at 20 weeks relative to 16 weeks (p=0.056), a change which was inconsistent in NT (p=0.633). In a sub-analysis, circulating L-arginine concentrations from 16 to 20 weeks of pregnancy decreased more in PE than in NT (p=0.040, shown in Figure 2). The other metabolites did not differ statistically significant between NT and PE. Likewise, NoSGA and SGA did not differ statistically significantly from one another (data not shown).

Table 2. Pregnancy outcome per subgroup. Data are presented as mean (SD) for continuous variables and number (%) for categorical variables.

	NT	HT	p
n	18	17	
Prophylactic antihypertensive treatment (< 20 wks GA)	n=9 (50%)	n=3 (18%)	0.088
GH	-	11 (65%)	
PE	-	6 (35%)	
GA at birth (wks)	39.3 (1.0)	37.3 (3.0)	0.009
Birth weight (grams)	3436 (503)	2749 (890)	0.008
SGA	n=2 (11%)	n=6 (35%)	0.089

NT = normotensive pregnancy, HT = hypertensive pregnancy, GH = gestational hypertension, PE = preeclampsia, SGA = small-for-gestational age, GA = gestational age

Figure 2. (Pre-)pregnant circulating concentrations of L-arginine in normotensive (NT) and preeclamptic (PE) women.



GA = gestational age (weeks)
* Significant decrease (p=0.040) in PE from 16 to 20 weeks of pregnancy

Table 3. (Pre-)pregnant mean arterial pressure (MAP) and circulating concentrations of ADMA, SDMA, L-arginine, L-citrulline and their ratios in normotensive and hypertensive women. Data are presented as estimated mean (SE).

		NP	12 wks GA	16 wks GA	20 wks GA	p for trend
MAP	NT	92.2 (2.3)	83.7 (2.0)†	82.9 (2.4)	83.5 (2.1)	0.407
(mmHg)	HT	93.1 (4.0)	86.3 (2.2)	80.8 (2.7)†	82.8 (2.3)	
ADMA	NT	0.50 (0.02)	0.41 (0.02)†	0.39 (0.02)	0.39 (0.03)	0.140
(µmol/L)	HT	0.42 (0.03)	0.37 (0.02)	0.40 (0.02)	0.39 (0.02)	
SDMA	NT	0.48 (0.02)	0.39 (0.02)†	0.40 (0.02)	0.39 (0.02)	0.145
(µmol/L)	HT	0.40 (0.03)*	0.35 (0.02)	0.39 (0.02)	0.37 (0.02)	
L-arginine	NT	86.3 (5.3)	83.5 (4.5)	85.5 (4.6)	82.2 (5.8)	0.132
(µmol/L)	HT	70.0 (6.9)	82.7 (4.5)	93.7 (4.6)	81.1 (5.3)	
L-citrulline	NT	24.4 (1.1)	16.5 (0.9)†	15.7 (1.0)	14.6 (1.1)	<0.001
(µmol/L)	HT	17.0 (1.3)*	14.5 (0.9)†	15.3 (1.0)	14.8 (1.1)	
ADMA/SDMA	NT	1.02 (0.05)	1.06 (0.04)	1.00 (0.04)	1.04 (0.05)	0.746
ratio	HT	1.09 (0.05)	1.07 (0.04)	1.05 (0.04)	1.08 (0.05)	
L-arginine/	NT	190.2 (13.9)	208.4 (12.1)	226.8 (12.4)	214.4 (15.0)	0.420
ADMA ratio	HT	171.9 (17.5)	224.1 (12.2)†	240.4 (12.5)	208.9 (14.0)†	

NT = normotensive pregnancy, HT = hypertensive pregnancy, NP = non-pregnant, GA = gestational age
P for trend gives the p-value for the overall difference in longitudinal pattern between both groups
* Significantly ($p<0.05$) lower pre-pregnant value in HT relative to NT
† Significant difference ($p<0.05$) within groups as compared to previous value.

At 12 weeks pregnancy, the variability in circulating levels of ADMA and related metabolites was larger than in the non-pregnant state. Therefore, we tested various correlations at this time point. Table 4 lists correlation coefficients of the measured parameters with placental markers at 12 weeks of pregnancy. ADMA levels correlated significantly inversely with the concomitantly measured placental associated plasma protein-A (PAPP-A), whereas ADMA/SDMA ratio showed a marginally significant inverse correlation with PAPP-A. ADMA/SDMA ratio was found to correlate negatively, and L-arginine/ADMA ratio marginally positively with β-hCG.

Table 4. Correlations of ADMA, SDMA, L-arginine, L-citrulline and their ratios with placental markers at 12 weeks of gestation.

	ADMA		SDMA		L-citrulline		L-arginine		ADMA/SDMA ratio		L-arginine/ ADMA ratio	
	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
PAPP-A	*-0.648*	*0.001*	-0.268	0.240	-0.399	0.073	-0.199	0.386	-0.432	0.051	0.325	0.151
β-hCG	-0.282	0.215	0.227	0.323	-0.170	0.461	0.253	0.268	*-0.466*	*0.033*	0.428	0.053

PAPP-A = placental associated plasma protein A; β-hCG = β-human chorionic gonadotrophin; r_s = correlation coefficient; p = p-value

Discussion

In this study, HT differed from NT by lower pre-pregnant values for SDMA and L-citrulline, though with a similar decline in response to pregnancy. Furthermore, HT differed from NT by a pregnancy-induced rise in L-arginine/ADMA ratio, a consequence of a trend towards a larger rise in L-arginine and a smaller decline in ADMA (along with SDMA) in HT. In neither group, we discerned a consistent trend in the metabolites with advancing pregnancy between 12 and 20 weeks, although in HT, circulating L-arginine levels tended to be lower at 20 weeks. The latter may be clinically relevant, more so as in a sub-analysis in women who had developed recurrent PE, we found that the fall in L-arginine levels at 20 weeks was statistically significant. Besides, at 12 weeks, we noticed that ADMA levels correlated inversely with PAPP-A and ADMA/SDMA ratio correlated inversely with β-hCG.

These findings differ from those reported by others, who found elevated rather than unchanged plasma levels of ADMA in the weeks preceding the onset of PE.[8,9] Possible explanations for this discrepancy are firstly, the higher prevalence of obesity in the reported study population,[8] a condition known to be associated with higher circulating ADMA levels,[14] and secondly, the measurements of ADMA being performed at a more advanced gestational age (about 24 weeks) in another study.[9] Additionally, our hypertensive group not only consisted of women with PE, but also of women with gestational hypertension.

Our results could be interpreted as follows. The lower pre-pregnant values for SDMA and L-citrulline, as well as the lower (although not statistically significant) L-arginine/ADMA ratio in the HT subgroup provide some circumstantial evidence for a preexistent lower activity of this important endothelial vasodilator system. The concomitant decline in plasma levels of ADMA and SDMA in early pregnancy and therefore unchanged ADMA/SDMA ratio, suggests no appreciable effect of pregnancy on ADMA metabolism and function. Therefore, this phenomenon seems a consequence of the pregnancy-induced physiologic hemodilution in this period of pregnancy. It is tempting to speculate that the data on blood pressure suggest that, initially, endothelial vasodilator activity may by higher in HT as compared to NT, possibly due to the slightly higher blood pressure in early pregnancy. PAPP-A correlates with placental volume[15,16] and β-hCG with placental function.[17,18] Assuming that tissue and plasma ADMA levels vary as a function of one another, it is conceivable that the inverse correlation between circulating ADMA levels and PAPP-A provides indirect support for the in-vitro observation that raised tissue ADMA levels interfere with extravillous trophoblast invasiveness[19] and that placental development may benefit from an increased tissue L-arginine/ADMA ratio (consistent with higher NO bioavailability). Although the latter provides support for a role of ADMA

and L-arginine/ADMA ratio in placental development, our data do not support predictive potential of these compounds as markers for disease severity later on in pregnancy, as proposed by others.[20,21]

The falling trend in L-arginine levels between 16 and 20 weeks pregnancy in the HT group and the consistent decline in the PE subgroup may be clinically relevant. As L-arginine has been shown to regulate ADMA metabolism dose-dependently by competing for DDAH,[22] it is conceivable that in high-risk pregnancies, L-arginine supplementation reduces the risk of hypertensive complications later on in pregnancy. In this context, it is interesting that several studies confirm beneficial effects of L-arginine supplementation on pregnancy outcome in gestational hypertension with and without proteinuria,[23,24] and when administered before the onset of clinical symptoms.[25] However, the latter requires confirmation in a sufficiently powered randomized controlled trial.

To the best of our knowledge, this is the first longitudinal study exploring both the effect of pregnancy on circulating ADMA levels, and the pattern of change in these levels in the first half of pregnancy in women at increased risk to develop recurrent hypertension. We realize that the modest group size limits the power of this study. Moreover, we followed the women for the first 20 weeks of pregnancy. In addition, only some women received prophylactic antihypertensive treatment (Table 2). Several authors have demonstrated antihypertensive drugs to be associated with a decline in circulating ADMA concentrations, although this has not been shown for all drugs and there does not seem to be a class effect.[26-28] The majority of women used methyldopa, for which the effect on ADMA levels has not been reported yet. Since most women who used antihypertensive drugs are in the NT-group, it is possible that this treatment diminished our group-effect on circulating ADMA levels. Yet, we were still able to demonstrate that HT differed from NT by a pregnancy-induced rise in L-arginine/ADMA ratio. Furthermore, all women had a severe hypertensive disorder in their history. Therefore, our group of women is not representative of all women with a (recurrent) hypertensive disorder. However, taking in mind the high recurrence risk, this is an important target group to study.

Conclusion

In conclusion, although the differences between both groups in the change in ADMA and related metabolites in response to pregnancy are small, our data do suggest at least a modest role of these compounds in the pathogenesis of hypertensive pregnancy in women at high risk for a recurrent hypertensive disorder.

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4

Circulating fibronectin and plasminogen activator inhibitor-2 levels as possible predictors of recurrent placental syndrome: an exploratory study

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Abstract

Objectives: Placental syndromes (PS) are characterized by endothelial dysfunction complicating placental dysfunction. Possible markers for endothelial dysfunction and amount of trophoblast are fibronectin and plasminogen activator inhibitor-2 (PAI-2), respectively. We aimed (1) to determine whether in women with recurrent PS (rPS), this complication is preceded by deviating fibronectin- and PAI-2-levels, and (2) whether this is dependent on pre-pregnant plasma volume (PV).

Methods: In 36 former patients, we determined fibronectin- and PAI-2-levels in blood-samples collected preconceptionally and at 12–16 weeks in their next pregnancy. Differences were analyzed between pregnancies with rPS (n=12) and without rPS (non-rPS, n=24) using linear mixed models, with sub-analyses based on pre-pregnant normal or subnormal PV.

Results: We observed higher fibronectin-levels at 12–16 weeks ($p<0.05$ and $p<0.01$, respectively) and lower PAI-2-levels at 16 weeks ($p<0.01$) in the rPS subgroup, the inter-group differences being larger in women with subnormal PV.

Conclusion: We showed that former PS patients who developed rPS have raised fibronectin- and reduced PAI-2-levels already in early/mid pregnancy. These deviations are even more prominent in women with subnormal pre-pregnant PV, supporting development of a 2-step screening program for former patients to identify the high-risk subgroup of women who may benefit from closer surveillance.

Introduction

Preeclampsia (PE) is an epitome of the placental syndrome (PS) constituting a major cause of maternal and perinatal morbidity and mortality worldwide. PE has an overall incidence of 4.6%,[1] with a higher chance of recurrence in about 25% of former preeclamptic patients.[2] Other clinical disorders within the spectrum of PS are gestational hypertension (GH), fetal growth restriction and/or fetal demise due to placental dysfunction. PS tends to recur in the next pregnancy in any of these different clinical entities.[3] Although the onset of PS is usually in the late second and third trimester, it is strongly associated with defects of placentation in early pregnancy,[4] characterized by defective remodeling of uterine spiral arteries[5] leading to placental dysfunction as indicated by both inadequate fetal supply of oxygen and nutrients and excessive release of placental endothelio-toxic microparticles.[6-8] Fibronectin is an extracellular matrix protein released by fibroblasts in the vessel wall in response to mechanical stimuli. Fibronectin promotes fibrin deposition in the extracellular matrix.[9] Therefore, this protein is regarded as a marker for endothelial stress.[10] On the other hand, plasminogen activator inhibitor-2 (PAI-2) is a placenta-specific protease inhibitor[11] released by trophoblast cells. The circulating PAI-2 level typically varies as a function of the total amount of trophoblast.[10] PAI-2 accelerates the conversion of plasminogen into plasmin, which in turn degrades a blood clot.[11] Increased fibronectin and decreased PAI-2 levels precede PE onset as early as at 20 weeks pregnancy.[12-14] Whether these abnormal peripheral levels also prevail earlier in pregnancy in women who later develop PE is unclear.

Previously, we identified a subnormal pre-pregnant plasma volume (PV) as an independent risk factor for recurrent PE in former patients.[15] We also found a higher cardiovascular sympathetic activity in women with a subnormal PV, which indicates a higher level of mechanical stress exerted upon their endothelium.[16]

We postulate that in former PS patients, abnormal circulating levels of fibronectin and PAI-2 at 12–16 weeks precede the development of recurrent PS (rPS) in their next pregnancy. To this end, we compared circulating fibronectin and PAI-2 levels before and at 12–16 weeks of the next pregnancy in a cohort of former PS patients who eventually did (n=12) and did not (n=24) develop rPS later on during their pregnancy. In addition, we explored whether the presence of a subnormal pre-pregnant PV enables to more accurately identify former patients who actually develop rPS in their next pregnancy.

Methods

This retrospective, longitudinal, observational cohort study was performed at the Maastricht University Medical Center, Maastricht, the Netherlands, between January 2002 and December 2010. Former 'severe' PS patients underwent extensive serial monitoring in a specialized obstetrical unit as part of their individual risk assessment. They underwent various examinations before and during the first half of their next pregnancy. PS was considered 'severe' when its clinical onset was early (<34 weeks) and/or the infant's birth weight was low (<2.3%) due to placental dysfunction as diagnosed on the basis of abnormal fetal biometry (asymmetric growth) and/or Doppler tracings of the uterine arteries. The study protocol was approved by the institutional Medical Ethics reviewing Committee (MEC 10–4-049). During the study period, we followed women prospectively. Deep-frozen serum samples from 50 women were available for further analyses. Since pre-existing hypertension is also associated with endothelial dysfunction,[17] we only enrolled normotensive women (excluding 14 women), all being primiparous, and subdivided them into 2 subgroups depending on whether they did (rPS, n=12) or did not develop recurrent maternal PS (non-rPS, n=24) later on during their pregnancy.

To determine the impact of the pre-existent endothelial reserve capacity, we stratified these 2 groups into subgroups based on their pre-pregnant PV as follows: (1) normal PV (NPV) and non-rPS in the next pregnancy (n=12); (2) NPV and rPS in the next pregnancy (n=5); (3) low PV (LPV) and non-rPS in the next pregnancy (n=12); and (4) LPV and rPS in the next pregnancy (n=7). All participants were Caucasian.

We defined PE as GH, as described in detail previously.[18] A newborn was considered small-for-gestational-age (SGA) when the birth weight was below the 10th centile, based on the most recent Dutch birth weight reference curves.[19] A PV below 48 ml/kg·lean body mass (kg·lbm) was defined as 'subnormal', as opposed to a 'normal' PV, which was ≥48 ml/kg·lbm.[20]

We estimated pre-pregnant PV by the I125-labeled albumin indicator dilution method.[20] We collected a venous blood sample after an overnight fast before pregnancy and again at 12-16 weeks in the next pregnancy. These samples were immediately centrifuged and the supernatant plasma stored at −80°C for the later measurement of fibronectin and PAI-2 levels. Fibronectin was determined nephelometrically (Image Beckman Coulter L02.2626. SE1, Beckman Coulter, Fullerton, USA). PAI-2 was determined by ELISA (IMUBIND PAI-2 ELISA, American Diagnostica Inc., Stamford, USA). PAI-2 measurement was only measured in the 2 samples obtained in pregnancy.

We performed statistical analyses using IBM SPSS Statistics for Windows version 20.0 (SPSS, Armonk, N.Y., USA). Data are presented as medians with interquartile range (IQR) for numerical variables and as numbers (with %) for categorical variables. IQRs are presented as 25th and 75th percentiles. Differences between the 2 subgroups were tested using the Mann-Whitney U test for numeric variables and the Fisher's exact test for categorical variables. The differences in longitudinal trends in outcome parameters between subgroups were assessed using linear mixed models with time (categorical: pre-pregnant, 12–16 weeks of pregnancy), group and group*time as fixed factors and an unstructured covariance structure for the repeated measurements. The interaction of time with the subgroups was assessed to evaluate whether the time course of a studied parameter differed significantly between the groups. Pregnancy outcome and serum levels of fibronectin and PAI-2 were also compared between subgroups based on either the development of rPS (hypertensive vs. normotensive pregnancy) or the presence of a normal or subnormal pre-pregnant PV (LPV vs. NPV). The latter included the following comparisons: NPV-non-rPS vs. NPV-rPS, LPV-non-rPS vs. LPV-rPS, NPV-rPS vs. LPV-rPS, and NPV-non-rPS vs. LPV-non-rPS. A p-value <0.05 was considered to indicate statistical significance.

Results

Table 1 lists baseline demography and Table 2 lists pregnancy outcomes of the study population. Both former PS patient groups were comparable, although the variation in most variables was large.

Table 1. Baseline demographic characteristics of the groups. Data are presented as median (IQR), unless noted otherwise.

	Non-rPS	rPS
n	24	12
Pre-pregnant BMI	26.9 (24.3-31.2)	26.3 (21.9-28.2)
Pre-pregnant PV	48.5 (44.0-52.8)	45.0 (43.0-49.8)
Age	34.8 (31.4-37.9)	32.5 (29.5-35.3)
Obstetric history:		
GH/PE	n=17 (70.8%)	n=12 (100%)
SGA/stillbirth	n=7 (29.2%)	n=0
Gestational age (weeks)	31.9 (30.1-34.2)	30.4 (28.6-33.8)
Birthweight	1280.0 (885.0-1817.0)	1252.5 (735.0-1643.8)

BMI = body mass index, PV = plasma volume (in mL/kg lean body mass), GH = gestational hypertension
PE = preeclampsia, SGA = small-for-gestational age, Non-rPS = no recurrent placental syndrome
rPS = recurrent placental syndrome
No significant differences were found

Table 2. Outcome of current pregnancy per group. Data are presented as median (IQR), unless noted otherwise.

	Non-rPS	rPS
n	24	12
MAP at 12 wks GA	81.0 (75.5-86.8)	88.0 (80.0-91.0)
GH	-	n=7 (58.3%)
GA at onset (weeks)		36.4 (29.6-38.3)
PE	-	n=5 (41.7%)
GA at onset (weeks)		36.0 (33.4-37.8)
GA at birth (weeks)	38.7 (37.7-40.3)	38.1 (37.2-40.1)
Birth weight	3280.0 (2986.3-3615.0)	3007.5 (2525.0-3310.0)
SGA	n=4 (16.7%)	n=3 (25%)

MAP = mean arterial pressure, GH = gestational hypertension, GA = gestational age, PE = preeclampsia, SGA = small-for-gestational age, Non-rPS = no recurrent placental syndrome, rPS = recurrent placental syndrome
No significant differences were found

Table 3 shows that the former patients in the rPS group differed from those in the non-rPS group by higher fibronectin levels at 12–16 weeks ($p<0.05$ and $p<0.01$, respectively) and by ≈30% lower PAI-2 levels at 16 weeks ($p<0.01$). These differences were similar when only the more homogeneous subgroup of former patients with a history of GH was included (data not shown). While fibronectin levels did not change over time, the PAI-2 levels increased consistently between 12 and 16 weeks ($p<0.001$ in both groups).

Table 3. (Pre-)pregnant circulating concentrations of fibronectin and PAI-2. Data are presented as estimated means (standard error), using linear mixed model.

		Pre	12 weeks GA	16 weeks GA
Fibronectin (mg/L)	Non-rPS	254.3 (19.1)	263.8 (15.7)	254.9 (17.2)
	rPS	317.8 (34.0)	336.4 (22.7)*	346.0 (24.7)*
PAI-2 (ng/mL)	Non-rPS	-	34.1 (4.0)	63.0 (4.1)†
	rPS	-	23.4 (5.9)	43.5 (5.8)*†

Non-rPS = no recurrent placental syndrome, rPS = recurrent placental syndrome, GA = gestational age
* Significant difference ($p<0.05$) compared to NT
† Significant within-group change ($p<0.05$) compared to 12 weeks GA

Table 4 shows that stratification of pregnancy outcome based on pre-pregnant PV did not lead to clustering of baseline demographic features in either of the former PS patient group.

Table 4. Pregnancy outcome in each subgroup based on pre-pregnancy plasma volume. Data are presented as median (IQR), unless noted otherwise.

	NPV-Non-rPS	NPV-rPS	LPV-Non-rPS	LPV-rPS
N	12	5	12	7
Pre-pregnant PV*	52.5 (52.0-56.8)	50.0 (48.5-52.5)	44.0 (42.3-46.5)	43.0 (41.0-45.0)
MAP at 12 weeks GA	79.5 (74.5-89.3)	80.0 (77.5-90.5)	82.0 (74.0-86.8)	88.0 (83.3-92.8)
GH	-	n=3 (60%)	-	n=4 (57.1%)
GA at onset (weeks)		35.3 (34.1-36.4)		38.0 (25.0-38.6)
PE	-	n=2 (40%)	-	n=3 (42.9%)
GA at onset (weeks)		36.8 (36.0-37.6)		34.0 (32.9-38.0)
GA at birth (weeks)	38.9 (37.8-42.0)	37.9 (37.1-39.4)	38.3 (37.7-40.4)	39.1 (37.3-40.3)
Birth weight (grams)	3225.0 (2597.5-3425.0)	3055.0 (2730.0-3290.0)	3300.0 (3097.5- 3685.5)	2760.0 (2230.0-3400.0)
SGA	n=2 (16.7%)	n=0	n=2 (16.7%)	n=3 (42.9%)

MAP = mean arterial pressure, GH = gestational hypertension, GA = gestational age, PE = preeclampsia, SGA = small-for-gestational age LPV = low plasma volume, NPV = normal plasma volume, Non-rPS = no recurrent placental syndrome, rPS = recurrent placental syndrome

* Per definition significant difference ($p<0.05$) between groups, no significant differences in other values found

Table 5 presents the results from the subgroup analysis after subdividing both the rPS and non-rPS groups into normal and subnormal PV subgroups. Despite comparable incidences of rPS in both PV subgroups (5 of 17 (29%) cases in the NPV subgroup and 7 of 19 (37%) cases in the LPV subgroup), this subdivision led to clear intergroup differences in fibronectin and PAI-2 levels. Circulating fibronectin levels in LPV-rPS subgroup were higher than in LPV-non-rPS subgroup ($p=0.001$). Even though pregnancy induced a decline in the fibronectin levels in LPV-rPS subgroup, these levels were still higher than in the 3 other PV subgroups at mid-pregnancy.

Meanwhile, in all subgroups except LPV-rPS, circulating PAI-2 levels increased between 12 and 16 weeks ($p<0.001$). As a consequence, PAI-2 levels at 16 weeks were lower in LPV-rPS than in both LPV-non-rPS and NPV-rPS ($p<0.01$ and $p<0.05$, respectively).

Table 5. (Pre-)pregnant circulating concentrations of fibronectin and PAI-2 for plasma volume-based subgroups. Data are presented as estimated means (standard error), using linear mixed model.

		Pre	12 weeks GA	16 weeks GA
Fibronectin (mg/L)†	NPV-Non-rPS	247.3 (22.3)	262.2 (19.7)	237.7 (20.3)
	NPV-rPS	218.9 (35.6)	266.4 (30.6)	262.6 (31.4)
	LPV-Non-rPS	262.5 (20.6)	265.3 (19.7)	273.3 (20.6)
	LPV-rPS	552.0 (70.0) *(vs LPV-Non-rPS)	370.7 (27.9) *(vs LPV-Non-rPS)**(vs NPV-rPS)	385.5 (28.7) *(vs LPV-Non-rPS)**(vs NPV-rPS)
PAI-2 (ng/mL)††	NPV-Non-rPS	-	40.3 (5.7)	65.5 (5.7)
	NPV-rPS	-	27.1 (9.2)	56.3 (8.6)
	LPV-Non-rPS	-	28.4 (5.5)	60.4 (5.7)
	LPV-rPS†	-	20.8 (7.6)	33.0 (7.6) *(vs LPV-Non-rPS)**(vs NPV-rPS)

GA = gestational age, LPV = low plasma volume, NPV = normal plasma volume, Non-rPS = no recurrent placental syndrome, rPS = recurrent placental syndrome
* Significant difference ($p<0.05$) between LPV-Non-rPS and LPV-rPS
** Significant difference ($p<0.05$) between NPV-rPS and LPV-rPS
† Within-group changes: LPV-rPS showed a significant rise in fibronectin levels at 12 and 16 weeks GA, compared to the pre-pregnant value
†† Within-group changes: except for LPV-rPS, all groups showed a significant rise in PAI-2 levels at 16 weeks GA.

Discussion

In this exploratory study, we found that in the subgroup of former PS patients with a subnormal PV, those who developed rPS in their next pregnancy differed from their counterparts who did not develop rPS by higher fibronectin and lower PAI-2 levels, mainly at 16 weeks of gestation. Our results are in line with our previous observations[21,22] that (1) both elevated fibronectin levels and subnormal PV prior to a next pregnancy predispose former PS patients to rPS in their next pregnancy and (2) rPS is preceded by indirect signs of placental insufficiency already at 12 weeks. Interestingly, in a previous study, we found that in the non-pregnant state endothelial activation seems to indicate a lower endothelial functional reserve capacity, and thus probably also a lower cardiovascular reserve capacity.[23]

Obviously, a major limitation of this study was the modest size of our study population which limits its statistical power. Therefore, we were unable to determine robust cutoff values for fibronectin and PAI-2, which are needed to estimate the positive and negative predictive values. In conjunction with the exploratory character of this study, we also decided not to correct for multiple testing or confounders but to perform subgroup analysis instead. The observed differences between non-rPS and rPS, and the results of the subgroup analysis support the view that former PS patients developing rPS in their next

pregnancy can be identified among their counterparts who do not develop rPS, on the basis of 3 parameters: (1) pre-pregnant PV, (2) circulating fibronectin and (3) PAI-2 levels at 16 weeks. In this context, it is relevant to mention that fibronectin is an easy, fairly accurate and non-invasive method to estimate endothelial function.[10] This also applies to PAI-2 levels, which vary as a function of total amount of trophoblast. In most cases, low peripheral PAI-2 levels in mid-pregnancy are consistent with the presence of a relatively small placenta, without providing information on placental function.[16] PAI-2 inhibits urokinase-type plasminogen activator and both are produced by trophoblast cells at the fetal-maternal interface. Reduced circulating PAI-2 levels at 12–16 weeks are often an early sign of imminent placental dysfunction possibly due to defective fibrinolytic activity in the intervillous space because of enhanced fibrin deposition, a common feature in pregnancies complicated by a PS. Lastly, our study groups show a heterogenous obstetrical history, which could be a source of bias or confounding factor. Yet, PE, GH, SGA and IUFD all predispose to each other in a subsequent pregnancy[3] and share a common placental etiology.[6,7] Nevertheless, despite the aforementioned limitations, this study is still unique because of the preconceptional and early- to mid-pregnant measurements.

Several other research groups reported that abnormal fibronectin[12,14,24-26] and PAI-2 levels[13,27-29] precede the onset of a PS, presumably from 20 weeks pregnancy onward.[12-14,26,27,29] One study measured fibronectin at 13 weeks, but the authors only performed a trend analysis based on 3 consecutive measurements in the first, second and third trimester.[30] Our data indicate that former PS patients - previously identified with a subnormal pre-pregnant PV - may already have raised fibronectin levels at 12 weeks.

Previously, we reported that a subnormal PV prior to pregnancy predisposes former PS patients to rPS in their next pregnancy.[15] Subnormal PV is a suitable surrogate for reduced venous capacitance and is accompanied by raised cardiovascular sympathetic activity,[16,20,31] both independently predisposing not only to rPS,[15] but also to premature cardiovascular morbidity.[32]

Conclusion

In conclusion, this study supports the concept that former PS patients with a subnormal PV destined to develop an rPS in their next pregnancy differ from their counterparts who do not develop rPS in their next pregnancy by elevated peripheral fibronectin levels together with reduced circulating PAI-2 levels at 16 weeks pregnancy. It follows that the former PS patients may benefit from a 2-step approach consisting of a pre-pregnancy PV measurement followed by a PAI-2 and fibronectin measurement at 16 weeks to determine

the risk of developing rPS. Obviously, our findings require confirmation in a much larger population to determine the predictive power of this model before being implemented in clinical practice. Currently, no suitable predictive tests are available to guide the management of former PS patients in future pregnancies. Thus, exploring novel strategies based on plausible biological molecules involved in the pathogenesis of PS women, could offer new perspectives to guide the management of these high-risk pregnancies.

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5

Early-pregnancy circulating antioxidant capacity in recurrent placental syndrome: an exploratory study

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Submitted

Abstract

Objective: Placental syndromes (PS), either maternal gestational hypertensive or fetal growth restricted, are characterized by increased oxidative stress. This study was designed to test the hypothesis that maternal (pre-)pregnant anti-oxidants levels relate to the maternal circulatory adaptation in PS relative to normal pregnancy.

Methods: Before, and at 12, 16 and 20 weeks pregnancy, we measured trolox equivalent antioxidant capacity (TEAC), uric acid (UA) and TEACC (TEAC corrected for UA) in maternal serum of former PS patients, who either developed recurrent PS (rPS; n=16) or had a normal next pregnancy (non-rPS; n=23). Concomitantly, we measured markers of vascular stress (fibronectin, CRP), and various circulatory variables (blood pressure, heart rate (HR), cardiac output (CO), creatinine clearance (CC), plasma volume and uterine artery Doppler velocimetry).

Results: rPS differed from non-rPS by higher pre-pregnant (p=0.02) and 20 weeks pregnant TEACC levels (p=0.04). Only non-rPS responded to pregnancy by significant rises in CO, HR, CC and CRP. Fibronectin levels were similar in both groups except for a higher level at 16 weeks in rPS.

Conclusion: rPS differed from non-rPS by absent hemodynamic adaptation to pregnancy and higher pre-pregnant and 20-weeks pregnant antioxidant levels. More research is needed to determine how these two phenomena are interconnected.

Introduction

Preeclampsia (PE) is a common placental syndrome (PS) and contributes importantly to maternal and perinatal morbidity and mortality worldwide. Its overall prevalence is about 4.6%[1] with a recurrence rate of about 25%.[2] Other PSs are gestational hypertension (GH), HELLP syndrome, eclampsia and fetal growth restriction (FGR) due to placental dysfunction. Although the clinical signs of PS usually develop after the late second trimester, PS is often preceded by impaired placental development in early pregnancy, consisting of defective uterine spiral artery remodeling along with poor migration of cytotrophoblast cells (CTBs) into the decidua.[3]

During early placentation, CTBs migrate upstream into the lumen of the spiral arteries, not only increasing their functional capacity, but also forming plugs that impede maternal blood to reach the intervillous spaces. The latter allows the embryo to develop in a low oxygen environment, providing protection of proliferating and differentiating STBs against damage by reactive oxygen species (ROS).[4] Defective spiral artery remodeling raises the risk of premature dislocation of trophoblastic plugs, and with it, a premature rise in local tissue pO_2.[5] Therefore, spiral artery remodeling is also pivotal for boosting the embryo's defense against ROS, more so as fetal antioxidant defenses are relatively weak in early gestation.[6] These inferences suggest that circulating maternal antioxidants levels in early pregnancy may be pivotal in the defense against ROS at the fetal-maternal interface.

Antioxidants have been shown to be decreased in both plasma[7-11] and placentas[9,12,13] of women with PE. Unfortunately, the few studies that explored antioxidant levels in the preclinical phase of PE, only reported data on a limited number of antioxidant markers instead of total antioxidant capacity.[9,14,15] Therefore, we evaluated total antioxidant capacity by measuring trolox equivalent antioxidant capacity (TEAC), in serum of former PS patients before pregnancy, and again at 12, 16 and 20 weeks pregnancy. Given that uric acid (UA) is a major determinant of TEAC[16,17] and also strongly associated with the development of PE,[18] we also measured UA concentrations and calculated antioxidant capacity in serum attributable to other antioxidants than UA (TEACC). Since oxidative stress not only impairs hemodynamic function[19] but also associates with increased vascular injury- and inflammatory markers,[20,21] we postulate that during early placental development, serum antioxidant capacity is reduced in women destined to develop recurrent PS compared to their counterparts without PS.

Methods

We performed a retrospective, longitudinal study at the Maastricht University Medical Center in Maastricht, the Netherlands. From 2002 onwards, women who had a severe PS in the preceding pregnancy underwent serial monitoring in a specialized obstetrical unit before, and during the first half of their next pregnancy. PS was considered severe, when its clinical onset was early (<34 weeks) and/or the infant's birthweight was low (< 2.3rd centile) due to placental dysfunction as diagnosed on the basis of abnormal fetal biometry (asymmetric growth) and/or Doppler tracings of the uterine arteries. We selected women with severe PS for two reasons: 1) in these women the underlying pathophysiology of inadequate trophoblast invasion and placental ischemia/reperfusion injury is thought to be more relevant than in milder cases as it is expected to lead to more contrast with our control group. This would provide a more solid base to test our hypothesis;[22] 2) by selecting women with a history of PS we can expect a higher recurrence rate of PS in our study population. This will raise the statistical power to this explorative study. The study protocol was approved by the institutional Medical Ethics reviewing Committee (MEC 10-4-049). All women gave informed consent. During the study period, we followed women prospectively with serial examination of various hemodynamic parameters and serum markers of the metabolic and the oxidative status. Since pre-existent hypertension not only is treated in most cases, but also associates with subnormal antioxidant levels,[23] we only enrolled women who were normotensive at initial screening. Post hoc, we subdivided these women into a subgroup who did (rPS) and a subgroup who did not develop recurrent PS (non-rPS) later on in that pregnancy. A technician blinded for the pregnancy outcome measured all serum TEAC and UA levels after completion of data collection.

We defined PE and gestational hypertension, as described in detail previously.[24] A newborn was considered Small-for-Gestational-Age (SGA), when birthweight was below the 10th centile, based on the most recent Dutch birth weight reference curves.[25] After an overnight fast, we collected a venous blood sample, which was immediately centrifuged and the serum stored at -30 °C for later analysis. We obtained ABTS (2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)) and UA from Sigma Chemical Co. (St. Louis, USA), and 2,2'-azino-bis(2-amidinopropane) dihydrochloride (ABAP) from Brunschwig Chemicals (Amsterdam, The Netherlands). Finally, Merck Biochemica (Darmstadt, Germany) provided $NaH_2PO_4{\cdot}H_2O$, $Na_2HPO_4{\cdot}2H_2O$, and trichloroacetic acid (TCA) and NaOH, which were of analytical grade purity.

For measuring total serum antioxidant status, we used the TEAC assay.[26] This assay determines the capacity of all antioxidants present in blood serum to scavenge $ABTS^{\cdot+}$. Serum deproteination was carried out by adding an equal volume of a 10% (w/v) TCA solution.

Serum samples were placed on ice for 5 minutes to complete deproteination, followed by centrifugation at 14.000g at 4°C for 5 minutes. In the TEAC decolorization assay, $ABTS^{\cdot+}$ $ABTS^{\cdot+}$ is generated chemically. $ABTS^{\cdot+}$ was produced by incubating a solution of 0.23mM ABTS and 2.3mM ABAP (2,2'-azino-bis(2-amidinopropane) dihydrochloride) in 100mM sodium phosphate buffer, pH 7.4 at 70°C until the absorption of the solution reached 0.70±0.02 at 734nm. The antioxidants in the serum react with $ABTS^{\cdot+}$ which leads to decolorization of the initially blue/green sample. The fall in the $ABTS^{\cdot+}$ concentration in a given time period, due to the reaction of $ABTS^{\cdot+}$ with the antioxidants present in the sample, is used to quantify the antioxidant capacity of the sample. So, the degree of decolorization reflects the amount of $ABTS^{\cdot+}$ that has been scavenged and can be determined spectrophotometrically. The resulting value was compared with the TEAC value for the synthetic antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman 2-carbonic acid). The TEAC value of a serum sample is expressed in mM and gives the level of a trolox solution, which corresponds with the antioxidant capacity in the serum sample.

For measuring UA in supernatant after denaturization, reversed-phase High-Performance Liquid Chromatography (HPLC) was used. TEACC was calculated by subtracting UA from TEAC to provide an indication of the change in other important contributors to overall antioxidant capacity.

PV was estimated as detailed previously.[27,28] Markers of vascular injury and inflammation (serum fibronectin and CRP, $mg.L^{-1}$), parameters of maternal hemodynamic adaptations (mean arterial pressure (MAP, mmHg), heart rate (HR, bpm), cardiac output (CO, $L.min^{-1}$), stroke volume (SV, mL), creatinine clearance (CC, $mL.min^{-1}$),[29] as well as uterine artery Doppler indices - as a measure of placental hemodynamic development - were performed as previously described.[29-31]

We performed statistical analyses using IBM SPSS Statistics for Windows (Version 23.0, Armonk, NY, USA). Data are presented as median with interquartile range (IQR) for numerical variables and as number (%) for categorical variables. Interquartile ranges (IQR) are presented as 25^{th} and 75^{th} percentiles. Intergroup differences were tested using Mann-Whitney U-test for numerical variables and Fisher's exact test for categorical variables. Spearman's rho correlations were used for associations between numerical variables. The differences in longitudinal trends in outcome parameters between groups (rPS vs. non-rPS) were assessed using linear mixed models (LMM) with time (categorical: pre-pregnancy, 12, 16 and 20 weeks of pregnancy), group and group*time as fixed factors. Different covariance structures for repeated measures ((heterogeneous) first-order auto-regression, compound symmetry, diagonal or independence) were considered, where the final structure was based on the smallest Akaike's information criterion (AIC). Missing outcome data are assumed to be miss-

ing at random (MAR), where the likelihood-based approach (no multiple imputation) was used. Results from LMM are presented as estimated means with standard error. A two-sided p-value smaller than or equal to 0.05 was considered to be statistically significant.

Results

Serum samples were available from 48 women with a severe PS in their preceding pregnancy and intending to conceive again. We excluded 9 women because of pre-existent hypertension, thus enrolling 39 women in our study. From these women, 16 developed rPS and 23 had a normal next pregnancy (non-rPS). Median time interval between pregnancies was in rPS 36 months (range 19-96 months) and in non-rPS 29 months (range 17-92 months). The difference was not statistically significant (p=0.866). Table 1 lists baseline characteristics and pregnancy outcomes of both study groups. All women were Caucasian. There were no statistically significant differences between the two study groups. On the other hand, outcome of the next pregnancy in rPS differed from that in non-rPS by an about two weeks shorter pregnancy duration and a 20% lower birthweight.

Table 2 shows the levels of TEAC, UA and TEACC at the four consecutive measurement points. There were no significant interaction effects of group with time. However, there was a consistently higher TEACC level at 12 weeks relative to pre-pregnancy, only in non-PS (p=0.001). TEACC levels in rPS tended to be higher than in non-rPS, the difference only being statistically significant before pregnancy (p=0.019) and at 20 weeks (p=0.041). Interestingly, the TEACC level in the rPS group had changed little throughout the measurement period. Meanwhile, UA and TEAC levels did neither differ appreciably between the two groups at the four measurement points, nor did they change in the course of the measurement period, except for temporarily lower levels relative to pre-pregnancy, in both groups at 12 and 16 weeks (all $p<0.05$).

Table 3 shows maternal parameters related to inflammation, and to the hemodynamic and placental adaptation to pregnancy in both study groups. At 16 weeks pregnancy, fibronectin levels were higher in r-PS than in non-rPS (p=0.008), this difference being only borderline significant at 12 and 20 weeks (p=0.059 and p=0.056, respectively). In both groups, we observed a rise in CRP in response to pregnancy (non-rPS $p\leq0.001$ at 12 weeks, rPS p=0.042 at 20 weeks of gestation), reaching a similar plateau by 12 weeks. However, because of only pre-pregnancy data of two women in rPS, it was not possible to determine, whether the pre-pregnant CRP level in rPS differed from that in non-rPS. The hemodynamic response to pregnancy was similar in both study groups. Nevertheless, the magnitude of the systemic changes was larger in non-rPS. Interestingly, the highest uterine PI and RI levels at 12 weeks

of pregnancy in the rPS group, were observed in women who also had the highest TEACC levels (r=0.736, p=0.028 and r=0.788, p=0.025, respectively, Table 4.).

Table 1. Baseline demographic characteristics and pregnancy outcome of the groups. Data are presented as median (IQR), unless noted otherwise.

	Non-rPS	rPS	p
n	18	21	
Age (years)	33 (30-36)	32 (29-36)	0.57
BMI (at 12 weeks GA)	27 (23-31)	27 (23-28)	0.47
Obstetric history:			
GH/PE	n=15 (83%)	n=20 (95%)	0.22
SGA/stillbirth	n=3 (17%)	n=1 (5%)	0.22
Birth weight (grams)	1630 (974-2509)	1283 (670-1841)	0.22
GA (weeks)	33 (31-36)	31 (28-34)	0.14
Current pregnancy:			
GH	-	n=10 (48%)	
GA at onset (weeks)		34.1 (27-38)	
PE	-	n=6 (29%)	
GA at onset (weeks)		36.0 (33-37)	
SGA	-	n=8 (38%)	
Birth weight (grams)	3417 (3149-3675)	2756 (2278-3365)	0.003
GA at birth (weeks)	39 (38-40)	37 (36-39)	0.01

BMI = body mass index, GA = gestational age, GH = gestational hypertension, PE = preeclampsia, SGA = small-for-gestational age, Non-rPS = no recurrent placental syndrome, rPS = recurrent placental syndrome

Table 2. (Pre-)pregnant circulating concentrations of TEAC, UA and TEACC. Data are presented as estimated means (standard error), obtained from linear mixed model analysis.

		Pre-pregnancy	12 weeks GA	16 weeks GA	20 weeks GA
	Non-rPS	***n=13***	***n=17***	***n=18***	***n=13***
	rPS	***n=9***	***n=20***	***n=20***	***n=16***
TEAC (mM)	***Non-rPS***	465 (19)	389 (15)***	394 (16)***	398 (23)**
	rPS	466 (23)	397 (15)***	405 (16)**	437 (24)
UA (mM)	***Non-rPS***	312 (20)	217 (14)***	233 (15)***	238 (19)***
	rPS	287 (243)	223 (15)**	230 (15)*	258 (20)
TEACC (mM)	***Non-rPS***	152 (7)	171 (6)***	161 (6)	160 (7)
	rPS	178 (8)†	181 (7)	179 (7)	180 (7)†

Non-rPS = no recurrent placental syndrome, rPS = recurrent placental syndrome, Pre = pre-pregnant, GA = gestational age

* Significant within-group change ($p<0.05$) compared to pre-pregnant value

** Significant within-group change ($p<0.01$) compared to pre-pregnant value

*** Significant within-group change ($p\leq0.001$) compared to pre-pregnant value

† Significant difference ($p<0.05$) compared to Non-rPS

Table 3. (Pre)pregnant maternal parameters for inflammation, hemodynamic, and placental adaptation to pregnancy in women with recurrent PS (rPS) and those without (Non-rPS). Data are presented as estimated means (standard error), obtained from linear mixed model analysis.

		Pre-pregnancy	n
Fibronectin (mg/L)	***Non-rPS***	262 (23)	10
	rPS	285 (31)	5
CRP	***Non-rPS***	1.3 (1.1)	6
	rPS	3.8 (2.4)	2
PV (mL)	***Non-rPS***	2357 (140)	10
	rPS	2073 (219)	5
MAP (mmHg)	***Non-rPS***	92 (3)	10
	rPS	94 (3)	5
Heart rate (beats/min)	***Non-rPS***	67 (3)	10
	rPS	71 (3)	5
Cardiac output (L/min)	***Non-rPS***	5.4 (0.2)	10
	rPS	5.3 (0.3)	5
Stroke volume (mL)	***Non-rPS***	78 (3)	10
	rPS	68 (4)	5
Creatinine clearance (mL/min)	***Non-rPS***	113 (8)	10
	rPS	124 (12)	5
Uterine artery PI	***Non-rPS***	2.9 (0.2)	9
	rPS	3.4 (0.3)	5
Uterine artery RI	***Non-rPS***	0.91 (0.0)	9
	rPS	0.92 (0.0)	5

CRP = C-reactive protein, MAP = mean arterial pressure, PI = pulsatility index, RI = resistance index
* Significant within-group change ($p<0.05$) compared to pre-pregnant value
** Significant within-group change ($p\leq0.01$) compared to pre-pregnant value
*** Significant within-group change ($p\leq0.001$) compared to pre-pregnant value
†† Significant difference ($p<0.01$) compared to Non-rPS

12 weeks GA	n	16 weeks GA	n	20 weeks GA	n
269 (21)	15	257 (19)	13	250 (22)	13
331 (24)	16	335 (20)††	16	313 (24)	15
8.6 (1.8)***	12	9.2 (1.5)***	13	7.5 (1.3)***	12
8.4 (1.9)	15	8.4 (1.5)	16	8.8 (1.3)*	15
2520 (124)	13	2723 (124)*	13	2845 (124)**	13
2667 (140)*	14	2733 (136)**	15	2644 (140)*	14
82 (2)***	15	82 (2)***	4	82 (2)***	13
85 (2)**	16	79 (2)***	8	82 (2)***	15
69 (3)	15	72 (2)**	15	74 (2)***	13
76 (3)	16	73 (2)	16	75 (2)	15
5.7 (0.2)	15	6.0 (0.2)**	15	6.1 (0.2)***	13
5.8 (0.2)	16	5.6 (0.2)	16	5.9 (0.2)	15
77 (3)	15	80 (3)	15	79 (3)	13
73 (3)	16	74 (3)	16	76 (3)	15
150 (7)***	14	144 (6)***	15	144 (7)***	13
135 (7)	16	135 (7)	16	135 (7)	15
1.9 (0.2)**	12	1.4 (0.2)***	13	1.0 (0.1)***	11
1.9 (0.3)**	12	2.0 (0.2)**	12	1.1 (0.1)***	14
0.72 (0.0)***	12	0.65 (0.0)***	13	0.58 (0.0)***	11
0.75 (0.0)**	12	0.75 (0.0)**	12	0.59 (0.0)***	14

Table 4. Correlations between TEAC, UA and TEACC and maternal parameters for endothelial stress, hemodynamic, and uterine adaptation 12 weeks of pregnancy in women with recurrent PS (rPS) and those without (Non-rPS). Data are presented as Spearman correlation coefficient.

		TEAC	UA	TEACC
	n	*Correlation coefficient*	*Correlation coefficient*	*Correlation coefficient*
Non-rPS				
Fibronectin	14	0.724*	0.484	0.209
CRP	12	0.560	0.522	-0.238
PV	9	-0.183	-0.300	0.533
MAP	14	-0.243	-0.161	-0.163
Heart rate	14	-0.018	-0.075	0.196
Cardiac output	14	-0.110	-0.093	-0.117
Creatinine clearance	14	-0.020	0.081	-0.147
Uterine artery Doppler PI	11	-0.364	-0.255	-0.427
Uterine artery Doppler RI	11	-0.264	-0.209	-0.291
rPS				
Fibronectin	15	-0.004	0.106	-0.221
CRP	14	0.486	0.504	0.002
PV	5	-0.100	0.500	-0.700
MAP	15	0.283	0.179	0.285
Heart rate	15	0.208	0.080	0.142
Cardiac output	15	0.203	0.103	0.229
Creatinine clearance	11	-0.297	-0.284	0.098
Uterine artery Doppler PI	11	0.300	-0.070	0.736*
Uterine artery Doppler RI	11	0.237	-0.165	0.788*

* Significant correlation ($p \leq 0.01$)

Discussion

The maternal hemodynamic changes in the first half of normal and PS-complicated pregnancy are accompanied by oxidative stress. In this study, we compared former PS patients, who developed rPS with those who had a normal outcome of their next pregnancy (non-rPS) with respect to pregnancy-induced changes in maternal circulating antioxidant capacity. To this end, we serially determined hemodynamic changes together with the circulating anti-oxidative capacity between pre-conception and 20 weeks pregnancy. Throughout this time interval, circulating TEACC levels in rPS were not lower, but even tended to be higher than in non-rPS, a difference that reached statistical significance in the pre-pregnant state and at 20 weeks. An increase was also seen in fibronectin levels, but these were only significantly higher in rPS at 16 weeks.

CRP levels in pregnancy did not differ appreciably between both groups. We had insufficient pre-pregnant CRP data in this study to enable intergroup comparison. Therefore, we mention in the context of this study our previous reports about a large fraction of apparently healthy, non-pregnant formerly PE women, who were found to have chronically raised plasma fibronectin and CRP levels suggesting subclinical vascular injury and chronic systemic inflammation.[32,33]

Changes in circulating biomarkers for oxidative stress and antioxidant capacity as indicators of normal pregnancy adaptation as well as predictors of the later development of PE, have been debated for decades.[13,34,35] The observation in the present study of higher pre-pregnant TEACC levels along with our previous observations of higher pre-pregnant CRP and fibronectin levels in a large fraction of former PS suggests that the higher pre-pregnant TEACC levels in rPS patients in this study may be an adaptive response to the chronic subclinical vascular damage and chronic low-grade inflammation.[33] This adaptive response is supported by the high positive correlation between TEACC and uterine artery Doppler PI/RI values, since increased uterine PI and RI are associated with defective spiral artery remodeling[36] and therefore with increased oxidative stress. Such a redox mediated, adaptive response is not unusual in nature.[37]

Reports on the anti-oxidant levels in relation to PS are conflicting with some studies showing decreased[8-11,38] and others increased circulating antioxidant levels[39-41] in PS pregnancies. Similarly, studies measuring antioxidant status in the pre-clinical phase of the disorder showed either decreased[14] or equal[15] levels. Some of the conflicting reports are probably related to the use of different methods to assess the anti-oxidant status, e.g. by determining distinct anti-oxidative enzymes or total antioxidant capacity, or by whether the anti-oxidant status was corrected for UA levels, as the endocrine environment of pregnancy is known to influence UA levels.[42] On the other hand, it is also conceivable that systemic circulatory stress has a much larger impact on TEACC levels than the changes in anti-oxidant status in the relatively small surface area of the fetal-maternal interface.

Our findings do not support a direct link between pregnancy-dependent changes in circulating antioxidant capacity and abnormal placental development in PS. At any rate, it is conceivable that the pre-existent subclinical vascular damage together with the chronic low-grade inflammation are responsible for both the trend to higher antioxidant capacity and the defective placental development, which precedes symptomatic rPS. Thus, rPS may continue to develop in spite of the already activated anti-oxidant system. Apparently, the activated anti-oxidant system in rPS pregnancies does not play a protective role but is merely an epiphenomenon of some other latent cardiovascular or metabolic

abnormality that interferes with early placental development. It is interesting to interpret these results in view of larger randomized controlled trials which did not show any effect of antenatal anti-oxidant therapy with vitamin E and/or C in preventing PE.[43,44]

Strengths of this study are the inclusion of longitudinal data on anti-oxidant capacity from preconception until mid-pregnancy, and the simultaneous measurement of various circulatory parameters. This allowed us to study the association between oxidative stress and maternal hemodynamic adaptation in a group of women at high risk of developing PS. Yet, we are aware of the modest sample size which limits the power of this study. On the other hand, the demonstration of statistically significant differences in this exploratory study is encouraging and may enable sample size calculations for larger prospective studies. We are also aware of the consequence of studying this particular high-risk population, which is known to contain a relatively large fraction of women with some subclinical disorder that may influence the endpoints of this study. Nevertheless, the high recurrence rate of PS in such a study population enabled us to explore in a relatively small group of patients.

Conclusion

In conclusion, this study showed that recurrent PS pregnancies are preceded by an increase in anti-oxidant capacity, presumably induced by subclinical vascular injury and low-grade chronic inflammation. More research is needed to determine how these phenomena are interconnected to improve the management strategy of high-risk pregnancies.

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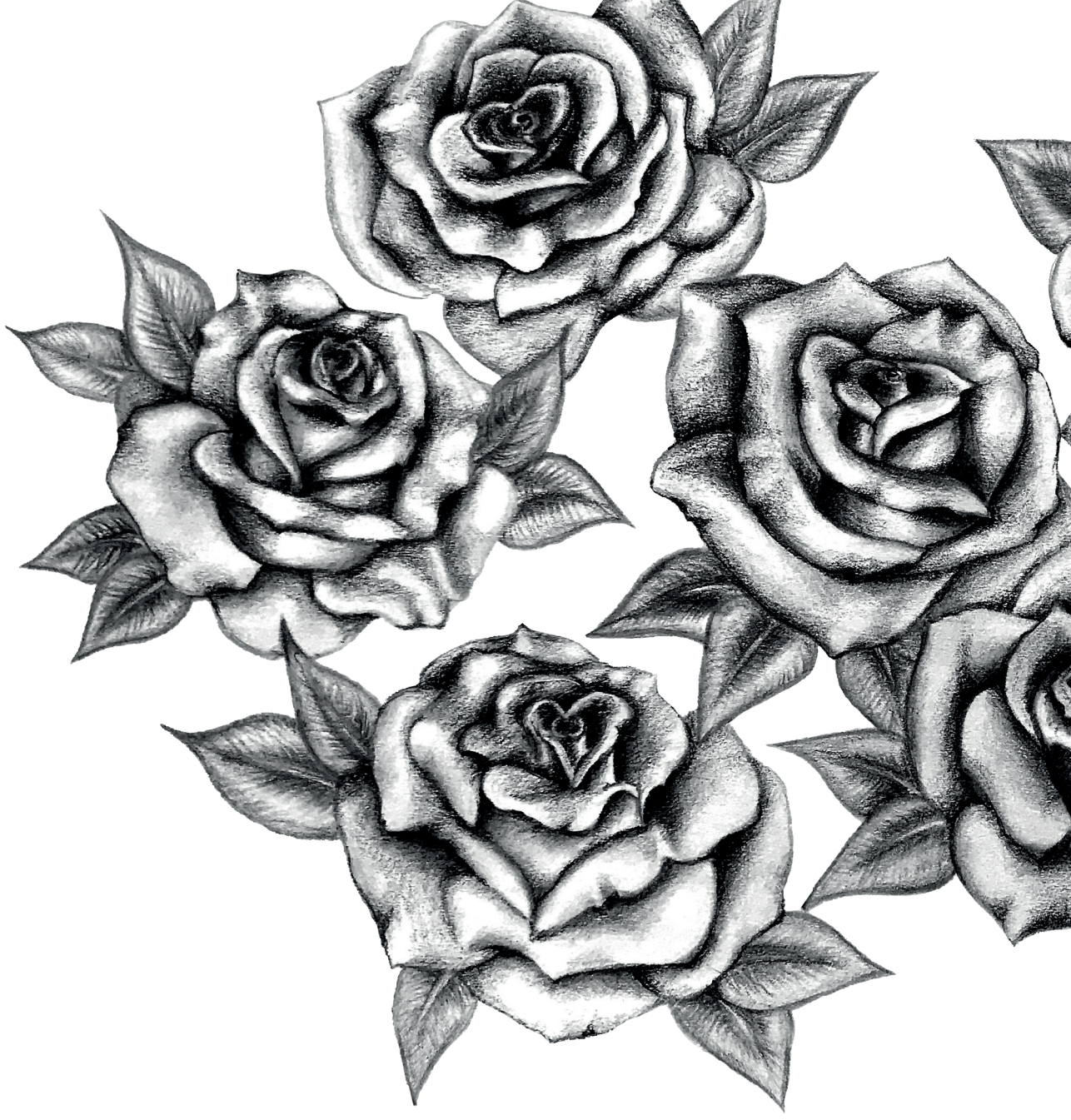

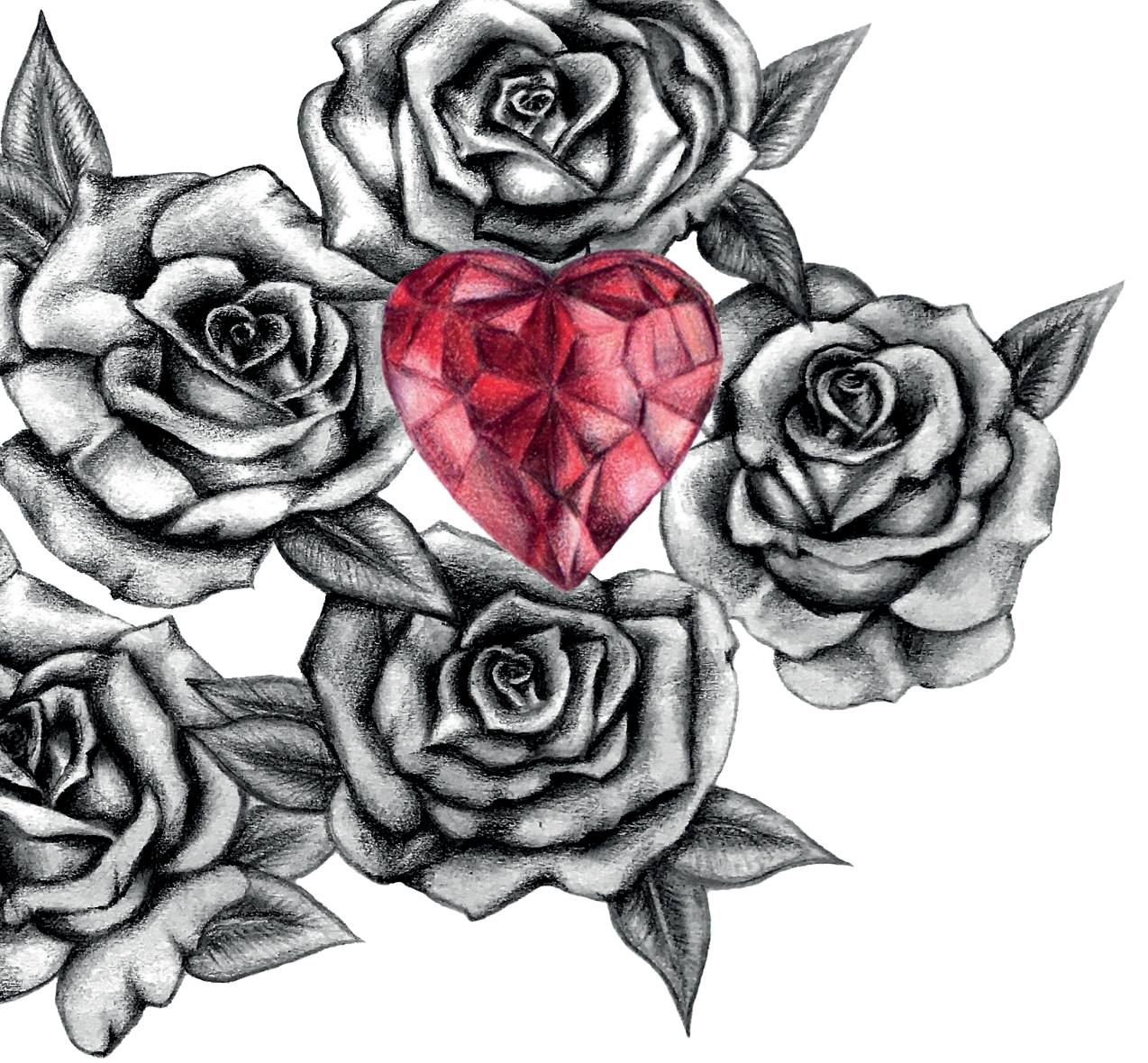

Part 2

Placental vascular development in placental syndrome


6

The microvasculature of the placenta: potential pathophysiological significance in placental syndrome

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In preparation

Abstract

Placental syndrome consists of a set of different pregnancy complications, all linked to each other by having a defective spiral artery remodeling as a common mechanism in the pathogenesis. However, it still remains largely unknown why this common factor can lead to different clinical entities. We hypothesize that (micro)vascular remodeling plays a crucial role in the pathophysiology of placental syndrome. In this review, we present 1) a survey of current knowledge about placental (vascular) development in both normal pregnancy and those complicated by placental syndrome; 2) our ideas on how defective spiral artery remodeling can result in different clinical entities; and 3) we reflect the current literature on quantitative morphological features of placental vascularization.

We believe that by studying oxygenic state and correlating this to villous vascularization, we could gain more insight into the pathophysiology of placental syndrome, which in turn could provide other perspectives for clinical management.

Introduction

The placenta is an essential organ to both fetus and mother, yet it is probably not fully appreciated due to its complex function and development. Abnormal placental development leads to a variety of pregnancy complications that can be grouped together under the term "placental syndrome" and can be divided into three compartments of interrelated disorders: maternal, fetal and placental compartments. The maternal compartment includes gestational hypertension (GH), preeclampsia (PE) and HELLP syndrome (acronym for Hemolysis, Elevated Liver enzymes and Low Platelet count). The fetal compartment includes fetal growth restriction (FGR) and intrauterine fetal demise (IUFD). And finally, the placental compartment includes placental abruption and some cases of otherwise unexplained spontaneous preterm delivery.[1-3] Although placental syndrome is a major cause of maternal and perinatal morbidity and mortality,[4-6] there is still much to be elucidated regarding its pathophysiology. The pathogenesis of placental syndrome can be traced to the early stages of placentation, characterized by defective maternal vascular remodeling, that subsequently affects microvascular development in placental villi and placental oxygenation and nutrients transport to the fetus.[1-3,7]

We hypothesize that vascular pathology from both the maternal and fetal side may play a crucial role in the development of placental syndrome. In this paper we will briefly discuss placental development, focusing on villous angiogenesis, and discuss our hypothesis on the pathophysiological mechanisms that may underlie aberrant microvascular development in placental syndrome, from both maternal and fetal perspectives. Furthermore, we reflect on current literature on placental morphometry, as an essential tool to quantitatively and qualitatively investigate villous vasculature to understand its role in placental development and its relevance to the pathophysiology of placental syndrome. We comment on areas of future research, where this hypothesis can help further our understanding of the placental contribution to a spectrum of pregnancy complications.

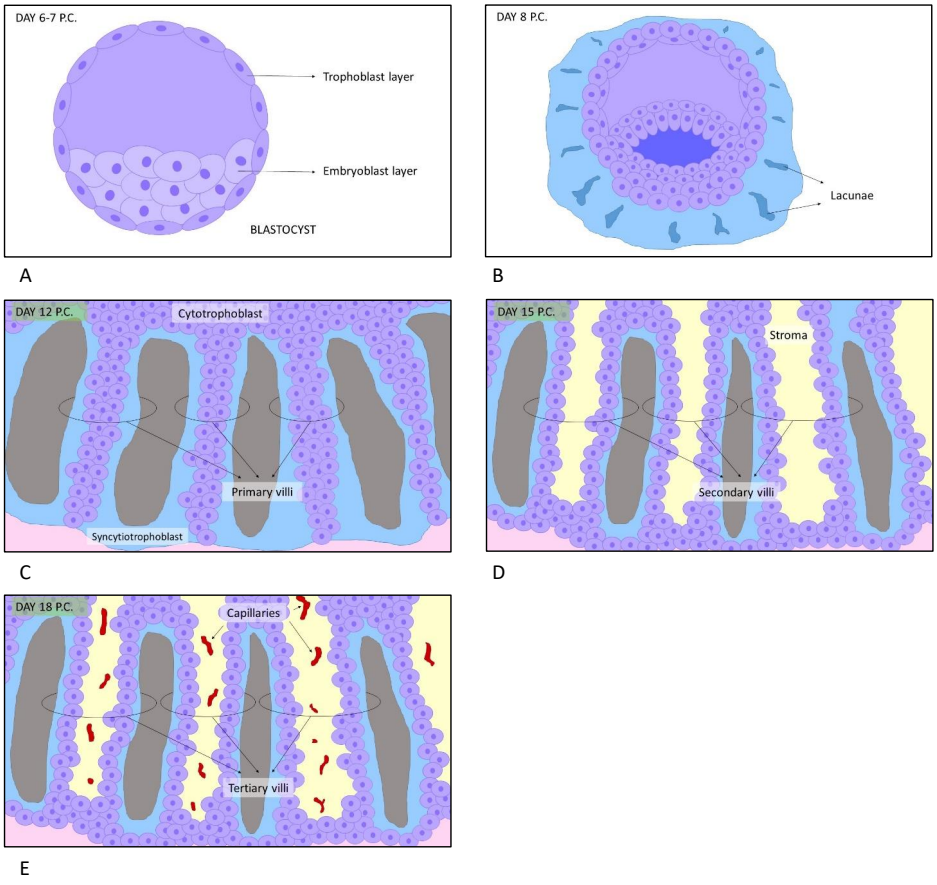
Vascular development in normal pregnancy

Maternal vascular development

The trophoblast layer surrounding the blastocyst develops into cytotrophoblast and syncytiotrophoblast, in which lacunae develop giving rise to primary villi.[8] Subsequently, a triple-layered chorionic plate develops composed of mesenchyme, cytotrophoblast and syncytiotrophoblast, and secondary villi are formed. When the first fetal capillaries arise, the encompassing villi are called tertiary villi (Figure 1). From the second month of gestation, a range of villous types are formed; stem villi, mesenchymal villi, immature

intermediate villi, mature intermediate villi and terminal villi, all with a different structure and function. These different villous types all originate from tertiary villi undergoing a complex process of villous differentiation,[8] which is substantially controlled by vascular development.[9]

Figure 1. Early placental development.



After fertilization, the zygote develops into a blastocyst surrounded by trophoblast, while the inner core consists of embryoblast (A). The blastocyst implants in the uterine wall, where the trophoblast proliferates and forms a double layer; an inner layer of cytotrophoblast and an outer layer of syncytiotrophoblast. Next, lacunae develop into the syncytiotrophoblast (B). The lacunae are separated from each other by bands of syncytiotrophoblast, in which the cytotrophoblast extends giving rise to primary villi (C). Subsequently villous stroma develops in the primary villi, giving rise to secondary villi (D). When capillaries arise, the villi transform into tertiary villi (E).
P.C. = post conception

Villous vascular development starts under the influence of the trophoblast cells of the blastocyst, which differentiate into a subpopulation of invasive extravillous trophoblast cells (EVTs),[10] that accumulate in the lumen of the spiral arteries to form plugs and block early maternal blood flow to the placenta (endovascular trophoblast),[11,12] in order to prevent precocious onset of the maternal-placental circulation, which would lead to oxidative damage due to high levels of oxygen and the generation of reactive oxygen species.[13]

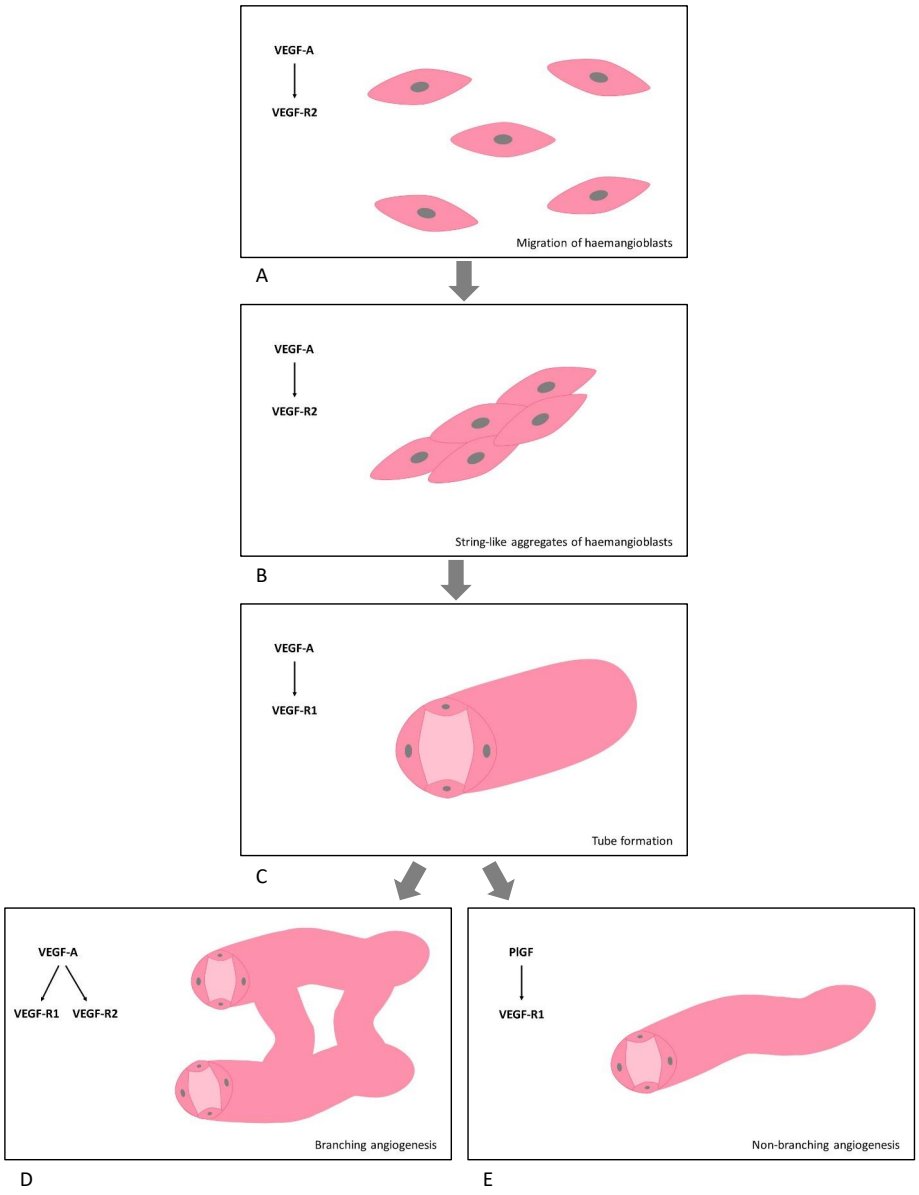
After the first trimester, EVTs induce spiral artery remodeling. The trophoblast cells invade the vessel walls to cause disruption of the vascular smooth muscle cells and extracellular matrix.[13] The vessel lumen diameter increases dramatically by appropriate remodeling, resulting in a lower vascular resistance.[14] This process ensures a high volume/low resistance circulation[15] and equally distributed perfusion needed for adequate feto-placental vascular development.

Placental microvascular development

Placental villous vascularization starts with vasculogenesis[16] and is driven by the release of angiogenic factors released by cytotrophoblast cells in response to changes in oxygen tension, shear stress and cyclic strain (Figure 2).[17,18] Subsequently, new blood vessels are generated from pre-existing blood vessels by a process called angiogenesis, which can be divided into branching and non-branching forms.[9]

Until mid-gestation, new blood vessels are formed by branching angiogenesis. From about 24 weeks of gestation to term non-branching angiogenesis predominates.[9] In branching angiogenesis, new vessels are generated by the formation of multiple short capillary loops, stimulated by VEGF and its receptors VEGF-R1 (flt-1, leading to endothelial tube formation) and VEGF-R2 (KDR, leading to endothelial cell proliferation).[9,19] As pregnancy progresses placenta-like growth factor (PlGF) rises, stimulating VEGF-R1 resulting in tube formation or non-branching angiogenesis. At the same time, expression of VEGF and VEGF-R2 decline steeply.[9,16,19] Feto-placental angiogenesis is a multistep process and involves abundant factors that can be modified in various ways, a major factor being oxygenation. Placental oxygenation in turn is largely dependent on spiral artery remodeling, highlighting the strong relationship between both the maternal and fetal vascular compartments. Placental oxygenation also plays a crucial role in the pathophysiology of placental syndrome, since it strongly influences maturation and behavior of the placenta during pregnancy.

Figure 2. Placental vasculogenesis and angiogenesis.



A) Placental vasculogenesis starts with the differentiation and migration of hemangioblasts during the third week post fertilization. B) String-like aggregates of hemangioblasts form. C) A lumen arises and the surrounding endothelial cells become flattened. D) New blood vessels are formed by branching angiogenesis until mid-gestation. E) In the second half of pregnancy, non-branching angiogenesis predominates.
VEGF = vascular endothelial growth factor
VEGF-R = vascular endothelial growth factor receptor
PlGF = placental growth factor

Vascular development in placental syndrome

Alterations in maternal vascular development

An impaired migration of EVTs towards the endometrial stroma and into the lumen of the spiral arteries is observed.[20] This aberrant trophoblast invasion leads to impaired trophoblastic plug formation in the first trimester of pregnancy causing early placental perfusion and oxidative stress. Subsequently, from the second trimester it results in impaired dilation of the terminal ends of the spiral arteries.[20,21] The absence of dilation provokes an increased local velocity of blood flow in the intervillous space, which will lead to villous shear stress, hypoxia (due to local malperfusion), and increased blood pressure in the intervillous space.[21] The malperfusion leads to a changed oxygenation and may be the primary trigger for a disturbed release of trophoblastic angiogenic factors, changing the feto-placental vascularization already in the first trimester of pregnancy. This is supported by serum measurements of PlGF and sFlt-1, which already deviate in the first trimester in women destined to develop preeclampsia.[22]

Alterations in placental microvascular development

Oxygenation plays a pivotal role in placental syndrome. There are two types of altered oxygenation described in placental syndrome; uteroplacental hypoxia and postplacental hypoxia/intervillous hyperoxia. Uteroplacental hypoxia is thought to be the result of a deficient maternal vasculature, associated with occult cardiovascular risk factors,[23] defective spiral artery remodeling and/or increased uterine artery resistance, leading to a decrease in the delivery of oxygen to the intervillous space.[1-3,7,24] Intervillous hyperoxia on the other hand, is seen in FGR with absent or reversed end diastolic flow of the umbilical artery (ARED).[24] This does not mean that maternal intervillous oxygen tensions are higher than maternal arterial tensions but recognizes that, in FGR+ARED, intervillous oxygen tensions may be closer to arterial than in age-matched normal controls.[24]

The pathophysiology of ARED has not been well characterized. However, one theory is that placental stem villus vessels play an important role in placental hemodynamics, minimizing ventilation-perfusion mismatch,[25] as is also seen in the lung.[26] In the lung alveolar hypoxia causes vasoconstriction, reducing perfusion of poorly oxygenated alveoli. The blood flow is diverted toward better ventilated areas.[26] In the placenta a prolonged reduction in intervillous blood flow/oxygenation (intervillous hypoxia), also leads to prolonged vasoconstriction of vessels. This results in secondary reduction of vessel luminal diameter, vascular medial hypertrophy and increased flow resistance.[25] The feto-placental circulation is compromised and causes reduced extraction of oxygen from the intervillous space, resulting in poor fetal oxygenation but higher than normal intervillous oxygen levels (relative hyperoxic state).[24] Intervillous hyperoxia thus results

from aggravating intervillous hypoxia. Herewith, the importance of oxygen homeostasis in placental syndrome is emphasized.

Oxygen homeostasis is a crucial factor in the development of fetal vasculature and villous differentiation. Reduced oxygen tensions lead to activation of the VEGF system and to attenuated trophoblastic PlGF secretion.[27-29] VEGF stimulates both VEGF-R1 and VEGF-R2 resulting in an overstimulation of branching angiogenesis with respect to non-branching angiogenesis.[9,24] Capillary diameter increases as a result of augmented VEGF-R2 stimulation.[30-32] The altered angiogenesis results in malformed terminal villi, which are more broad and show indented surfaces.[9,24] Conversely, hyperoxia as seen in ARED results from an attenuation of the VEGF system and enhanced PlGF secretion. Overstimulation of VEGF-R1 subsequently results in more non-branching angiogenesis[24] and relatively small capillary diameter.[30-32] These changes in turn result in elongated slender villi, recognized in histological sections as distal villous hypoplasia (DVH).[24]

Different clinical entities in placental syndrome

Placental syndrome consists of many different clinical entities but all have been shown to be associated with defective spiral artery remodeling. This raises the question how this defective remodeling can result in such diverging clinical diseases. To complicate the understanding of its pathophysiology further, histological data of the matching placentas show variations as well. This question has been addressed earlier, yet, the answer still remains hypothetical.

It has been proposed by others that the severity of spiral artery adaptation is responsible for the differences in clinical presentations.[2,33] Ness and Sibai[34] state that both women with FGR and PE enter pregnancy with some degree of endothelial dysfunction, which predisposes to spiral artery maladaptation. They further hypothesize that PE develops when spiral artery maladaptation interacts with maternal metabolic syndrome, comprised of adiposity, insulin resistance/hyperglycemia, hyperlipidemia, and coagulopathy. FGR develops in the absence of maternal metabolic syndrome, but with other risk factors for endothelial dysfunction such as high blood pressure and smoking. This is in line with the fact that FGR is more common in women of lower weight and none of the lipoproteins appear to be elevated antepartum in pregnancies complicated by FGR. Also, hyperglycemia is not associated with FGR. Indeed, diabetes mellitus and gestational diabetes are associated with macrosomia.[34] This hypothesis is also in conjunction with maternal cardiovascular risk in later life. Preterm birth, FGR and PE are all associated with an increased risk of developing later ischemic heart disease, in ascending order.[35] Yet, they do not take PE without FGR (late-onset PE) into account. Furthermore, this assumption still does not explain why DVH is seen irrespective of PE.

We hypothesize that the interaction of the severity of defective spiral artery remodeling – which influences the timing of spiral artery remodeling – together with pre-existing maternal risk factors plays a role in the observed placental histological changes (Figure 3). We believe that more severe and earlier defective spiral artery remodeling leads to earlier malperfusion with subsequent changes in angiogenic factors, resulting in a different histological image. The earlier the malperfusion occurs, the more influence angiogenic factors have on placental villous development and therefore also on fetal development. Early malperfusion with a high pressure of incoming blood leads to arteriovenous shunting in the intervillous space, which causes the maternal intervillous blood to be relatively hyperoxic. This in turn might cause higher levels of PlGF leading to excessive non-branching angiogenesis, resulting in long slender villi compatible with the histological picture of DVH. Furthermore, higher PlGF levels stimulate smooth muscle cell recruitment in fetal stem vessels,[36] leading to increased vasoconstriction of these vessels eventually resulting in ARED. In case of early malperfusion without such a high pressure of incoming blood, the maternal intervillous blood is relatively hypoxic due to a diminished maternal-placental circulation. This leads to higher levels of VEGF, resulting in excessive branching angiogenesis with indented villous surfaces, leading to a histological picture of accelerated placental villous maturation. In moderate defective spiral artery remodeling and later onset of malperfusion, angiogenesis starts normally and later on alters according to oxygenation, leading to a less severe histological picture in a gradual scale. One can partially compare this situation with placentas from women who live at high altitudes and thus in a relative hypoxic environment, which often show the histological picture of chorangiosis and higher rates of FGR.[37] According to timing of onset of malperfusion, villous maturation shifts from DVH or accelerated villous maturity towards normal maturation. Furthermore, we believe that the degree of malperfusion also reflects in lower placental weight. In normal pregnancies,[38] villous development in the first and second trimester occurs by producing villous sprouts along the surfaces of mesenchymal and immature intermediate villi. The sprouts are transformed into mesenchymal villi and the circle repeats itself. In the third trimester, however, the mesenchymal villi differentiate into mature intermediate villi, which then produce terminal villi. The source of new sprouts is reduced and the growth capacity of the villous tree therefore gradually slows. In case of malperfusion leading to DVH or accelerated villous development the villi develop into terminal villi much earlier in pregnancy, skipping a great deal of villous sprout production leading to a reduced villous growth capacity and therefore decreased placental weight.

Figure 3. Our hypothesis for the pathophysiology of placental syndrome.

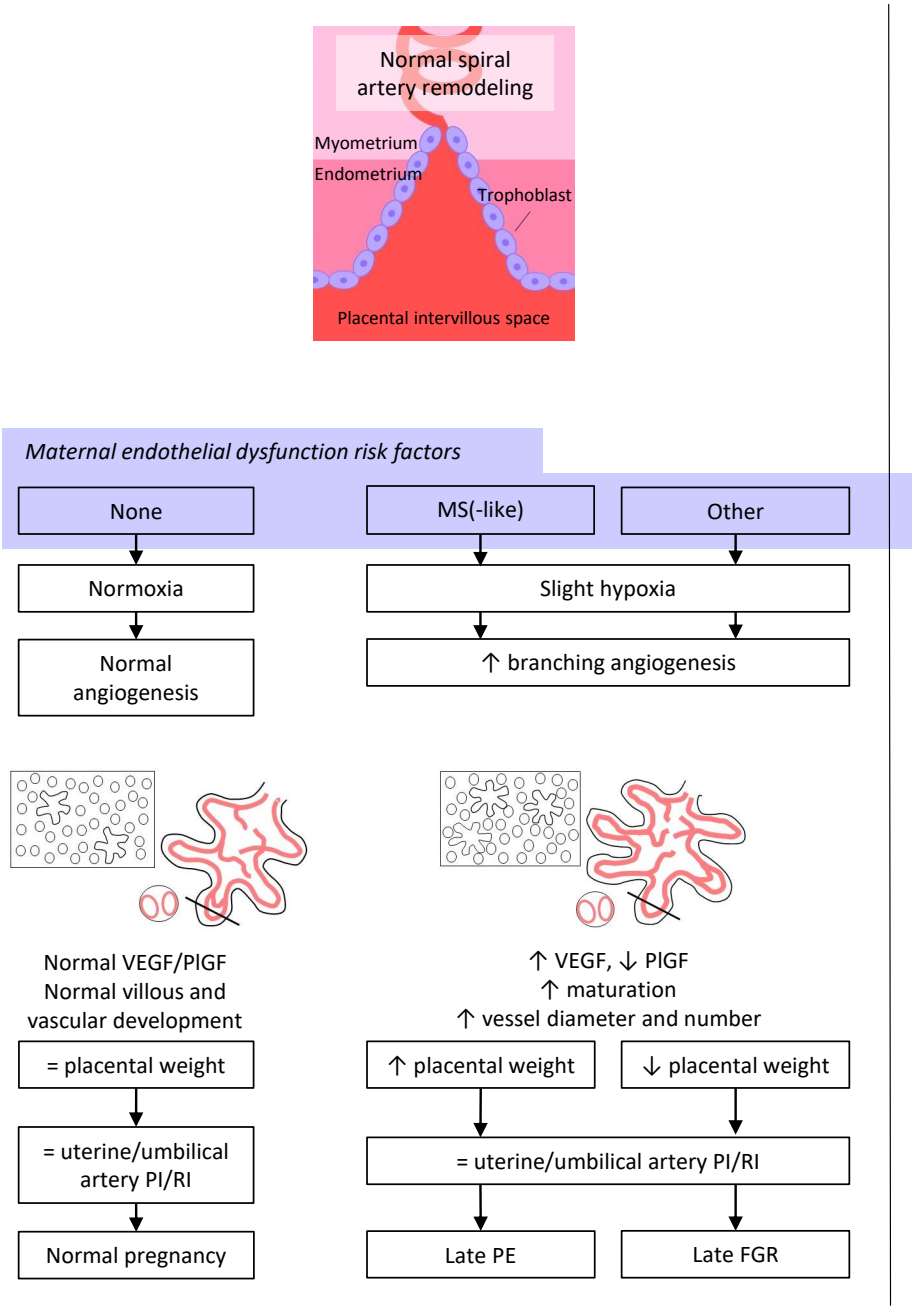
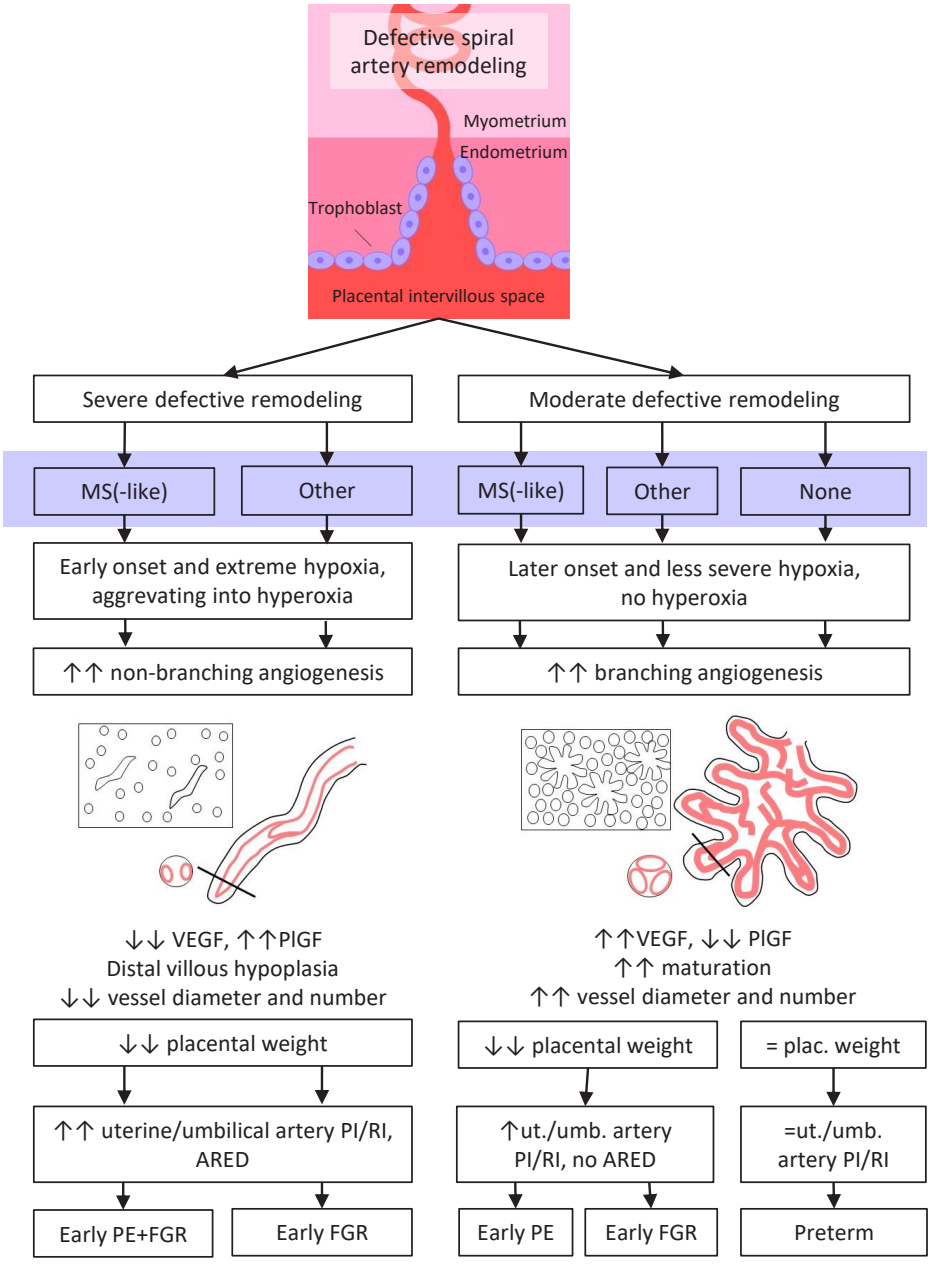


Figure 3. Continued.



We believe that the current placental syndrome can be divided into two main groups, namely those women with normal spiral artery remodeling (mature placental syndrome) and those with defective spiral artery remodeling (premature placental syndrome).

Figure 3. Continued.

The group of women who show normal spiral artery remodeling are those who develop late-onset PE or FGR (mature placental syndrome). Both groups of women have pre-existing risk factors for endothelial dysfunction. Women with late-onset PE have risk factors compatible with metabolic syndrome (such as adiposity, insulin resistance/hyperglycemia, hyperlipidemia, and coagulopathy) and women with late-onset FGR have other risk factors for endothelial dysfunction (such as hypertension or smoking). These risk factors result in a slightly hypoxic intervillous circulation, following in a subtle increase in branching angiogenesis. These placentas show a subtle increase in villous maturation, with a small increase in villous vessel count and have a relatively high placental weight. Uterine and umbilical artery Doppler measurements are somewhat increased.
On the other hand, there is the premature placental syndrome with defective spiral artery remodeling. In this group, there is also a subdivision into the different types of risk factors for endothelial dysfunction, but first there is a subdivision into the severity/timing of the defective remodeling. Women with early-onset and/or severe defective spiral artery remodeling are destined to develop early-onset PE with FGR or early-onset FGR, depending on the type of endothelial endothelial dysfunction risk factors. Again, women with risk factors compatible with metabolic syndrome will develop PE (in the severe/early defective remodeling group combined with FGR) and those with other risk factors will develop early-onset FGR. The risk factors result in an extremely hypoxic intervillous circulation, which aggravates into intervillous hyperoxia (as previously explained). The intervillous hyperoxia leads to an increase in non-branching angiogenesis, resulting in DVH. There is a decrease in villous vessel count and placental weight. Furthermore, there is an increase in uterine artery Doppler PI and RI and eventually ARED develops of the umbilical artery. The group of women with moderate/later onset defective spiral artery remodeling can be subdivided into three groups according to pre-existing risk factors, namely risk factors associated with: 1) metabolic syndrome, developing into early-onset PE; 2) other risk factors for endothelial dysfunction, developing into early-onset FGR; and 3) no risk factors for endothelial dysfunction, developing into early preterm birth (<34 weeks gestational age). In the latter, other factors may play a role such as inflammatory and immunological factors. All three groups develop a hypoxic intervillous circulation due to the defective spiral artery remodeling. The intervillous hypoxia leads to an increased branching angiogenesis, resulting in an accelerated villous maturation. There is an increase in villous vessel count and a decreased placental weight. The uterine artery Doppler PI and RI are increased and there is a preserved umbilical artery flow. However, other factors such as inflammatory, immunological and not yet identified factors may also play an important role in the pathophysiology of placental syndrome. Furthermore, there remains much overlap between the different entities. Yet, we believe that our hypothesis takes us one step closer to unravelling the pathophysiology of placental syndrome and deserves further study.

Next to the processes described above, we believe that late-onset PE and FGR are mainly caused by maternal risk factors, those as stated by Ness and Sibai, and that spiral artery remodeling plays a subordinate role in these entities. Defective spiral artery remodeling may be a secondary phenomenon. Instead of a causative factor, we believe defective spiral artery remodeling plays an interactive role between pre-existing risk factors for endothelial dysfunction and the clinical/histological picture. The metabolic syndrome risk factors are associated with endothelial dysfunction and make the spiral arteries more sensitive to the same risk factors, developing a vicious circle. Indeed, this vicious circle is also described for endothelial dysfunction and insulin resistance.[39] The subordinate role of spiral artery remodeling is supported by previous studies investigating uterine artery Doppler measurements. Resistance to blood flow within the uteroplacental circulation is transmitted upstream to the uterine arteries and can be measured as an increased pulsatility index (PI) or resistance index (RI).[40] In normal pregnancies, uterine artery PI and RI values decrease with increasing gestational age, a change that is thought to be secondary to a fall in impedance in uterine vessels following trophoblastic invasion.[40] Defective spiral artery remodeling, however, leads

to increased uterine artery PI and RI values. To come back to our perspective of the etiology of late-onset disease, it has been shown that late-onset PE and IUGR is less well predicted by early Doppler measurements than early-onset disease.[40] Furthermore, late-onset pre-eclamptic placentas have a higher weight than controls,[41] supporting the theory of Ness and Sibai that (late-onset) PE is associated with the metabolic syndrome risk factors including insulin-resistance, given that diabetic placentas also have a higher weight. This leaves us with the final category of preterm birth without maternal hypertensive disease. We believe this category results from spiral artery maladaptation without previous maternal cardiovascular risk factors. In this category trophoblast function and its ability to sufficiently invade maternal spiral arteries may play a more important role. This would indicate that these mothers have a lower cardiovascular risk in later life compared to mothers who developed GH/PE and/or who delivered a baby with FGR. However, studies investigating later cardiovascular risk of mothers who delivered prematurely also include premature deliveries resulting from GH, FGR and PE. Although we describe our hypothesis fairly straightforward, we recognize that this might not be the case in practice. There still remains much overlap between the different clinical entities. Further research, including pathological research on placentas is needed.

Our hypothesis could be prospectively investigated by performing uterine artery Doppler measurements at different gestational weeks, starting in very early pregnancy. Guzin *et al*.[42] confirmed that Doppler ultrasonography of the uterine arteries is a reliable, non-invasive method correlating with partial trophoblastic invasion in spiral arteries. However, we realize that performing such a study will be challenging to manage. An alternative method to gain more insight into the pathophysiology would be to quantitatively investigate villous vasculature.

Morphometrical quantification of villous vasculature

The villous vasculature can be quantified during gestation by means of stereological quantification on placental tissue sections. In Table 1 we give an overview of morphometrical data obtained in placentas on this topic until now. Total volume, surface area and length represent net growth of the fetal capillaries. Villous vascularization is described by estimating capillary volume, surface and length densities within villi, and capillary:villus surface and length ratios.[16]

Normal pregnancies

In order to critically assess morphometrical data on the subject of placental syndrome, information is required for normal pregnancies at different periods of gestation. Although several research groups stereologically investigated normal pregnancies,[43-46] only Mayhew[45]

and Stoz *et al*.[46] provided data at diverse gestational ages throughout pregnancy (Table 1). Both demonstrated increasing villous capillary growth throughout pregnancy and a biphasic nature to placental angiogenesis.

The two studies explore placental angiogenesis extensively, nevertheless some remarks should be made regarding the study design. Since normal pregnancy is technically defined as a term pregnancy without complications, it is impossible to study placentas at different gestational ages from normal pregnancies. Ideally, placentas should be selected from induced abortions and preterm deliveries due to cervical incompetence, ruling out any other pathology, the so called idiopathic preterm deliveries. Both studies, however, included also spontaneous[45] and therapeutic abortions.[46] In addition, the exclusion criteria for preterm deliveries were not thoroughly enough explained. More studies are needed to explore villous angiogenesis in normal pregnancies at diverse gestational ages with explicit inclusion and exclusion criteria.

Pregnancies complicated by placental syndrome

Various authors have investigated stereological data in the entities associated with placental syndrome, with highly varying results as shown in Supplementary Table 1 and summarized in Table 1.

In order to explain the differences between the studies, we first need to review the different factors that influence stereological placental data (Table 1). Since vascularization increases with gestational age,[45,46] differences in gestational age between study groups could be an important confounder. In several studies gestational age is lower in the study groups than in controls,[24,47-49] or insufficiently described.[50-55] However, early-onset disease generally leads to premature delivery, thereby making it impossible to match these placentas for gestational age in healthy term controls. Furthermore, increasing maternal age in healthy pregnancies shows decreased villous vascularization,[56] which is also often not or not sufficiently depicted.[24,47,48,50-55,57-59] Smoking status alters villous vascularization as well,[57] which is only mentioned in two studies.[48,60] Additionally, mode of delivery (vaginal delivery versus caesarean section) shows impact on villous vascular stereology, as shown by Guiot *et al*.[58] Another possible confounding factor is the used methodology. Some investigators consider villi in general,[48,50,52,61-65] while the others subdivide the tertiary villi. Some studies exclude vessels without a lumen or a lumen smaller than 1.02 μm in diameter, while investigating capillary count.[50,61] Storage and tissue fixation also alters stereological data,[66] but this should not raise any problems since those methods are similar for all placentas within a study. Lastly, and perhaps most importantly, PE/HELLP is considered to be a heterogeneous disorder that can be classified into early-onset (<34 weeks gestation) and late-onset (≥34 weeks gestation) disease.[67,68] Both groups are thought to have different

etiologies, in which we believe the type of hypoxia plays a crucial role (as explained before). To gain more insight into the type of hypoxia involved in early-onset disease, uterine and umbilical artery blood flow can be measured.[24,40]

Table 1. Summary of morphometric parameters studied in placental syndrome. In case of multiple villous types studied, terminal villi were selected.

Morphometric parameters		Study groups	Available uterine and/or umbilical artery Doppler
Villous area/ volume	↑	Term CH[50] Altered Doppler[55,61(villous area)] FGR[70] PE[47,64]	[47]No significant differences in UtA PI, RI not known [60]ARED: FGR 50%, PE+FGR 50%, PE 7% [61]UtA RI increased in altered Doppler group [71]AED: PE (no FGR) n=0, FGR n=2, PE+FGR n=6, Controls n=0 [72]Early cases: PE (no FGR): all PED; FGR: AED n=2, PED n=2, missing n=3; PE+FGR: AED n=4, PED n=5; Controls: all PED Late cases: PE (no FGR): all PED; FGR: all PED; PE+FGR: AED n=2, PED n=5; Controls: all PED [55]Groups based on increasing severity of UtA and UmA RI [62]FGR: AED n=1, PED n=2, missing n=2; PE: PED n=3, missing n=2; PE+FGR: PED n=3, missing n=2; Controls: PED n=5, missing n=4 [58]PE+FGR: AED n=3, RED n=1, PED n=4
	↓	GH[73] Altered Doppler[55(villous volume)] FGR[52,62,63,71,73] PE[73], PE (no FGR)[60], early PE (no FGR)[72] PE+FGR[52,60,62,63], early PE+FGR[72] (Pre)term PSCH[50]	
	=	Preterm CH[50] (Pre)term GH[50] FGR[60], late FGR[72], early FGR[72] PE[49,53], PE (no FGR)[6,52,63,71], late PE (no FGR)[72] (Pre)term PE[50] Late PE+FGR[72]	
Capillary diameter	↑	-	
	↓	Altered Doppler[55] Placental hypoplasia[54] PE[49]	
	=	PE[64] Early and late PE/PE+FGR/FGR[72]	
Capillary count	↑	PE[64] Altered Doppler[61] Term CH[50] (Pre)term PE[50]	
	↓	FGR[70]	
	=	PE[53] Preterm CH[50] (Pre)term GH[50] (Pre)term PSCH[50]	
Capillary area	↑	Altered Doppler[55] PE[64]	
	↓	FGR[62,70,71,73], early and late FGR[72] Severe PE[53], PE+FGR[62,71], early and late PE+FGR[72]	
	=	GH[73] FGR[60,63] PE[48,73], PE (no FGR)[60,63,71], moderate PE[53], PE+FGR[60,63]	

Table 1. Continued

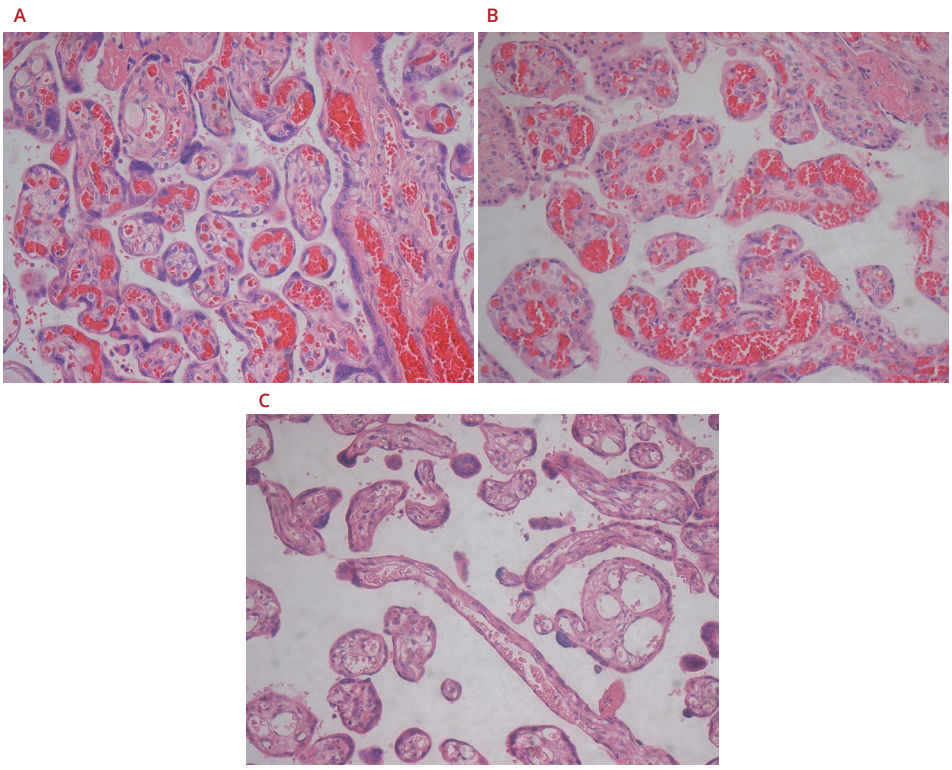
Morphometric parameters		Study groups	Available uterine and/or umbilical artery Doppler
Capillarization index	↑	FGR[60], early FGR[72] PE[49,64], PE+FGR[60], early PE+FGR[72]	
	↓	ARED[60] FGR[70] PE[65]	
	=	GH[51] FGR[63], late FGR[72] PE[53], PE (no FGR)[60,63], early and late PE (no FGR)[72], PE+FGR[63], late PE+FGR[72]	

CH = chronic hypertension, FGR = fetal growth restriction, PE = preeclampsia, PSCH = PE superimposed on CH, AED = absent end diastolic flow, RED = reversed end diastolic flow, PED = preserved end diastolic flow, RI = resistance index, PI = pulsatility index, UtA = uterine artery, UmA = umbilical artery Late-onset Controls

Hypoxic versus hyperoxic status may explain large differences in morphometrical data in the literature

As previously stated, placental syndrome is associated with different types of hypoxia; uteroplacental hypoxia and postplacental hypoxia/intervillous hyperoxia. These different types of hypoxia lead to different changes in angiogenesis, and therefore villous development. In addition, uteroplacental hypoxia can also develop into intervillous hyperoxia. To explain the differences between the studies, we first need to review what kind of changes we would expect in disorders associated with placental syndrome. These changes need to be translated into a two-dimensional view, as in microscopic slides (Figure 4). In intervillous hypoxia, we would expect a larger villous surface, as well as a larger capillary count and diameter than in normoxic pregnancies. Conversely, in intervillous hyperoxia we would expect a smaller villous surface, as well as a smaller capillary count and diameter. Regarding stem villi we would expect to see thicker vessel walls in hyperoxia and thinner walls in hypoxia, since smooth muscle cell recruitment is stimulated by PlGF.[36] Hypertensive pregnancy is likely associated with hypoxia due to altered uteroplacental blood flow, whereas FGR is associated with a hyperoxic intervillous state in case of impaired umbilical blood flow. However, this is not present in all cases of FGR, often there is still a preserved end diastolic flow (PED). In case of intervillous hyperoxia, there is a gradual shift from intervillous hypoxia to hyperoxia, which complicates the interpretation of results.

Figure 4. H&E staining of placentas, taken with 20x objective.



A) Placenta from uncomplicated term pregnancy at 37+6 weeks gestational age (GA). Placental weight p50, birth weight p50. Normal mean uterine artery PI and RI. Preserved enddiastolic flow (PED) of the umbilical artery. B) Placenta from pregnancy complicated by preeclampsia (PE) at 26+4 weeks GA. Placental weight p10-25, birth weight p50-80. Uterine artery PI and RI >p95. PED of the umbilical artery. Note larger villous surface and increased capillary number. C) Placenta from pregnancy complicated by PE with fetal growth restriction (FGR) at 28+3 weeks GA. Placental weight <p10, birth weight <p2.3. There was absent enddiastolic flow of the umbilical artery. Uterine artery PI and RI >p95. Note the long and slender villi with a decrease in villous surface and capillary number.

We believe that one of the main issues in the highly diverging results is caused by neglecting uterine and umbilical artery blood flow, which are indirect measurements of oxygenation. There are only a few studies analyzing these Doppler values.[55,60,61] Van Oppenraaij *et al*.[60] demonstrated a lower terminal villous capillarization index in the FGR subgroup with ARED, compared to those with PED. They state that the effect was not influenced by PE. Yet, they do not provide data regarding this statement, while there were only 5 FGR cases and 9 cases of FGR with PE. Unfortunately, they also did not investigate terminal villous area, capillary count or diameter in this subgroup. Kuzmina *et al*.[55] investigated subgroups with increasing severity of uterine, spiral, umbilical and stem villi arteries RI. They found a decreased terminal villous volume, while terminal villous area was increased. Terminal

villous capillary volume aggravatingly declined in increasing severity of the subgroups. In the most severe subgroup, capillary diameter was diminished, whereas capillary area was enhanced. The other subgroup did not show significant changes in these parameters. However, 78% of the patients in the most severe subgroup also had PE. Numbers for the other subgroups are not mentioned in their article. In addition, there is no mentioning of ARED. Lastly, Gilio *et al*.[61] investigated normal pregnancies with an increased uterine artery RI. They showed increased terminal villous area and capillary count, in line with what we would expect based on the molecular mechanisms we explained. Although there is no mentioning of umbilical artery Doppler, we can assume there are no cases of ARED since they selected clinically normal pregnancies while ARED is strongly associated with FGR. Interestingly, placental hypoplasia was also associated with decreased terminal villous capillary diameter.[54]

In summary, although various authors have investigated stereological data in PE and FGR, results vary tremendously and should be interpreted with great discretion.

Areas for future research

Placental angiogenesis is a key step in villous differentiation to ensure optimum placental structure and function during pregnancy. There is insufficient information regarding the role of placental angiogenesis in the pathophysiology of different clinical disorders within placental syndrome. By studying placental angiogenesis and villous differentiation morphometrically in placental syndrome, we can gain more insight into the common underlying mechanisms, as well as the divergent steps leading to the de development of different clinical phenotypes within placental syndrome. Furthermore, we believe that data on uterine and umbilical artery blood flow should be taken into account when conducting research into placental disorders. A recently published study protocol by Duan *et al*. describes this intention in PE and/or FGR.[69] Moreover, additional data are needed at various gestational ages in normal pregnancies. For preterm deliveries, placentas should be obtained from induced abortions or preterm delivery solely due to cervical incompetence. Lastly, stereological villous vascularization data could be combined with circulating maternal angiogenic factors, which may provide us with important biomarkers for placental syndrome. For instance, Jeevaratnam *et al*.[51] demonstrated a positive correlation between antepartum PlGF levels and villous surface area, whereas there was an inverse correlation for antepartum sFlt-1 levels. Villous vascularization was positively correlated to antepartum sFlt-1 levels and inversely correlated to postpartum PlGF levels.

Conclusion

In conclusion, we hypothesize that placental vascular development plays a crucial role in the pathophysiology of placental syndrome. Available morphometrical data of villous vessels in placental syndrome show highly varying results, which we believe can be explained by differences in the definitions of the study groups, in clinical characteristics and in used methods. Moreover, we provide support for our hypothesis that villous vascularization in placental syndrome is influenced by the degree and timing of spiral artery maladaptation, the kind of hypoxic state (uteroplacental hypoxia versus postplacental hypoxia/intervillous hyperoxia) and the type of pre-existing cardiovascular risk factors. We believe that by studying oxygenic state and correlating this morphometrically to villous vascularization, we could gain more insight in the pathophysiology of placental syndrome, which in turn could provide other perspectives for clinical management.

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Supplementary Table 1. Studies with characteristics examining villous angiogenesis in normal pregnancies and those complicated by placen

Study	Study groups and total patients (N)	GA at delivery (weeks, mean ± SD)	Umbilical / uterine artery Doppler	Maternal age (years, mean ± SD)
Normal pregnancies				
Mayhew *et al.* (2002)[45]	-GA 10-13 n=4 -GA 14-17 n=11 -GA 18-21 n=6 -GA 22-25 n=9 -GA 26-29 n=11 -GA 30-33 n=13 -GA 34-37 n=20 -GA 38-41 n=18 Total N=92	10-41, see study groups	?	?
Stoz *et al.* (1988)[46]	N=60	7-37	?	?
Pregnancies complicated by placental syndrome				
Gansburgskii *et al.* (2015)[54]	-Placental hypoplasia n=36 (low placental weight) -Controls n=15	39-40	?	26-32
Mukherjee *et al.* (2014)[64]	-Preeclampsia (PE) n=40 -Controls n=35 Total N=75 Sub-analysis matching for GA: PE n=10 Controls n=8	-PE: 34.6±2.5 -Controls: 37.9±2.2 p=0.0002 Sub-analysis matching for GA	?	-PE: 28.4±4.7 -Controls: 27.5±4.2 NS
Sankar *et al.* (2013)[49]	-PE n=59 -Controls n=59 Total N=112	-PE: 33.79±4.3 -Controls: 36.61±1.5 p=0.0001	?	-PE: 26.2±4.4 -Controls: 24.4±3.9 p=0.0194

ntal syndrome.

	Methods	Definition villi	Results
	-LM with test grid -Magnification x250 -Connective tissue procedure *-Number of fields?* *-Blinded?*	-Terminal villi (TV): <80µm Ø -Intermediate villi (IV): >80µm Ø	-Capillary total length continued to increase during pregnancy without changes in mean cross-sectional area -Longitudinal growth was due to endothelial cell proliferation and increases in mean cell area -Angiogenesis is linked to villous growth and maturation -Angiogenesis is biphasic with an inflection point at about mid-gestation for capillary:villus length ratios and capillary shape factors -No evidence for branching versus non-branching angiogenesis
	-LM with software -Magnification? -Staining? -50 villi/case *-Blinded?*	?	↑ cross-sectional surface, number and circumference of villous capillaries until 20 weeks gestation ↑ capillary:villous surface ratio until 28-29 weeks gestation
	-LM with ocular micrometer *-Magnification?* -H&E *-Number of fields?* *-Blinded?*	?	↓ TV capillary diameter in placental hypoplasia ↑ smooth muscle cells in placental hypoplasia Deviating smooth muscle cell distribution in placental hypoplasia
	-LM with software -10x objective -H&E -'Numerous photomicrographs' *How many?* *-Blinded?*	-	↑ villous area and diameter in PE ↑ capillary count and area in PE ↑ capillarization index (area of capillaries / area of villi) = capillary diameter Matching for GA: same results
	-LM with micrometers -40x objective -H&E -200 terminal villi (TV) per placenta *-Blinded?*	TV: smallest villi containing capillary loops without any histological artifacts	= TV area ↓ TV diameter in PE ↑ TV density in PE ↓ TV capillarization index in PE ↓ TV capillary diameter

Supplementary Table 1. Continued

Study	Study groups and total patients (N)	GA at delivery (weeks, mean ± SD)	Umbilical / uterine artery Doppler	Maternal age (years, mean ± SD)
Almasry *et al.* (2012)[70]	-Idiopathic fetal growth restriction (FGR): n=50 -Controls: n=25 (matched for GA, but only term placentas) Total N=75	-FGR: 38.11±0.13 -Controls: 38.88±0.19 p=0.001	?	-FGR: 28.6±0.6 -Controls: 29.8±0.8 NS
Shchyogolev *et al.* (2012)[53]	-Moderate PE: n=9* -Severe PE: n=6* -Controls n=10 Total N=25 *No definitions given	38-40 *Difference between groups?*	?	-PE: 25-41 -Controls: 26-43 *p-value?*
Szewczyk *et al.* (2012)[65]	-PE n=11 -Controls n=11 Total N=22	-PE: 37.5 (range 35-40) -Controls: 39 (range 37-40) *p-value?*	?	-PE: 30 (range 27-42) -Controls: 30 (range 23-37)
Ducray *et al.* (2011)[47]	-Normotensive (NT) n=30 -PE n=30 Total N=60	-N: 38.9±1.4 -HT: 36.6±2.8 p<0.001	?	18-40 *Difference between groups?*
Odibo *et al.* (2011)[73]	-FGR n=7 -PE n=13 -Gestational hypertension (GH) n=7 -Controls n=20 Total N=45	-Adverse outcome: 37.1±3.3 -Controls: 38.9±1.1 p=0.06	UtA PI: -Controls: 1.5±0.5 -Abnormal morphometry: 1.6±0.5 -Normal morphometry: 1.6±0.4 NS	-Adverse outcome: 28.9±6.0 -Controls: 31.5±5.4 NS

Methods	Definition villi	Results
-LM with software -20x objective -H&E -5 fields/slide; 50 slides FGR, 25 slides controls -1 blinded investigator	TV: <80µm Ø	↓ TV number in FGR = TV area ↓ TV capillary count and area in FGR ↓ TV capillarization index in FGR Positive correlation between capillarization index and birth weight
-LM with software *-Which objective?* -CD31 *-Number of fields?* *-Blinded?*	?	= TV area = syncytiotrophoblast area ↓ TV capillary area in severe PE = TV capillary count per villus = TV capillarization index = TV vascularization in moderate PE
-LM with software -20x objective -H&E -50 fields/case -2 blinded investigators	-	↓ capillarization index in PE ↑ histamine concentration and mast cells in PE
-LM with software -Various magnifications -H&E -'Numerous photomicrographs' *How many?* *-Blinded?*	Stem villi (SV): as outlined by Bernischke and Kaufmann[#]	↑ villous area in PE ↓ SV vessel lumen area in PE ↑ SV vessel wall thickness in PE ↑ fibrin deposition in PE
-LM with test grid -20x objective -H&E -20 fields/case *-Blinded?*	-SV: >80µm Ø with tunica media in vessels -IV: >80µm Ø without tunica media -TV: <80µm Ø	↓ TV volume in FGR, PE and GH ↓ IV volume in PE = SV volume ↓ TV area in FGR and PE = IV area ↑ SV area in GH ↑ intervillous space volume in FGR and GH ↓ TV capillary area in FGR = IV/SV vessel area ↑ SV vessel volume in PE

Supplementary Table 1. Continued

Study	Study groups and total patients (N)	GA at delivery (weeks, mean ± SD)	Umbilical / uterine artery Doppler	Maternal age (years, mean ± SD)
Van Oppenraaij *et al*. (2011)[60]	-FGR n=16 -PE+FGR n=19 -PE n=18 -Controls n=19 Total N=72	-FGR: 29.3±2.2 -PE+FGR: 29.7±2.4 -PE: 30.5±2.2 -Controls: 29.5±3.0 NS	UmA ARED: -FGR: n=5 -PE+FGR: n=9 -PE: n=1 p=0.011 for FGR UmA PI elevated: -FGR: n=7 -PE+FGR: n=13 -PE: n=7 NS	-FGR: 29.8±4.8 -PE+FGR: 28.7±5.6 -PE: 30.7±4.7 -Controls: 31.4±5.2 NS
Jeevaratnam *et al*. (2010)[51]	-NT n=51 -GH n=32 Total N=83	?	?	?
Gilio *et al*. (2009)[61]	-Normal Doppler: n=13 -Altered Doppler: n=14 Total N=27	-Normal Doppler: 39.2±0.9 -Altered Doppler: 38.6±2.2 NS	UtA RI: -Normal Doppler: 0.5 (0.5-0.6) -Altered Doppler: 0.6 (0.6-0.7) p=0.009	-Normal Doppler: 23.3±5.3 -Altered Doppler: 20.0±4.4 NS
Correa *et al*. (2008)[50]	-Chronic hypertension (CH) n=6 -PE superimposed on CH (PSCH) n=8 -GH n=23 -PE n=36 -Controls n=18 Total N=91	?	?	?
Mayhew *et al*. (2007)[52]	-FGR n=5 -PE n=5 -PE+FGR n=5 -Controls n=9 Total N=24	?	?	26-40 *Difference between groups?*

Methods	Definition villi	Results
-LM with software -10x and 40x objective -H&E -15 IV and 15 TV -2 blinded investigators	-IV: >80µm Ø -TV: <80µm Ø	↓ TV area in PE = IV area ↑ IV and TV capillarization index in FGR, no effect for PE = capillary area ↓ TV capillarization index in ARED
-LM with software *-Magnification?* -H&E -9 fields/case *-Blinded?*	-TV: <80µm Ø	= intervillous space area = TV capillarization index
-LM with software -Magnifications SV x400, IV x600, TV x1000 -H&E -80 villi per case *-Blinded?* -Vessels with no lumen, imprecise limits and oblique sections (internal circumferences with radii <1.02 02µm Ø) were not considered	-SV: vessels with media or adventitia -IV: vessels without media or adventitia -TV: high degree of capillarization and highly dilated sinusoids	↑ TV area in altered Doppler ↑ SV vessel count in altered Doppler = SV vessel wall thickness ↑ IV and TV capillary count in altered Doppler
-LM with software -Various magnifications -H&E -Various number of fields; 80/90 villi – 80 fields/case *-Blinded?* -Vessels with absent lumen or lumen <1.02µm Ø were not considered	?	↓ villus area in PSCH ↑ villus area in term CH ↑ syncytiotrophoblast area in term CH and PSCH, and in preterm GH, CH and PSCH ↓ intervillous space area in term GH, PE and CH ↑ intervillous space area in term PSCH = SV vascularization ↑ TV capillary count in term CH and both preterm and term PE ↑ fibrin deposits in term CH, PSCH and GH ↓ fibrin deposits in preterm PSCH
-LM with test points -Magnification x364 -Masson trichrome -25-50 fields/case -1(?) blinded investigator	-	↓ villus area in FGR and PE+FGR ↓ diffusive conductance in FGR and PE+FGR

Supplementary Table 1. Continued

Study	Study groups and total patients (N)	GA at delivery (weeks, mean ± SD)	Umbilical / uterine artery Doppler	Maternal age (years, mean ± SD)
Egbor *et al.* (2006)[71]	-PE n=20 -FGR n=17 -PE+FGR n=16 -Controls (matched for GA) n=16 Total N=69	-PE: 34.3 ± 1.0 -FGR: 35.0 ± 0.9 -PE+FGR: 32.9 ± 0.9 -Controls: 34.9 ± 1.1 *p-value?*	UmA AED: -PE: n=0 -FGR: n=2 -PE+FGR: n=6 -Controls: n=0 NS	-PE: 34.3±1.6 -FGR: 26.3±1.2 -PE+FGR: 27.5±1.1 -Controls: 29.2±1.8 NS
Egbor *et al.* (2006)[72]	-PE n=20 -FGR n=17 -PE+FGR n=16 -Controls n=16 (matched for GA) Sub-analysis early and late-onset: -Early-onset: PE n=9, FGR n=7, PE+FGR n=10, Controls n=6 -Late-onset: PE n=11, FGR n=10, PE+FGR n=6, Controls n=10 Total N=69	-PE: 29.9±0.9 -FGR: 31.3±1.1 -PE+FGR: 30.8±1.0 -Controls: 30.0±1.2 NS	Early-onset group UmA: -PE: all normal -FGR: 2 AED, 2 normal, 3 missing -PE+FGR: 4 AED, 5 normal -Controls: all normal NS Late-onset group UmA: -PE: all normal -FGR: all normal -PE+FGR: 2 AED, 5 normal -Controls: all normal NS	Early-onset group: -PE: 32.2±1.2 -FGR: 23.7±3.0 -PE+FGR: 26.7±1.1 -Controls: 30.8±3.3 NS Late-onset group: -PE: 35.4±2.4 -FGR: 27.1±1.4 -PE+FGR: 28.3±2.0 -Controls: 28.4±2.3 NS
Kuzmina *et al.* (2005)[55]	Groups based on increasing severity of Doppler RI -Group 1: n=29 -Group 2: n=18 -Group 3: n=11 -Control: n=10 Total N=68 *Groups combined with PE; 78% PE in group 3, other groups unknown*	38-40	All groups significantly higher RI's for UtA, SpA, UmA and SvA compared to controls	?

Methods	Definition villi	Results
-LM with software *-Magnification?* -CD34 -50 fields/case -1 blinded investigator	-IV: >80µm Ø without tunica media -TV: <80µm Ø	↓ intervillous space area in PE, FGR and PE+FGR ↓ IV and TV area in FGR and PE+FGR ↓ IV and TV capillary area in FGR and PE+FGR
-LM with software *-Magnification?* -CD34 -50 fields/case -1 blinded investigator	-IV: >80µm Ø without tunica media -TV: <80µm Ø	↓ intervillous space area in early PE and PE+FGR ↓ TV area in early PE and PE+FGR ↓ IV capillary volume in FGR and PE+FGR ↓ IV and TV capillary length in FGR and PE+FGR ↑ TV capillarization index in early FGR and PE+FGR = TV capillary diameter
-LM with test grid *-Magnification?* -H&E -30 fields/case *-Blinded?*	?	↑ SV volume in groups 1-3 ↓ TV volume in groups 2 and 3 ↑ SV area in groups 1-3 ↑ TV area in groups 1 and 2 = intervillous space volume ↑ fibrin volume in groups 1-3 ↓ SV vessel volume in groups 2 and 3 ↓ SV vessel area in groups 1-3 = SV vessel diameter ↓ TV capillary volume in groups 1-3 ↑ TV capillary area in group 3 ↓ TV capillary diameter in group 3

Supplementary Table 1. Continued

Study	Study groups and total patients (N)	GA at delivery (weeks, mean ± SD)	Umbilical / uterine artery Doppler	Maternal age (years, mean ± SD)
Maly *et al*. (2005)[48]	-PE (no FGR) n=23 -PGDM (pregestational diabetes mellitus) n=10 -Controls n=13 Total N=46	-PE: range 37-41 -PGDM: range 39-42 -Controls: range 38-41 *p-value?*	?	Age in decades: -PE: 3rd: 14, 4th: 7, 5th: 2 -PGDM: 3rd: 7, 4th: 3 -Controls: 3rd: 9, 4th: 4 *p-value?*
Mayhew *et al*. (2004)[63]	-FGR n=5 -PE n=5 -PE+FGR n=5 -Controls n=9 Total N=24	Only descriptive: GA similar in FGR, PE and controls, but about 4 weeks shorter in PE+FGR	?	?
Guiot *et al*. (2003)[58]	-PE+FGR n=8 -Controls n=5 Total N=13 *All FGR cases delivered by cesarean section, controls vaginally*	Matching for GA Controls: week 30, 32, 33, 35 and 36	Dopplers from PE+FGR: 3 AED, 1 RED, 4 PED but PI >95th percentile for gestation	?
Mayhew *et al*. (2003)[62]	-FGR n=5 -PE n=5 -PE+FGR n=5 -Controls n=9 Total N=24	-FGR: 37 -PE: 36 -PE+FGR: 33 -Controls: 39 *Adverse outcome significantly lower GA than controls*	UmA: -FGR: 1 AED, 2 PED, 2 missing -PE: 3 PED, 2 missing -PE+FGR: 3 PED, 2 missing -Controls: 5 PED, 4 missing	-FGR: 36.2 -PE: 31.0 -PE+FGR: 27.6 -Controls: 30.9 *p-value?*

	Methods	Definition villi	Results
	-LM with software -Magnification x60 -H&E -6 fields/case *-Blinded?*	-	↓ capillary area in PE (although NS) and PGDM
	-LM with test points -Magnification x410 -Masson trichrome -25-30 fields/case *-Blinded?*	-	↓ villous area in FGR and PE+FGR = capillary area = capillarization index No interaction effects detected in PE+FGR
	-LM with software *-Magnification?* -α-SMA -Total of 9,132 vessels *-Blinded?*	ASMA positive vessels	↑ SV vessel diameter in AED and RED vs PED ↑ SV vessel internal diameter in cesarean section vs vaginal deliveries ↑ SV vessel internal diameter in increasing GA and increasing placental weight (practically independent from birth weight) ↑ SV vessel wall thickness in AED vs PED and RED ↑ SV vessel wall thickness in increasing GA, in heavier placentas and in cesarean section
	-LM with test points -Magnification x364 -Masson trichrome -25-50 fields/case -1 blinded investigator	?	↓ villous area in FGR and PE+FGR ↓ intervillous space in FGR and PE+FGR ↓ trophoblast volume in FGR and PE+FGR ↑ trophoblast depth in FGR ↓ capillary area in FGR and PE+FGR

Supplementary Table 1. Continued

Study	Study groups and total patients (N)	GA at delivery (weeks, mean ± SD)	Umbilical / uterine artery Doppler	Maternal age (years, mean ± SD)
Mitra *et al*. (2000)[74]	-FGR n=45 (10 with CH, 18 unexplained FGR, 3 collagen vascular disease, 14 chronic substance abuse) -Non-FGR n=27 (cases with hypertension, diabetes, collagen vascular disease in remission, renal disease) -Controls n=78 Total N=150	-FGR: 28-38 -Non-FGR: 33-38 -Controls: 28-40 *p-value?*	RI of UmA varied between >2 standard deviations and RED	-FGR: 21-39 -Non-FGR: ? -Controls: ?
Mitra *et al*. (1997)[59]	-Preterm deliveries at 23-36 weeks GA n=33 -Spontaneous abortion at 19-22 weeks GA n=14 -Normal deliveries at 37-40 weeks GA n=16 Total N=63	See study groups	RIs of UmA all within two standard deviations for GA	?

GA = gestational age, N/A = not applicable, NS = not significant, UtA = uterine artery, UmA = umbilical artery, SpA = spiral artery, SvA = stem villi artery, PI = pulsatility index, RI = resistance index, ARED = absent or reversed end diastolic umbilical flow Doppler, PED = preserved end diastolic umbilical flow Doppler

Methods	Definition villi	Results
-LM with software -40x objective -Periodic acid-Schiff *-Number of fields/ case?* *-Blinded?*	?	↑ SV vessel wall thickness in FGR vs controls and non-FGR With advancing GA ↓ vessel wall thickness in all groups ↓ SV vessel lumen in FGR vs controls and non-FGR Positive correlation between UmA RI and capillary wall thickness
-LM with software and tracing with stylus -40x objective -H&E, Periodic acid-Schiff -32-26 arteries per placenta *-Blinded?* -Exclusion of arteries with 1) no lumen, 2) ill-defined vessel-wall boundaries, or 3) obliquely sectioning	?	↓ SV vessel wall thickness and UmA RI with advancing GA = SV vessel diameter with advancing GA Strong correlation between the decrease in vessel wall thickness and decrease in UmA RI Spontaneous abortions were suggestive of cervical incompetence

#Stem villus characteristics: thick trophoblastic cover with identifiable cytotrophoblast (±20% of surface), stroma consists of condensed bundles of collagen fibers, adventitia of the vessels continues into surrounding stroma


7

Morphometric placental villous and vascular characteristics differ in early- and late-onset placental syndrome

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Submitted

Abstract

Objective: Preeclampsia (PE) and fetal growth restriction (FGR) are common pregnancy-complications, that are linked to abnormal placental development and associated with biochemical dysfunction and mechanical damage of the placenta. We investigated terminal villous and vascular growth in placentas of women with and without PE/FGR using a novel morphometrical method on placental tissue obtained in a routine clinical setting.

Methods: Fifty-seven placentas of various gestational ages (GA) were investigated on villus area, vessel area, lumen area and vessel number. The degree of villous capillarization was assessed by total vessel area/villus area-ratio and total vessel lumen area/villus area-ratio. Groups were divided into Early-onset (n=29, delivery <34 weeks GA) and Late-onset Delivery (n=28, delivery ≥34 weeks GA). Additionally, groups were divided by clinical phenotype: 1) Early-onset PE/FGR, n=20; 2) Late-onset PE/FGR, n=18; 3) Early-onset Controls, n=9; and 4) Late-onset Controls, n=10. Sub-analyses were performed based on umbilical artery Doppler velocimetry in PE/FGR.

Results: We demonstrated reduced villus area in PE/FGR compared to No PE/FGR, with a significant interaction effect of GA (larger effect in early-onset versus late-onset PE/FGR), while capillarization was not significantly affected by GA. Early-onset Delivery placentas showed less capillarization than Late-onset Delivery, irrespective of PE/FGR. Interestingly, the extent of capillarization increased gradually with decreasing severity of umbilical artery Doppler velocimetry from absent/reversed enddiastolic flow (ARED) to increased pulsatility-index (PI) towards normal in preserved enddiastolic flow (PED).

Conclusion: Our results suggest that abnormal villous growth is relevant to Early-onset PE/FGR only, while capillarization is affected in this stage independent of PE/FGR. This morphometrical approach can be used to further investigate the relationship between capillarization and villus development in PE and FGR, as well as with umbilical artery Doppler velocimetry. Our approach may aid in unravelling the pathophysiological role of placental villous and capillary interaction in these important obstetric syndromes and ultimately provide novel prognostic and therapeutic opportunities.

Introduction

Preeclampsia (PE) and fetal growth restriction (FGR) are common complications of pregnancy associated with a high risk of maternal and perinatal morbidity and mortality. PE and FGR, despite apparently being separate entities, often coincide with each other and have similar risk profiles, suggesting a common etiological background. Hence, there is a growing standpoint that both entities are covered by the spectrum of placental related disorders, colloquially termed "placental syndrome".[1] These heterogeneous diseases may have a different etiology based on the gestational age in which they are encountered and can be divided into an early-onset (<34 weeks gestational age) and a late-onset group (>34 weeks gestational age). The underlying pathophysiology still remains controversial, although it is now widely accepted that early-onset disease starts during early placentation with absent or defective spiral artery remodelling,[1,2] whereas late-onset disease is associated with pre-existing maternal cardiovascular or metabolic risk factors generating endothelial dysfunction.[3]

Normal spiral artery remodeling is accomplished by invading extravillous trophoblast (EVT) cells into the vessel walls, thereby replacing the arterial medial layer with fibrinoid material, ensuring low resistance blood flow needed for adequate feto-placental vascular development.[4] In the case of early-onset PE and/or FGR, impaired migration of EVTs towards the myometrial stroma and into the lumen of the spiral arteries is observed,[5] leading to a lack of dilatation of the vessels and causing placental malperfusion. The small-diameter vessels provoke an increased velocity of blood flowing into the intervillous space, leading to villous shear stress, hypoxia (due to reduced perfusion time) and increased blood pressure in the intervillous space.[6] The originated resistance to blood flow within the spiral arteries is transmitted upstream to the uterine arteries and can be measured by Doppler ultrasonography as an increased pulsatility index (PI) or resistance index (RI).[7] In the placenta a prolonged reduction in intervillous blood flow/oxygenation, leads to prolonged vasoconstriction of vessels within placental villi. This results in secondary reduction of vessel luminal diameter, vascular medial hypertrophy and increased flow resistance of mainly stem villi. The stem villi develop increased pressure, which may eventually extend upstream in the umbilical artery leading to abnormal Doppler velocimetry indices and eventually ARED (absent or reversed enddiastolic flow).[8] The hypoxic mechanisms involved in placental syndrome were described as uteroplacental and postplacental hypoxia by Kingdom and Kaufmann in 1997,[9] and they proposed a scheme of villous maldevelopment in these types of hypoxia. We hypothesize that all the above described changes are associated with altered placental microvasculature, that can be investigated morphometrically.

Morphometric placental microvascular measurements in different obstetric complications that can be categorized as placental syndrome, have been previously performed by various authors. However, results are divergent, which may be related to morphometrical approach and study design.[10-13] In hypertensive pregnancies, several investigators demonstrated increased terminal villous capillarization in PE[14,15] or chronic hypertension.[14] On the other hand, some investigators found decreased terminal villous capillarization in PE.[11,16] Similarly, Shchyogolev *et al*.[12] found decreased vascularization of the villi in severe PE, but not in moderate PE. Unfortunately, no definitions were given for the subgroups. Others found no significant differences in PE[10] or gestational hypertension.[33,44,17] In addition, several investigaters[18-21] stated that FGR, but not PE, is associated with deprived villous development and feto-placental angiogenesis. There was no interaction effect between PE and FGR demonstrated, while others[22] provide evidence that PE and FGR may have a cumulative effect. When comparing FGR to normal pregnancies there is also evidence of reduced villous capillarization.[23] Regarding terminal villi and villi in general, villus area was decreased in PE,[14,21,24] early-onset PE,[13] FGR[18-22] and PE with FGR.[13,18-20,22] On the other hand, some investigators found increased villous area in PE,[15,25] chronic hypertension[14] and altered Doppler measurements,[26,27] whereas others were unable to demonstrate any differences in placental syndrome.[11,12,23]

Thus, clinical characteristics vary between studies and are often not sufficiently depicted. Gestational age, which is positively correlated with placental vascularisation,[28] is often lower in cases than in controls[10,11] or inadequately described.[12] Furthermore, most studies do not differentiate between early-onset and late-onset disease, and thereby do not investigate the assumed different pathophysiology of these two groups. We realize that in order to investigate the value of all these clinical variables in placental pathology large numbers of patients are necessary. Therefore, in the current study we aimed to develop a morphometric system to quantify villous and vascular growth in placentas obtained in a clinical setting. We focused specifically on vasculature in terminal villi, as alterations have been detected in this region in previous studies,[13,20,24] albeit using a more extensive morphometric approach. We applied our method on placentas of women with and without PE/FGR and assessed whether we could discern differences in relation to gestational age (early- versus late-onset) and clinical phenotype (PE/FGR versus no PE/FGR).

Methods

Placentas were retrospectively obtained from women who delivered at Maastricht University Medical Center+ (MUMC+, Maastricht, The Netherlands). We selected a total of 57 placentas between 2011 and 2015, of which umbilical artery Doppler velocimetry values

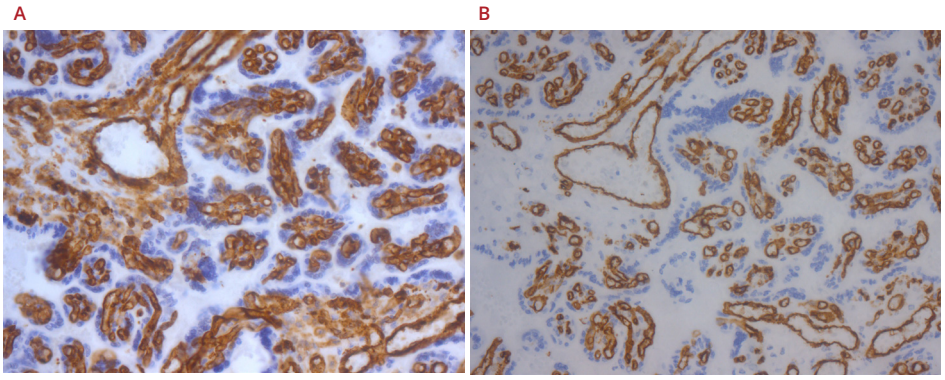
were available for the PE/FGR-cases. We included 38 PE/FGR-placentas and 19 placentas without PE/FGR of various gestational ages (both preterm and term). Groups based on gestational age were divided in two groups: Early-onset Delivery (delivery <34 weeks of gestation) n=29 and Late-onset Delivery (delivery ≥34 weeks of gestation) n=28. Subsequently, groups were based on both gestational age (delivery <34 vs ≥ 34 weeks of gestation) and clinical phenotype: 1) Early-onset PE/FGR, n=20; 2) Late-onset PE/FGR, n=18; 3) Early-onset Controls, n=9; and 4) Late-onset Controls, n=10.

Umbilical artery Doppler velocimetry data from the patients with PE or FGR were subdivided into 1) absent or reversed end diastolic umbilical flow: 'ARED', n=6; 2) preserved end diastolic flow (PED) with increased (>p90) pulsatility index (PI): 'PED↑PI', n=14; and 3) PED with normal PI: 'PED=PI', n=18. Doppler velocimetry was presumed normal in the control group (placentas delivered ≥34 weeks of gestation from pregnancies without PE/FGR).

PE was defined as described in detail previously.[29] A new-born was considered growth restricted when birth weight was below the 10th centile, based on the most recent Dutch birth weight reference curves.[30] Early-onset PE/FGR was defined as delivery before 34 weeks gestational age. Umbilical artery Doppler velocimetry was performed in the PE/FGR pregnancies as described in detail previously.[31]

After delivery, placentas were placed in 4% buffered formalin and allowed to fix for at least 48 hours. The cord was measured and inspected. The membranes were inspected as well and both the cord and membranes were removed. The placenta was weighed without the cord and membranes. Subsequently, the placenta was cut in 1 cm thick slices and the cut surface was inspected. At least two sections of normal central placental tissue were sampled. The samples were embedded in paraffin and 4 µm sections were cut. The sections were stained with H&E (hematoxylin and eosin) after which one paraffin block was selected for further immunohistochemical staining with CD31 and CD 34 to assess the fetal capillaries (Figure 1). We stained both CD31 and CD34 so we could choose which staining features the fetal capillaries best. 3 µm paraffin sections were cut onto Starfrost adhesive microscope slides. A monoclonal antibody was used to stain paraffin embedded tissue sections for endothelial markers using a CD 34 staining (CD 34 RTU DAKO clone QBEnd10). Then, sections were incubated with primary antibody for 20 minutes, after which they were incubated with Envision flex/HRP for 20 minutes. Slides were counterstained with hematoxylin. In Figure 1 the contrast and color saturation were slightly adjusted. In Figure 4 scanned images were used without any adjustments.

Figure 1. CD31- and CD34-stained slides of the same placenta.



A) CD31-stained placental slide. Note the increased background staining and staining of macrophages compared to B) CD-34-stained placental slide.

Morphometrical analyses were performed on CD34 stained paraffin sections of one tissue block per case by pathologist CS. Pictures were taken using a 20x objective. Morphometry was performed using Leica Qwin (V3) software (Leica, Cambridge UK). Villi were selected only if they were completely present in the pictures. Completely avascular trophoblastic knots were excluded, but knots in vascularized villi were included for the determination of villus surface. Since terminal villi are most important for gas-exchange we decided to include only terminal villi. A villus was defined as a terminal villus using a morphometrical threshold of <80 μm in diameter, according to the classification described by Mayhew and co-workers.[28] Per villus, total villus area, endothelial wall area and vessel lumen area were determined by setting a color threshold level for these individual parameters. First, each villus was manually traced to obtain villus area (including villous stroma, vessels and trophoblast). Then endothelial area was selected by a color threshold. Lumen area was automatically selected as the inner surface of vessels, and manually corrected if necessary. Vessel area was calculated by adding endothelial wall area and vessel lumen area. Finally, vessels were manually counted by clicking on the vessels. The degree of villous capillarization was assessed by calculating the following ratios per villus surface: total vessel area/villus area (capillarization index) and total vessel-lumen area/villus area (lumen index). At first, 20 pictures were taken from 20 different placentas and analyzed. We then tested the data using fewer pictures with T-tests and found that similar results per placenta for 20 pictures and eight pictures per placenta (results not shown). Therefore, we continued by analyzing 8 pictures of one block per placenta sample.

Statistical analyses were carried out using IBM SPSS Statistics for Windows (version 23; Armonk, NY: IBM Corp.). Means, 95% confidence interval (CI) and standard error (SE) were calculated for each group (PE/FGR and No-PE/FGR; Early preterm and Controls; ARED and

PED↑PI and PED=PI). Categorical data were analyzed using Chi-square tests. For numerical outcomes groups were compared using two-way analysis of variance, in which the factors analyzed were PE/FGR (yes versus no) and onset (early-onset versus late-onset). The interaction between PE/FGR and onset was first tested and removed from the model if it was not significant. If the interaction was significant, then the effects of PE/FGR were reported separately for early and late onset as well as the effects of onset for PE/FGR and no PE/FGR separately. Differences between the Doppler-based groups were analyzed using one-way ANOVA with 4 groups (ARED, PED↑PI, PED=PI, Late-onset Controls). A two-sided p-value ≤0.05 was considered statistically significant.

Results

Patient characteristics and morphometrical data in early- and late-onset PE/FGR

Table 1 describes clinical data of the study groups. As expected, the PE/FGR group demonstrated lower birth weight (mean difference 758 g, 95% CI 233-1284 g) and placental weight (mean difference 140 g, 95% CI 65-215 g) than the No PE/FGR group (p=0.006 and p<0.001, respectively). The Early-onset Delivery group showed a lower gestational age at delivery (mean difference 8.8 weeks, 95% CI 7.7-10.0 weeks) than the Late-onset Delivery group (p<0.001). In addition, Early-onset Delivery demonstrated lower birth weight (mean difference 1584 g, 95% CI 1281-1885 g; p<0.001) and placental weight (mean difference 175 g, 95% CI 112-238 g; p<0.001), as well as a higher amount of pregnancies complicated by PE (with or without FGR, p=0.011) and pregnancies complicated by FGR only (p=0.014).

Table 1. Clinical data for the study groups based on clinical phenotype and gestational age. Data are presented as means (SE), unless otherwise noted.

Total N=57	**PE/FGR**	**No PE/FGR**	**Early-onset Delivery (<34 weeks GA)**	**Late-onset Delivery (≥34 weeks GA)**
n	38	19	29	28
Maternal age (years)	30.6 (0.9)	30.6 (1.2)	29.6 (0.9)	31.6 (1.1)
Body mass index	25.0 (1.1)	27.8 (2.5)	24.5 (1.5)	26.5 (1.5)
Gestational age (weeks)	32.9 (0.8)	33.8 (1.2)	28.9 (0.4)†	37.7 (0.4)
Birth weight (g)	1700 (148)*	2459 (224)	1161 (95)†	2745 (116)
Placental weight (g)	279 (20)*	418 (35)	239 (21)†	414 (24)
PE (with or without FGR)	n=24 (63.2%)	-	n=15 (51.7%)†	n=9 (32.1%)
Only FGR	n=14 (36.8%)	-	n=5 (17.2%)†	n=9 (32.1%)

GA = gestational age (at delivery), PE = preeclampsia, FGR = fetal growth restriction
* Significant difference (p<0.05) between PE/FGR and No PE/FGR
† Significant difference (p<0.05) between Early-onset Delivery and Late-onset Delivery

Table 2 shows that in the presence of PE/FGR, villus area had a significant interaction effect with gestational age (p=0.029). Early-onset PE/FGR had a borderline significant lower villus surface than Late-onset PE/FGR, the mean difference being 116 µm (95% CI -17-249; p=0.086). Early-onset PE/FGR also showed a lower villus surface than Early-onset Controls, with a mean difference of 247 µm (95% CI 77-416; p=0.006). Late-onset PE/FGR was similar to Late-onset Controls (mean difference -1.7 µm, 95% CI -153.5-150.0; p=0.981). The deviation between Early-onset versus Late-onset Controls did not show a statistical significance (mean difference -133 µm, CI -313-48, p=0.140; Figure 2). There were no significant interactions with gestational age for the other parameters and no significant differences between PE/FGR and No PE/FGR. On the other hand, when comparing by gestational age only, the Early-onset Delivery group demonstrated a lower vessel area (p=0.002), vessel lumen area (p<0.001), vessel number (p=0.047), as well as a decreased capillarization index (p=0.005) and lumen index (p<0.001) compared to the Late-onset Delivery group.

Table 2. Morphometrical data for the study groups based on clinical phenotype and gestational age. Data are presented as means (SE), unless otherwise noted.

Total n=57	**Int. effect p-value**	**PE/FGR**	**No PE/FGR**	**Early-onset Delivery (<34 weeks GA)**	**Late-onset Delivery (≥34 weeks GA)**
n		38	19	29	28
Villus area (µm²)	0.029	2313 (34)*$^{p=0.032}$	2435 (44)	2335 (43)$^{p=0.879}$	2373 (35)
Vessel area (µm²)	0.975	723 (34)$^{p=0.386}$	685 (47)	631 (26)†$^{p=0.002}$	793 (43)
Vessel lumen area (µm²)	0.920	138 (15)$^{p=0.606}$	131 (22)	92 (10)†$^{p<0.001}$	182 (19)
Vessel number	0.646	4.3 (0.1)$^{p=0.341}$	4.5 (0.2)	4.2 (0.1)†$^{p=0.047}$	4.6 (0.1)
Capillarization index	0.447	0.32 (0.01)$^{p=0.126}$	0.29 (0.02)	0.28 (0.01)†$^{p=0.005}$	0.34 (0.02)
Lumen index	0.875	0.06 (0.01)$^{p=0.555}$	0.05 (0.01)	0.04 (<0.01)†$^{p<0.001}$	0.07 (0.01)

GA = gestational age (at delivery), Int. effect = interaction effect with GA (early-onset versus late-onset), PE = preeclampsia, FGR = fetal growth restriction

* Significant difference (p<0.05) between PE/FGR and No PE/FGR

† Significant difference (p<0.05) between Early-onset Delivery and Late-onset Delivery

Relationship between morphometrical data and Doppler measurements of the umbilical artery

We then analyzed the relationship between our morphometry data in patient subgroups based on Doppler velocimetry values (Table 3). As expected, umbilical artery Doppler PI was significantly higher in ARED than in PED↑PI (mean difference 1.31, 95% CI 0.45-2.17; p=0.007) and PED=PI (mean difference 1.89, 95% CI 0.68-3.11; p=0.015). Clinical data show a decreasing severity of clinical outcome with decreasing severity of abnormal Doppler data. Gestational age at delivery was significantly lower in ARED (mean difference 9.3 weeks, 95% CI 6.7-11.9 weeks; p<0.001) and PED↑PI (mean difference 7.5 weeks, 95% CI 4.6-10.4 weeks; p<0.001) compared to Late-onset Controls. A similar pattern was observed

for birth weight and placental weight in ARED (mean difference 2376 g, 95% CI 1831-2920 g and mean difference 347 g, 95% CI 262-432 g, respectively; both p<0.001), PED↑PI (mean difference 1841 g, 95% CI 1276-2407 g and mean difference 271 g, 95% CI 185-358 g, respectively; both p<0.001) and PED=PI (mean difference 888 g, 95% CI 313-1462 g and mean difference 139 g, 95% CI 43-234 g, respectively; p=0.004 and p=0.006, respectively) as compared to Late-onset Controls. Furthermore, all cases with ARED were derived from early-onset PE/FGR (6/6), as were most cases with PED↑PI (11/14), whereas cases with PED=PI were mostly stemming from late-onset PE/FGR (15/18). Significantly more cases of late-onset PE/FGR were observed in PED↑PI and PED=PI (both p<0.001) and fewer cases of early-onset in PED=PI (p<0.001) as compared to ARED.

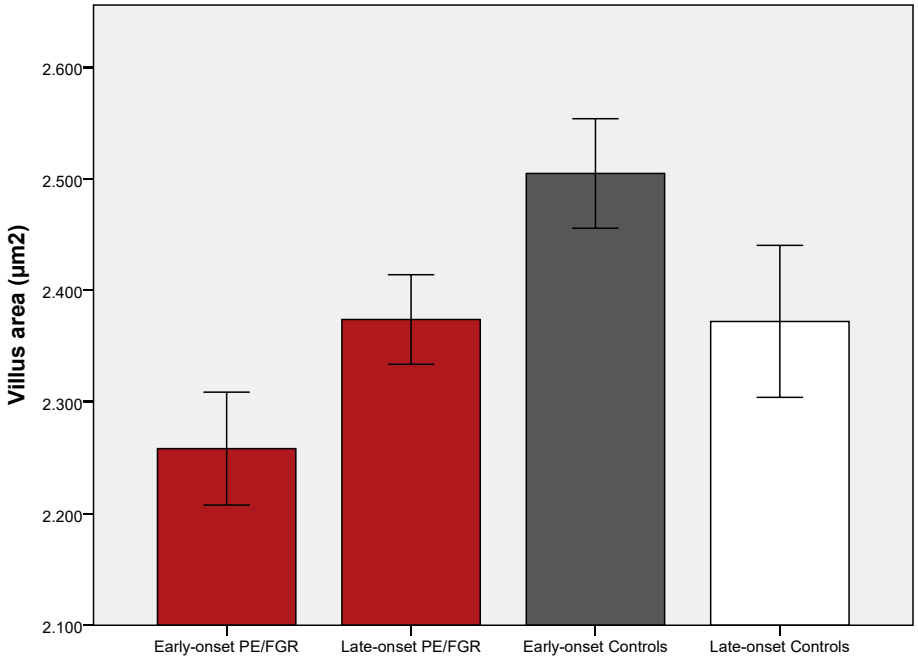
Table 3. Demographics for study groups based on Doppler velocimetry values. Data are presented as means (SE), unless otherwise noted.

Total n=48	**ARED**	**PED↑PI**	**PED=PI**	**Late-onset Controls (≥34 weeks GA)**
n	6	14	18	10
Maternal age (years)	26.0 (1.7)	30.8 (1.4)	32.0 (1.4)	30.8 (1.9)
Body mass index	25.5 (2.6)	26.5 (2.2)	23.9 (1.6)	28.8 (2.7)
Gestational age (weeks)	28.8 (0.7)*	30.6 (1.0)*	36.0 (1.0)	38.1 (0.9)
Birth weight (g)	778 (71)*	1312 (190)*	2266 (179)*	3154 (190)
Placental weight (g)	152 (13)*	228 (17)*	360 (28)*	499 (36)
Early-onset PE/FGR	n=6 (100%)	n=11 (79%)	n=3 (17%)†	-
Late-onset PE/FGR	n=0 (0%)	n=3 (21%)†	n=15 (83%)†	-
Highest Umbilical artery PI	2.9 (0.4)	1.6 (0.2)†	1.0 (0.1)†	-

GA = gestational age (at delivery), PE = preeclampsia, FGR = fetal growth restriction, ARED = absent or reversed enddiastolic flow, PED = preserved enddiastolic flow, PI = pulsatility index
* Significant difference (p<0.05) compared to Late-onset Controls
† Significant difference (<p0.05) compared to ARED

Table 4 shows that vessel lumen area, lumen index and vessel number were all (borderline) significantly lower in the ARED subgroup compared to Late-onset Controls (p=0.055, p=0.043 and p=0.043, respectively). Interestingly, the data show a gradual scale of increasing values from ARED to PED↑PI to PED=PI, whereas the PED=PI subgroup is comparable to the Late-onset Controls. This is graphically clarified for lumen index as an example in Figure 3 and illustrated in CD34 stained placentas in Figure 4.

Figure 2. Mean villus area for subgroups based on both clinical phenotype and gestational age (early = <34 weeks gestational age; late = ≥34 weeks gestational age).



Error bars represent +/- 1 SE.

Table 4. Morphometrical data for the PE/FGR group based on Doppler velocimetry indices and term control group (≥34 weeks GA). Data are presented as means (SE).

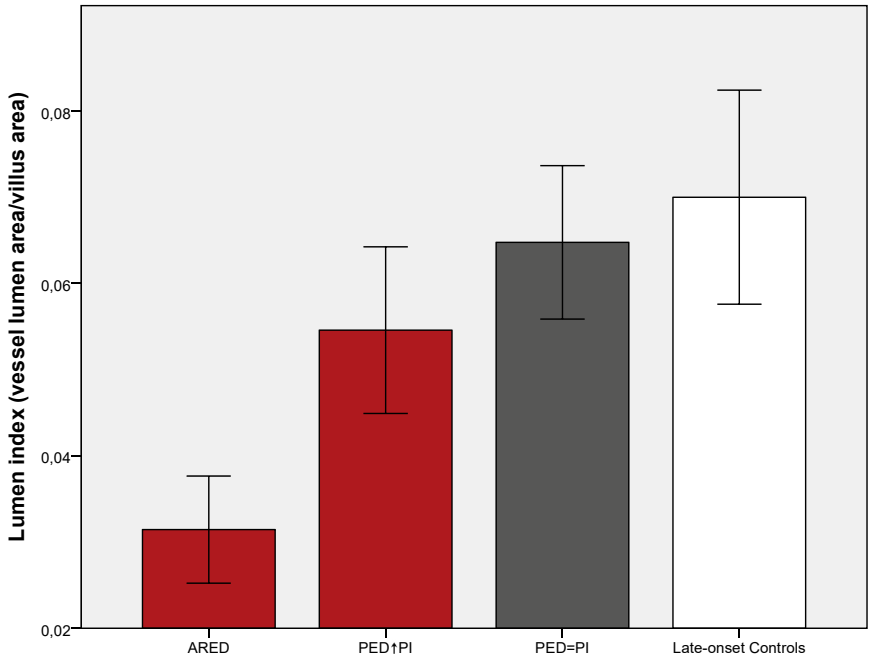
Total n=48	**Overall test**	**ARED**	**PED↑PI**	**PED=PI**	**Late-onset Controls (≥34 weeks GA)**
	p-value	**n=6**	**n=14**	**n=18**	**n=10**
n		6	14	18	10
Villus area (µm²)	0.199	2293 (77)$^{p=0.457}$	2234 (67)$^{p=0.109}$	2381 (37)$^{p=0.908}$	2372 (68)
Vessel area (µm²)	0.520	652 (69)$^{p=0.329}$	688 (42)$^{p=0.413}$	774 (58)$^{p=0.884}$	761 (77)
Vessel lumen area (µm²)	0.178	82 (14)$^{p=0.055}$	129 (23)$^{p=0.230}$	164 (23)$^{p=0.761}$	176 (33)
Vessel number	0.203	4.1 (0.2)*$^{p=0.043}$	4.3 (0.2)$^{p=0.124}$	4.4 (0.1)$^{p=0.224}$	4.7 (0.3)
Capillarization index	0.803	0.29 (0.03)$^{p=0.392}$	0.32 (0.02)$^{p=0.836}$	0.33 (0.02)$^{p=0.992}$	0.33 (0.03)
Lumen index	0.175	0.03 (0.01)*$^{p=0.043}$	0.05 (0.01)$^{p=0.303}$	0.06 (0.01)$^{p=0.712}$	0.07 (0.01)

GA = gestational age (at delivery), ARED = absent or reversed enddiastolic flow, PED = preserved enddiastolic flow, PI = pulsatility index

p-values indicate differences compared to Late-onset Controls

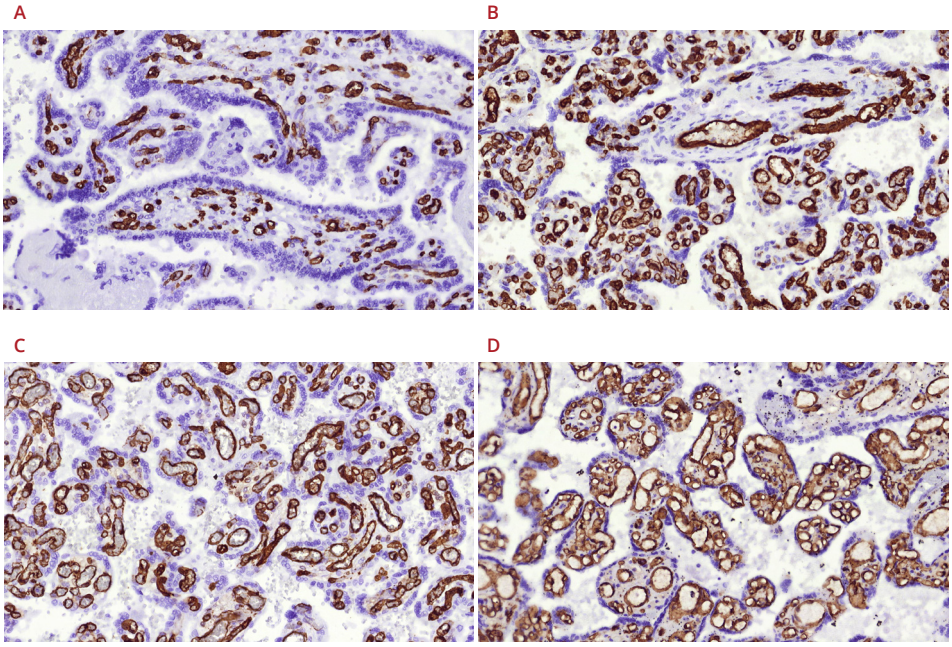
* Significant difference (p<0.05) compared to Late-onset Controls

Figure 3. Mean lumen index for subgroups with PE/FGR based on Doppler velocimetry compared to controls. Error bars represent +/- 1 SE.



Error bars represent +/- 1 SE.

Figure 4. Examples of CD34-stained placental slides indicating differences in terminal villus area, lumen area and vessel number. Pictures taken at same magnification (20x objective).



A) Placenta from a pregnancy complicated by early-onset FGR and ARED, GA 28+4 weeks. B) Placenta from a pregnancy complicated by late-onset FGR with PED and increased PI, GA 38+5 weeks. C) Placenta from a pregnancy complicated by late-onset FGR with PED and normal PI, GA 38+1. D) Control placenta at 37+1 weeks GA. Note the smaller terminal villus area, lumen area and vessel number in the early-onset FGR placenta compared to the other placentas and the gradual transition of these parameters with normalization of the Doppler values (ARED → PED with increased PI → PED with normal PI) towards the control placenta.

Discussion

In this pilot study we demonstrated that villous capillarization is lower in placentas from women who delivered before 34 weeks of gestational age compared to placentas from women who delivered beyond this gestational age. When not correcting for gestational age at delivery, there were no significant differences in villous capillarization in PE/FGR placentas and No PE/FGR placentas. However, there was a significant interaction effect between PE/FGR and gestational age (early- versus late-onset PE/FGR) for villus surface: Early-onset PE/FGR showed a decreased villus surface compared to Early-onset Controls and Late-onset PE/FGR, although the latter only reached borderline significance, and Late-onset PE/FGR demonstrated similar values compared to Late-onset Controls. Interestingly, when making subgroups based on the severity of deviating umbilical artery Doppler indices, the data showed a gradual scale. Capillarization parameters increase

with decreasing severity of umbilical artery Doppler indices towards the levels of control placentas.

We used a morphometric method specifically focusing on terminal villi and using an immunohistochemical endothelial staining that can be employed on one tissue block per placenta. With this method we validated earlier findings by other authors on decreased villous capillarization in PE[11,16] and in FGR.[19,22,23] In these previous studies more extensive morphometric analysis was performed, meanwhile for instance also taking placenta volume, systematic random-block sampling and perfusion-fixation into account. Although we cannot exclude a bias in our results based on the "reference trap" (as described by Mayhew *et al*.),[32] our data are in line with the data acquired using these more stringent morphometrical methods, and our method may thus allow comparison between patient groups. Our findings are also in line with Egbor *et al*.[13] who also had taken gestational age in account and formed subgroups of early-onset and late-onset disease, using the same cut-off value of 34 weeks. They also demonstrated decreased terminal villus area in early-onset PE and PE with FGR and reduced vessel area of terminal villi in late-onset FGR and PE with FGR, compared to controls and PE without FGR. However, in contrast to our findings, Egbor *et al*. suggested that capillary surface area of terminal villi show markedly lower values in all early-onset cases compared to the late-onset cases. However, their manuscript did not provide statistical analyses between these groups and they did not take Doppler velocimetry values into account as we did (see below). We recognize this current study is a pilot study and needs to be extrapolated to larger patient groups, in which also a subdivision between the PE/FGR group needs to be made. However, our results do indicate that the current morphometrical approach can be used in these future studies. Further research may focus on explaining the varying results regarding placental morphometrical measurements in placental syndrome.

Our results indicate that gestational age at delivery may play an important role in the observed differences between previous studies on placenta microvasculature.[10-27,33] With increasing gestational age, placental vascularization increases.[28] Since PE and FGR are often iatrogenically related to lower gestational ages at delivery, this could be an important confounder in studies morphometrically investigating placental vascularization. Gestational age at delivery is actually often lower in placental syndrome than in controls[10,11,19,23] or insufficiently depicted,[12,16] which could lead to incorrect assumptions of decreased vascularization in (late-onset) placental syndrome. Thus, our data underscore the importance of taking gestational age into consideration when interpreting data on stereological placental measurements. Villus area showed a significant interaction with gestational age (early- versus late-onset) and demonstrated similar values for late-onset PE/FGR and late-onset controls, indicating that early- and late-onset diseases are different entities

with different etiologies. Egbor *et al*. also demonstrated a similar morphology of placentas from late-onset PE and controls.[13] Furthermore, our results showed a lower villus area in Early-onset Delivery than in Late-onset Delivery, demonstrating that early-onset preterm birth cannot be considered as an accurate control-group. This is in accordance with the literature on placental syndrome in which early-onset preterm birth is also considered part of the syndrome,[1] making it challenging to correct for gestational age and meriting further research into the placental contribution in different phenotypes of preterm birth.

In Early-onset Delivery (with or without PE/FGR) we found a decreased vessel number and lumen area, which may have important pathophysiological consequences. In placental angiogenesis, there are two forms described: branching and non-branching angiogenesis.[34] In the first half of normal pregnancies branching angiogenesis predominates, whereas from about 24 weeks of gestation until term non-branching angiogenesis is the dominant type. In a two-dimensional view as in microscopic slides, non-branching angiogenesis results in a smaller number of vessels as we demonstrated in early-onset birth, indicating that these placentas show an accelerated maturation. Placental perfusion may be decreased due to the reduced vessel number and lumen area, which leads to a decrease in blood flow according to Poiseuille's equation. In this formula, blood flow (Q) through a vessel is indirectly proportional to the length of the vessel (L) and the viscosity of the blood (μ) and is proportional to the pressure drop across the vessel (ΔP) and the vessel radius (r) to the fourth power: $Q=(\Delta P\pi r^4)/(8\mu L)$.[35] In this equation vessel radius is related to vessel lumen area in our measurements, considering the surface of a circle is calculated by πr^2. In addition, there is a greater difference in lumen area among our subgroups then there is in vessel number (which is indirectly related to vessel length). Hence, lumen area carries the most influence in the formula and our observed decrease in lumen area will therefore result in a decreased placental blood flow ensuing in placental perfusion. The formula also underlines the difference between capillarization index and lumen index, in which the same numbers in these indices result in a greater blood flow for lumen index as compared to capillarization index. Furthermore, placental syndrome is associated with absent or defective spiral artery remodeling, leading to increased blood pressure in the intervillous space[6] and making it is presumable that the ΔP is also increased leading to an even greater effect on placental malperfusion. Moreover, vascular resistance (R) is incorporated in Poiseuille's equation, for which the formula is $R=(8\mu L)/(\pi r^4)$, indicating that our observed decrease in lumen area may also result in an increase in (upstream) vascular resistance.

An increase in placental blood flow resistance is mirrored by worsened umbilical artery Doppler waveforms, as was put forward by Kingdom *et al*.[36] This may be reflected by an increased PI and RI and even ARED. Our sub-analyses according to Doppler velocimetry

of the umbilical artery indeed show a gradual scale in which capillarization parameters increase with decreasing severity of umbilical artery Doppler indices towards the levels of late-onset control placentas. Our results are in line with those of Van Oppenraaij *et al*.[24] who demonstrated a lower terminal villous capillarization index in the FGR subgroup with ARED, compared to those with PE. However, they only compared ARED with PED, while they did not make subgroups of Doppler velocimetry values in decreasing severity, which we did by adding a subgroup of increased PI and RI. By doing so, we found a gradual scale in capillarization parameters, and we also demonstrated that clinical severity decreased with decreasing severity of the Doppler indices. These observations could indicate that placental resistance plays a crucial role in the pathophysiology of placental syndrome. There are some other reports on patients with altered Doppler values that found increased stem villus vascularization,[26,33] while Kuzmina *et al*.[27] found a decreased vascularization. Our current results merit further studies on the relationship of morphometric placental microvascular and villous capillary measurements in relation to umbilical Doppler velocimetry values, to aid in unravelling the underlying pathophysiological mechanism and ultimately develop prognostic biomarkers and therapeutic interventions during pregnancy.

Conclusion

To conclude, we developed a reliable method to perform morphometric placental measurements which seems to be accurate in helping to unravel the pathophysiology of placental syndrome. Our pilot study underlines the importance of taking gestational age into consideration when interpreting data on morphometric placental measurements in placental syndrome, and we suggest that placentas should be separated into early- and late-onset cases in future studies. Our results show abnormal villous growth in early-onset PE/FGR and normal villous growth in late-onset PE/FGR, without differences in microvascular parameters, confirming these are two separate entities with different underlying etiologies. Furthermore, we believe that investigating stereological placental measurements combined with umbilical artery Doppler velocimetry values could provide more insight into the pathophysiology of placental syndrome and warrants further investigation in larger patient groups.

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8

General discussion

Pathogenesis of placental syndrome

Placental syndrome originates at the placental bed as a result of impaired trophoblast invasion of the maternal spiral arteries located in the decidua and myometrium. Trophoblastic invasion normally takes place from 8 to 18 weeks of pregnancy,[1] and leads to physiological adaptation of the spiral arteries to allow unrestricted flow of maternal blood under unstressed and stressed situations into the intervillous space of the placenta. Maladaptation leads to persistent uterine autoregulatory flow and with it a generally increased vascular resistance and attenuated blood flow.[2] These changes in turn lead to rapidly cycling normoxic and hypoxic states in the intervillous space of the placenta and to ischemia-reperfusion injury caused by free radicals such as reactive oxygen species (ROS). [3,4] In chronic (uteroplacental) hypoxia, the production of certain angiogenic factors is stimulated, namely vascular endothelial growth factor-A (VEGF-A) and its receptors VEGF-R1 and VEGF-R2, resulting in branching-angiogenesis.[4] Chronic uteroplacental hypoxia can aggravate into postplacental hypoxia, resulting from arteriovenous shunting in the intervillous space, which causes the maternal intervillous blood to be relatively hyperoxic.[5] This type of hypoxia leads to non-branching angiogenesis and occurs through stimulation of VEGF-R1 by Placental Growth Factor (PlGF).[5] The different types of angiogenesis result in different histological pictures of these placentas. Branching angiogenesis leads to the formation of villi with indented surfaces compatible with the histological image of accelerated maturation, whereas non-branching angiogenesis results in the formation of long slender villi compatible with distal villous hypoplasia (DVH). Thus, the decreased oxygen supply is compensated by inducing angiogenesis. Another compensatory mechanism is the increased nitric oxide (NO) production in hypoxia, which stabilizes the endothelium.[3] NO is a strong vasorelaxant and anticoagulant factor and produced by the activation of NO synthase (NOS).[3]

In placental syndrome these compensatory mechanisms to hypoxia eventually fail and the balance tips over to the production of anti-angiogenic factors, influenced by oxidative stress.[2,3] ROS also suppress the expression and function of endothelial NOS.[3] The vascular endothelium of the uteroplacental circulation is damaged and the damage can spread to the maternal circulation as well, leading to widespread endothelial dysfunction.[2] The endothelial lesions are classified as acute atherosis. Acute atherosis is characterized by subendothelial lipid-filled foam cells, fibrinoid necrosis and leukocyte infiltration. It resembles not only early stages of atherosclerosis,[6] but also lesions seen in acute rejection in renal transplants.[7] In fact, analysis of immune reactions of placental syndrome and graft rejection show that both disorders share many mechanisms.[8] Not surprisingly, placental syndrome leads to an increased maternal cardiovascular risk in later life[9] and may show symptoms comparable to atherosclerosis and graft rejection.[10]

The next stage after widespread endothelial damage is the appearance of clinical symptoms. Endothelium destruction leads to increased vascular permeability and coagulation system activation with platelet consumption and development of infarctions. Maternal symptoms include hypertension, liver cell damage, renal glomerular damage or symptoms referred to as eclampsia. If uteroplacental gas and nutrient exchange is greatly reduced, FGR is observed. In case of the development of massive placental lesions, extremely compromising uteroplacental circulation, placental abruption symptoms or even fetal death may occur.[2] Currently, treatment is targeted on symptom-control. The only curing treatment is delivery of the placenta.

Heterogeneity of placental syndrome

Placental syndrome consists of a variety of clinical syndromes and although the common denominator is considered defective spiral artery remodeling, the exact mechanism is still unknown. Several factors play a role in this defective remodeling, such as abnormal genetic variations, biology of the trophoblasts or defective trophoblast differentiation acting together with extrinsic factors (such as maternal constitution factors), impaired action of maternal endothelial cells and immunological and inflammatory factors such as action of macrophage defense mechanism and impaired action of decidual natural killer cells.[11] Furthermore, even within a clinical disease entity there are differences regarding etiology. While early- and late-onset preeclampsia were first considered the same disease, there is now increasing evidence that they differ in pathophysiology.[12] Early-onset PE develops before 34 weeks of pregnancy and is by some considered a fetal disorder. It is associated with placental dysfunction and reduction in volume, FGR, abnormal uterine and umbilical artery Doppler evaluation, and adverse maternal and neonatal outcomes. Late-onset PE has an onset after 34 weeks of gestation and could be viewed upon a maternal disorder. It is often associated with a normal placenta, normal fetal growth, normal Doppler evaluation and less severe adverse outcomes.[12]

However, many investigators still fail to take this notion into consideration, allocating both types into the same group of PE. There are several reasons why investigators do not differentiate between early- and late-onset disease. Although early-onset PE has the most severe symptoms and consequences, the incidence is much lower than late-onset PE, making it methodologically more difficult to study early-onset disease. Often there is more interest to cover the entire spectrum, otherwise women with late-onset PE would be missed in the clinic. Lastly, not everyone is aware of the difference in pathophysiology between early- and late-onset PE.

Recurrent placental syndrome

Patients and their partners often perceive a pregnancy complicated by placental syndrome as a traumatic life event, particularly when there are serious consequences for mother and/or fetus. Although PE is mostly regarded a disease that affects first pregnancies,[13] there is a high recurrence risk approaching 50% in some studies for preeclamptic women with severe features in the initial pregnancy.[14] Not only PE shows an increased risk of recurrence, but also preterm birth, FGR and fetal death.[14,15] The conditions are strongly interrelated and each condition predisposes women to the other outcomes in their subsequent pregnancy.[15] Other than an increased risk based on obstetric history, the risk factors for incident and recurrent placental syndrome are fairly similar.[16,17] Given the low prevalence of placental syndrome in first pregnancies (5%), but the higher recurrence rates in subsequent pregnancies (30%), placental syndrome is more easily studied in women highlighted as high-risk due to a previous pregnancy complicated by placental syndrome. Although we do not know for certain whether the pathophysiology of recurrent PS and the role of serum biomarkers can be extrapolated to all women, including primigravida's, these data will help elucidate certain mechanisms of the disorder, that need to be confirmed in larger more inclusive studies.

Given the high recurrence rate, proper counselling in a next pregnancy is of high importance. Placental syndrome is one of the most challenging diseases of pregnancy. The etiology is rather diverse. Therefore, there is no simple unifying marker for prediction. Moreover, treatment is currently solely based on symptom control, the only curing treatment is delivery of the placenta. To ensure early detection of symptoms, more frequent clinical follow-up is needed. In addition, more research is needed in early pregnancy to detect risk factors before the clinical onset of the disease.

Recurrent placental syndrome is associated with biomarkers for endothelial dysfunction in early pregnancy

Many risk factors for placental syndrome are also risk factors for endothelial dysfunction and there is growing evidence that women with a history of placental syndrome are at increased risk of cardiovascular disease later in life.[18] Endothelial dysfunction plays an important role in the pathophysiology of both placental syndrome and atherosclerosis, in which oxidative stress has a central part.

Oxidative stress generates ROS, which are a group of small reactive molecules that play critical roles in the regulation of various cell functions and biological processes. Although

ROS are indispensable for endothelial homeostasis, abundant production is involved in endothelial injury. ROS are scavenged by endogenous anti-oxidants.[19] We demonstrated increased total anti-oxidant capacity in early pregnancy in women destined to develop recurrent placental syndrome, suggesting that during early pregnancy these women are still able to adapt to the increased production of ROS.

ROS also interfere with the production of NO by endothelial NOS, leading to endothelial dysfunction.[3] Another mechanism by which NO production could be influenced in placental syndrome is through impeding NOS by asymmetric dimethyl arginine (ADMA). Raised circulating levels of ADMA are generally regarded as a risk factor for endothelial dysfunctioin.[20] Although we were not able to demonstrate raised ADMA concentrations in women with recurrent placental syndrome, we did find a trend towards a rise in L-arginine/ADMA ratio at 12 weeks pregnancy in recurrent placental syndrome, indicating higher NO production and thus an effective adaptive response to pregnancy. By mid-pregnancy, however, L-arginine concentrations were declining speculating that after this time-point the scale will tip towards a decreased NO production eventually resulting in endothelial dysfunction.

Another important factor in endothelial dysfunction is cholesterol. Hypercholesterolemia is linked to endothelial dysfunction, whereas increased HDL concentrations exert a protective effect on the endothelium. Furthermore, LDL is prone to oxidation and oxidized LDL may even be more toxic to the endothelium than native LDL.[21] In this thesis we describe an absent transient decline of LDL concentrations in the first trimester of pregnancy in women prone to develop recurrent placental syndrome and a stagnation in the rising HDL levels from 12 weeks onwards. Interestingly, the before mentioned risk factors for endothelial dysfunction also play an important role in placental angiogenesis, as will be explained in the next section.

Recurrent placental syndrome is associated with angiogenic factors in early pregnancy

As previously explained there are two types of hypoxia in placental syndrome, namely uteroplacental and postplacental hypoxia, resulting in excessive branching and non-branching angiogenesis respectively. In uteroplacental hypoxia, the production VEGF-A and its receptors VEGF-R1 and VEGF-R2 is stimulated.[4] In order for branching angiogenesis to take place, endothelial cells need to migrate and break down the surrounding matrix to form new branches of vessels. Chronic uteroplacental hypoxia can aggravate into postplacental hypoxia, resulting in non-branching angiogenesis through stimulation of VEGF-R1 by PlGF.[5]

In branching angiogenesis cell migration is stimulated by upregulation of fibronectin.[22,23] Indeed, we demonstrated higher fibronectin concentrations during early pregnancy in women with recurrent placental syndrome. Furthermore, fibronectin is known to interact with fibrin and increased perivillous fibrin depositions are a common finding in PE.[24] The proteolytic activity is stimulated through upregulation of urokinase (uPA), which is part of the uPA system and inhibited by plasminogen activator inhibitors type 1 and 2 (PAI-1 and PAI-2).[25] We also showed lower PAI-2 concentrations in women with recurrent placental syndrome. PAI-2 is also considered a marker for total amount of trophoblast, since it is only produced by trophoblast cells. Lower PAI-2 levels would therefore suggest a lower placental weight, which is often the case in placental syndrome.[24]

In addition, LDL has been shown to dose-dependently reduce both VEGF-R1 and VEGF-R2.[26] Furthermore, acute atherosis (presence of lipid laden foam cells in maternal spiral arterioles) as seen in placental syndrome resembles the early stages of atherosclerosis,[27] which in turn has also been shown to be majorly influenced by hypoxia.[28]

An important factor in activating VEGF and VEGF-R's during hypoxia is HIF-1 (hypoxia inducible factor 1).[29] HIF-1 is a transcription factor and a heterodimer consisting of an alpha and a beta subunit. HIF-1α accumulates under hypoxic conditions, dimerizes with HIF-1β and binds to the nucleus to act as a transcription factor. Its accumulation is enhanced by ROS.[30] In contrast, PlGF gene expression is not influenced by HIF.[31] The exact mechanism of increased PlGF production in hypoxia still remains to be elucidated.

The importance of villous angiogenesis in the development of "early" placental syndrome: a new hypothesis

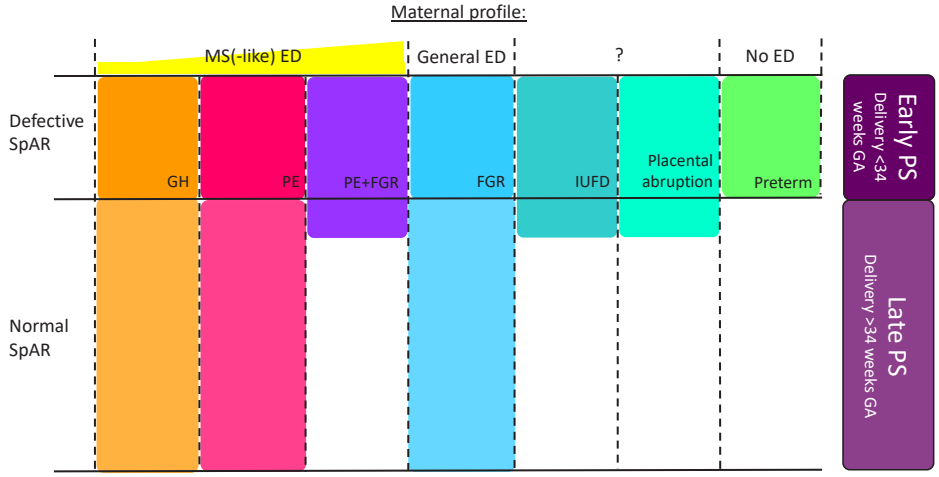
Many investigators have addressed the pathophysiology of the clinical entities associated with placental syndrome before, but the corresponding placental histology is disregarded. We believe, however, that the placental histology is very important and is even able to reflect the pathophysiology of placental syndrome.

Based on our findings, we propose to divide placental syndrome into two main groups, namely those women with normal spiral artery remodeling – "late" placental syndrome – and those with defective spiral artery remodeling – "early" placental syndrome (Figure 1). The group of women who show normal spiral artery remodeling are those who develop late onset PE or FGR. Both groups of women often have pre-existing risk factors for endothelial dysfunction. Women with late onset PE have risk factors consistent with metabolic

syndrome (such as adiposity, insulin resistance/hyperglycemia, hyperlipidemia, and coagulopathy) and women with late onset FGR have other risk factors for endothelial dysfunction (such as hypertension or smoking). These risk factors result in a slightly hypoxic intervillous circulation, following in a subtle increase in branching angiogenesis. These placenta's show a slightly increased villous maturation, with a small increase in villous vessel count and have a relatively high placental weight. Uterine and umbilical artery Doppler measurements of flow resistance are somewhat increased.

On the other hand, in the early placental syndrome with defective spiral artery remodeling, there is a subdivision into the different types of risk factors for endothelial dysfunction, but first there is a subdivision into the severity and timing of the defective remodeling. Women with early occurring and severe defective spiral artery remodeling are destined to develop early-onset PE with FGR or early-onset FGR, depending on the type of endothelial dysfunction risk factors. These women may also show (more extensive) decidual vasculopathy. Again, women with risk factors compatible with metabolic syndrome will develop PE (in the severe/early defective remodeling group combined with FGR) and those with other risk factors will develop FGR. The risk factors result in extreme uteroplacental hypoxia, which aggravates into postplacental hypoxia resulting in relative intervillous hyperoxia. The intervillous hyperoxia leads to an increase in non-branching angiogenesis, resulting in the histological picture of DVH. There is a decrease in villous vessel count and placental weight. Furthermore, there is an increase in uterine artery Doppler pulsatility- and resistance index and eventually ARED (absent or reversed end-diastolic flow) develops of the umbilical artery. The group of women with moderate/later onset defective spiral artery remodeling can be subdivided into three groups according to pre-existing risk factors, namely risk factors associated with 1) metabolic syndrome, developing into early-onset PE; 2) other general risk factors for endothelial dysfunction, developing into early-onset FGR; and 3) no risk factors for endothelial dysfunction, developing into early preterm birth (<34 weeks of gestation). In the latter, other factors may play a role such as inflammatory and immunological factors. All three groups develop a hypoxic intervillous circulation due to the defective spiral artery remodeling. The intervillous hypoxia leads to an increased branching angiogenesis, resulting in the histological picture of increased villous maturation. There is an increase in villous vessel count and a decreased placental weight. The uterine artery Doppler PI and RI are increased and there is a preserved umbilical artery flow.

Figure 1. Schematic presentation of our hypothesis. We believe that only early placental syndrome (PS), defined as delivery <34 weeks gestational age (GA), is associated with defective spiral artery remodelling (SpAR).



The type of clinical entity which then develops depends on the maternal profile. In case of pre-existing risk factors for endothelial dysfunction (ED) related to metabolic syndrome (MS), hypertensive pregnancy complications develop. The more severe the risk factors, the more severe the clinical syndrome ranging from early-onset gestational hypertension (GH) to preeclampsia (PE) to preeclampsia with fetal growth restriction (FGR). Other risk factors for ED, such as pre-existing hypertension or smoking, are associated with early-onset FGR without PE. Preterm delivery within the context of PS is considered idiopathic preterm delivery, thereby excluding preterm delivery by other causes such as ascending infection, increased intra-uterine pressure (polyhydramnion or multiple gestation) or cervical incompetence. We believe that in the case of idiopathic preterm delivery there are no maternal risk factors for endothelial dysfunction. Perhaps other factors play a role, such as inflammatory, immunological or yet to be identified factors. It is difficult to determine whether intra-uterine fetal death (IUFD) and placental abruption are also associated with these other factors or with endothelial dysfunction, since these entities usually develop as a complication of a hypertensive pregnancy disorder or FGR. Possibly IUFD and placental abruption should not be considered separate entities in PS, but rather complications of the other entities within PS.

Our hypothesis extends the hypothesis of Ness and Sibai[32] who state that both women with PE and FGR enter pregnancy with some degree of endothelial dysfunction; women with metabolic syndrome risk factors develop PE whereas women with other endothelial dysfunction risk factors develop FGR. Indeed, FGR is not associated with risk factors related to the components of metabolic syndrome; it is more prevalent in women with lower weight, lipoproteins are not elevated and there is no relationship with hyperglycemia. In fact, diabetes is associated with macrosomia.[32] The degree of increased maternal risk of ischemic heart disease in later life also supports their hypothesis as preterm birth, FGR and PE show an ascending order.[33] The associations are even additive, women with a pregnancy complicated by PE and FGR combined had a higher risk of ischemic heart disease than alone.[33] Women with placental syndrome whose placentas demonstrate decidual

vasculopathy show an even more increased cardiovascular risk.[34,35] Yet, Ness and Sibai do not take PE without FGR (late-onset PE) into account and there is no explanation why DVH is seen in FGR-placentas irrespective of the presence PE. This is where we believe placental angiogenesis takes a central place, resulting in the different histological pictures and differences in placental weight as described above. Early and severe defective spiral artery remodeling results in extreme hypoxic changes, an increased non-branching angiogenesis and therefore DVH. The placental villi mature more rapidly into terminal villi, ending placental growth since formation of terminal villi is the last differentiation step in placental villous development. There is a decreased placental weight and the placenta is incapable of providing sufficient nutrients to the fetus resulting in FGR. Early and moderate defective spiral artery remodeling leads to increased branching angiogenesis because the hypoxic changes are less extreme. These changes result in the histological picture of accelerated maturation with an increased number of villous capillaries, comparable to chorangiosis occurring in hypoxic environments such as high altitudes.[36] Again, the placental villi mature more rapidly and placental weight is also decreased, although less severe than in DVH. The placenta may be able to provide sufficient nutrients for normal fetal growth, although fetal growth can also be comprised as is also often the case in at high altitudes.[36] Late onset PE and FGR only show slight hypoxia and therefore a subtle increase in maturation. Late-onset preeclamptic placentas have a higher weight than controls,[37] supporting the theory of Ness and Sibai that (late-onset) PE is associated with the metabolic syndrome risk factors including insulin-resistance, given that diabetic placentas also have a higher weight.

We believe that gestational hypertension (GH) is associated with the same risk factors as PE, those related to metabolic syndrome, but the abnormalities are less severe. This thought is supported by the smaller increased cardiovascular risk in later life after a pregnancy complicated by GH as compared to PE.[38]

The case of idiopathic preterm birth without PE or FGR requires some elaboration. The term idiopathic is of utmost importance here, since preterm birth is often caused by other disorders not related to placental syndrome, such as increased intra-uterine pressure (for example in multiple gestation or polyhydramnion), infection or cervical incompetence. We believe that in idiopathic preterm birth trophoblast function and its ability to sufficiently invade maternal spiral arteries may play a more important role, possibly influenced by other factors such as immunological, inflammatory or yet to be identified factors. Indeed, an obstetric history of preterm birth demonstrates a lower risk of later maternal ischemic heart disease than PE or FGR.[33]

Intra-uterine fetal demise (IUFD) and placental abruption are more difficult to study, since these entities often occur as complications of severe PE or FGR. This is also reflected by conflicting results on the impact of cardiovascular risk in later life.[39,40] Two questions arise regarding IUFD and placental abruption without PE and/or FGR. The first question is which maternal risk profile is associated to these entities? Are these women also prone to endothelial dysfunction or do other factors play a (more important) role? The second question is whether to consider placental abruption and IUFD as separate entities within placental syndrome or to classify them as complications of placental syndrome. More research is needed to answer these questions.

Although we describe our hypothesis fairly straightforward, we recognize that reality may be more complicated and that other factors such as inflammatory, immunological and not yet identified factors may also play an important role in the pathophysiology of placental syndrome. Furthermore, there remains much overlap between the different entities. Yet, we believe that our hypothesis takes us one step closer to unravelling the pathophysiology of placental syndrome and deserves further study.

Conclusions

The central aim of this thesis is to elucidate the pathophysiology of placental syndrome, with a focus on recurrent placental syndrome. Our data provide evidence that women with recurrent placental syndrome differ from their counterparts who remain normotensive during their subsequent pregnancy already in the first to mid-second trimester. We demonstrated differences in concentrations of circulating substances which are not only considered risk factors for endothelial dysfunction, but also play important roles in placental angiogenesis. We hypothesize that feto-placental vascular development plays a central role in the pathophysiology of placental syndrome, with close relations to the kind of hypoxia. In placental syndrome there are two types of placental angiogenesis: branching and non-branching, linked to uteroplacental- and postplacental hypoxia, respectively. The importance of these two types of angiogenesis is underscored by the distinctions in placental villous development which result in different histological pictures: accelerated maturation and DVH, respectively. We provided morphometrical placental villous and vascular data supporting the importance of angiogenesis, although we must acknowledge the small sample size. Nevertheless, we were also able to demonstrate an evident trend of increasing vascularization parameters with decreasing severity of umbilical artery Doppler indices as a measure of postplacental hypoxia, supporting the relevance of the kind of hypoxic state in the pathophysiology of placental syndrome.

Future perspectives

To provide further support for our hypothesis, additional studies should be performed using larger patient groups. A retrospective study could be performed correlating morphometrical placental vascularization data with placental histology (including maturation, decidual vasculopathy and placental lesions) and immunohistochemistry of VEGF, PlGF and HIF-1. A prospective study could be conducted correlating morphometrical placental vascularization data with both uterine and umbilical artery Doppler velocimetry measurements, angiogenic biomarkers (such as VEGF and PlGF) in the maternal circulation, and clinical phenotype (different clinical entities and differentiating between early- versus late-onset).

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9

Summary

Summary

Emerging evidence on how placental structure and function contribute to successful pregnancy has changed our view in pregnancy-related complications. Many great obstetrical syndromes have recently been shown to share a common etiology, which is associated with disorders of deep placentation starting with defective spiral artery remodeling. These disorders have been put together in one umbrella group named 'placental syndrome', as previously explained. This thesis focusses on the role of placental angiogenesis in the pathophysiology of placental syndrome. The findings of this thesis conclude that not only biomarkers for endothelial dysfunction but also angiogenic factors measured in the maternal circulation deviate already in early pregnancy before the clinical onset of placental syndrome. Results are even more pronounced in early-onset disease. Furthermore, we illustrate the differences in angiogenesis by morphometrically investigating placental villous and vascular development, bringing us one step closer to unravelling the pathophysiology of placental syndrome. The main findings of each chapter will be summarized below.

Part 1: Circulating biomarkers during early pregnancy and the recurrence of placental syndrome.

The first part of this thesis investigates possible biomarkers in the maternal circulation during early pregnancy, in women who develop recurrent placental syndrome and their counterparts who do not. **Chapter 2** describes early pregnancy adaptation of the lipid profile in women who developed recurrent preeclampsia (PE) and women who remained normotensive during their subsequent pregnancy, as dyslipidemia relates to biochemical stress upon the vasculature. In normal pregnancy, low-density-lipoprotein (LDL) cholesterol declines transiently in the first trimester, whereas in women destined to develop recurrent PE this decline is absent. This observation points to an abnormal early adaptation of lipid metabolism to pregnancy preceding clinical manifestation of PE. LDL has also been shown to reduce important angiogenic receptors, namely vascular endothelial growth factor receptor-1 and -2 (VEGF-R1 and VEGF-R2, respectively), implying that placental vascular development could be either increased or decreased depending on which receptor predominates in placentas from preeclamptic women compared to women without PE. Furthermore, the observed rise of high-density-lipoprotein (HDL) cholesterol during early normal pregnancy stagnates in women with imminent PE indicating these women may have an unfavorable lipid profile that relates to endothelial dysfunction. One of the central hallmarks of healthy pregnancy is the early-pregnancy drop in vascular resistance along with the rise in arterial compliance. In **Chapter 3** the role of early preg-

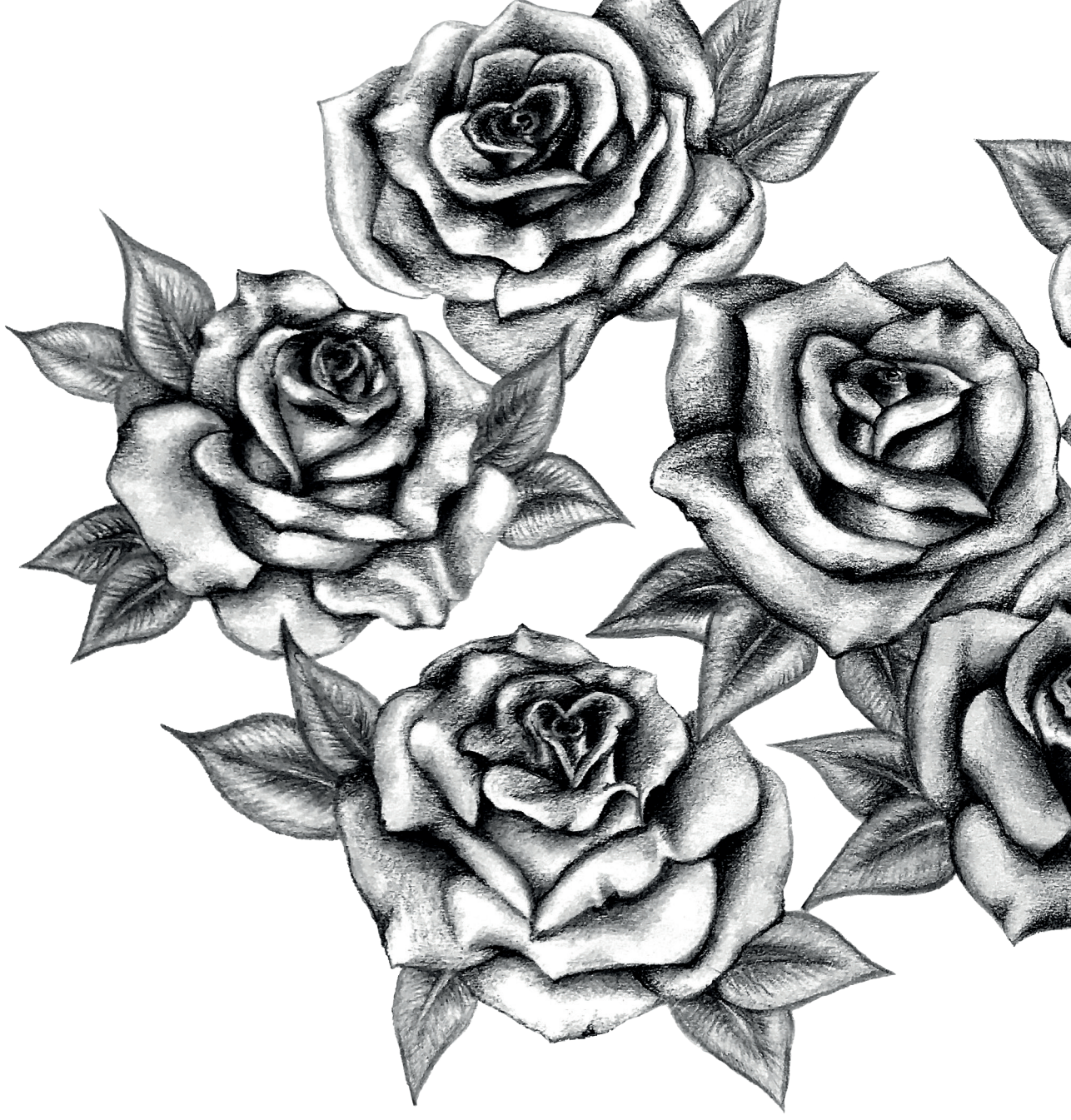
nancy adaptation to endothelium homeostasis is investigated by measuring asymmetric dimethyl arginine (ADMA) and its related counterparts-metabolites. ADMA is an endogenous inhibitor of one of the major endothelium-derived vasodilator substances, nitric oxide (NO). ADMA and its metabolites symmetric dimethyl arginine (SDMA), L-citrulline and L-arginine are studied in women with recurrent hypertensive pregnancy and in women who remained normotensive in their subsequent pregnancy. Even before pregnancy, lower SDMA and L-citrulline concentrations are found in recurrent hypertensive women. The lower L-citrulline levels suggest that there is a lower NO production, which is not only associated with an increased risk for endothelial dysfunction but may also affect placental angiogenesis. Moreover, by mid-pregnancy, L-arginine (a precursor of NO) tends to be lower in the recurrent hypertensive group, a finding that along with the lower levels of L-citrulline may suggest reduced NO production and availability along with insufficient available NO-precursor in these women. **Chapter 4** depicts early pregnancy adaptation to the fibrinolytic system in relation to endothelial integrity in women who developed recurrent hypertensive disease in their next pregnancy and those who remained normotensive. To this end, concentrations of plasminogen activator inhibitor-2 (PAI-2) and total fibronectin are measured, and the relationship with pre-pregnant plasma volume as measure of cardiovascular reserve is investigated. PAI-2 functions as placenta-specific protease inhibitor released by trophoblast cells and accelerates the conversion of plasminogen into plasmin, thereby degrading a blood clot. Since it is released by trophoblast cells, PAI-2 is also considered a marker for the total amount of functioning trophoblast. Fibronectin on the other hand promotes fibrin deposition in the extracellular matrix as a response to mechanical stimuli and is therefore regarded as a marker for endothelial stress. In addition, it decreases the stimulatory effect of fibrin and fibrinogen on plasmin formation by tissue plasminogen activator resulting in the preservation of a blood clot, which is the opposite effect of PAI-2. Fibronectin concentrations are consistently higher during pregnancy in women with recurrent hypertensive disease. In the subgroup of women with pre-pregnant subnormal plasma volumes, fibronectin levels are even higher already before pregnancy. In contrast, PAI-2 levels are lower in mid-pregnancy in women with a recurrent hypertensive pregnancy. This change is mostly caused by recurrent hypertensive women who also had subnormal pre-pregnant plasma volumes. These results indicate that the degree of pregnancy adaptation to the fibrinolytic system is at least partly related to a pre-existent increased risk profile for endothelial dysfunction. Moreover, placental angiogenesis may be affected in women with recurrent hypertensive disorders, since both fibronectin and PAI-2 are involved in branching angiogenesis in the placenta. Another important factor in branching angiogenesis is oxidative stress. In **Chapter 5** total antioxidant capacity is studied in early pregnancy of women with a pregnancy complicated by a recurrent hypertensive disease or by small for gestational age infancy (recurrent PS) and compared to women who had a normal subsequent pregnancy. Women with recurrent

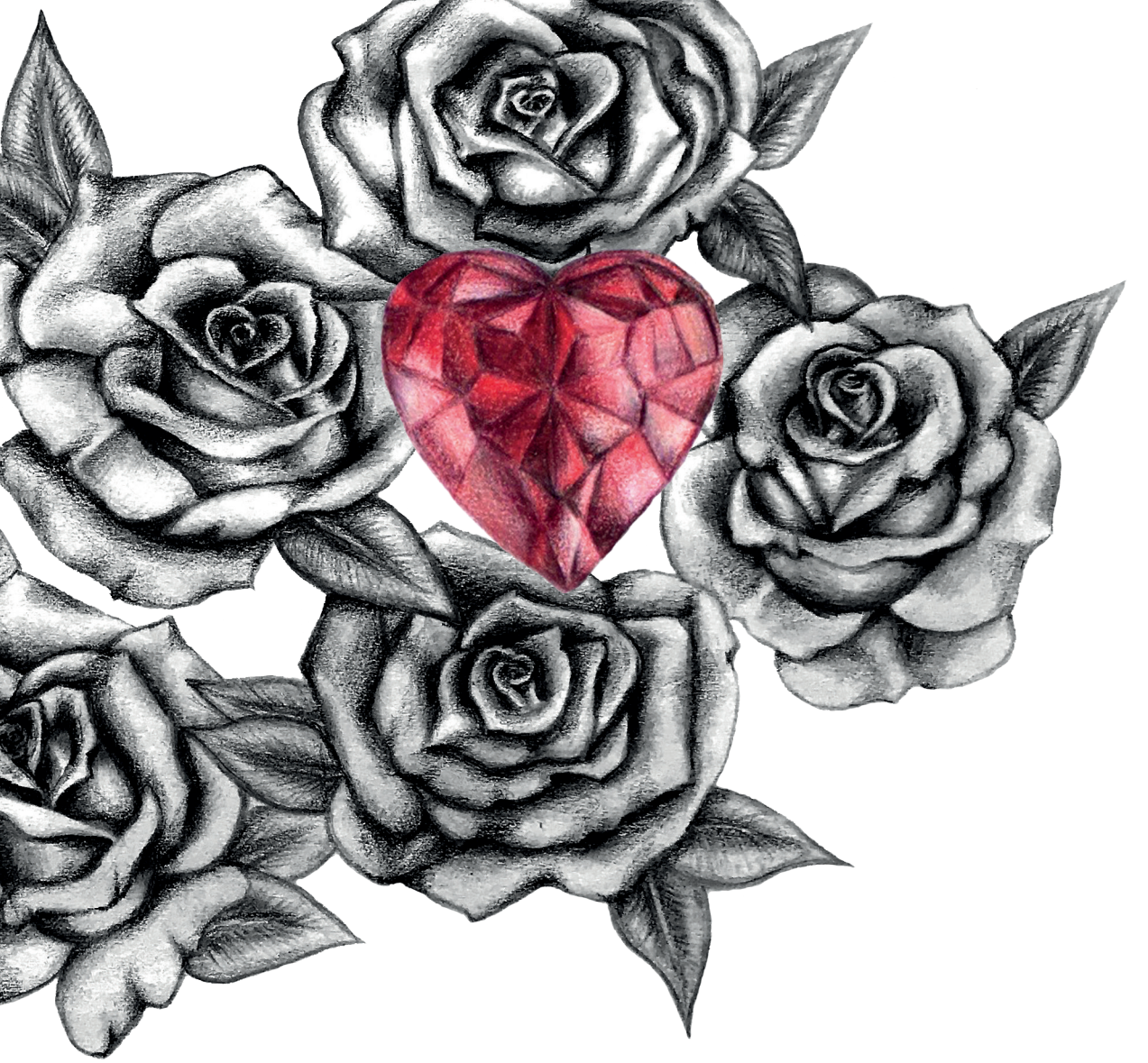
PS show higher antioxidant capacity measured as TEACC (Trolox Equivalent Antioxidant Capacity Corrected for uric acid) before pregnancy and again at 20 weeks of pregnancy. Furthermore, women with recurrent PS show a diminished hemodynamic adaptation to pregnancy. These findings suggest that the higher pre-pregnant antioxidant activity as indicated by TEACC levels in recurrent PS are an adaptive response triggered by chronic subclinical vascular damage and chronic low-grade inflammation. This suggests that in recurrent PS the anti-oxidant system is at first triggered to counterbalance these vascular and inflammatory changes, before ultimately being overwhelmed by these changes leading to the clinical stage of the disease.

Part 2: Placental vascular development in placental syndrome.

The second part of this thesis investigates placental vascular development in order to provide more insight into the pathophysiology of placental syndrome. In **Chapter 6** we review normal placental angiogenesis and villous development and compare this to changes observed in placental syndrome. We hypothesize that placental vascular development plays a crucial role in the pathophysiology of placental syndrome and that villous vascularization is influenced by the degree and timing of spiral artery maladaptation, the type of hypoxic state (uteroplacental hypoxia versus postplacental hypoxia/intervillous hyperoxia) and the type of pre-existing risk factors for endothelial dysfunction. We believe that severe spiral artery maladaptation occurring in early pregnancy eventually results in postplacental hypoxia/intervillous hyperoxia and leads to the histological picture of distal villous hypoplasia, in which the clinical picture depends on the type of maternal endothelial dysfunction profile: risk factors associated with metabolic syndrome result in severe early-onset PE with FGR, whereas other risk factors for endothelial dysfunction (such as pre-existing hypertension, smoking) result in severe early-onset FGR only. Moderate spiral artery maladaptation occurring somewhat later in pregnancy leads to uteroplacental hypoxia and the histological picture of accelerated placental maturation. Again, the clinical picture (PE or FGR) depends on the type of maternal endothelial dysfunction profile. We contemplate that this hypothesis could be investigated by studying umbilical and uterine artery Doppler velocimetry as a measure for the type of hypoxia and correlating these with morphometric villous vascularization measurements and clinical data. Finally, in **Chapter 7** we test our hypothesis that placental villous vascularization is correlated with umbilical and uterine artery Doppler velocimetry and clinical data using a pilot study. The hypoxic state is derived from umbilical artery Doppler velocimetry indices, in which absent or reversed enddiastolic flow (ARED) is associated with postplacental hypoxia/intervillous hyperoxia, while preserved enddiastolic flow (PED) combined with an increased pulsatility index of the uterine artery is associated with uteroplacental hypoxia. The data show a gradual

scale where vascularization parameters increase with decreasing severity of umbilical artery Doppler indices towards the levels in control placentas, thereby providing support for our hypothesis that the hypoxic state plays an important role in the pathophysiology of placental syndrome. This finding could provide perspectives for clinical management resulting in an even more important role for umbilical artery Doppler velocimetry indices.





Appendix


N

Nederlandse Samenvatting

Nederlandse Samenvatting

Er komen steeds meer onderzoeksresultaten bij over hoe de opbouw en functie van de placenta (moederkoek) bijdragen aan een succesvolle zwangerschap. Deze resultaten hebben ons inzicht in zwangerschapsgerelateerde complicaties drastisch veranderd. Van veel grote verloskundige aandoeningen is onlangs aangetoond dat ze een gemeenschappelijke etiologie delen die gepaard gaat met stoornissen in de placentatie, de vroege ontwikkeling van de placenta. Dit begint met een afwijkende aanpassing aan de zwangerschap van de spiraalarteriën van de moeder. Dit zijn de bloedvaten in de baarmoeder die de placenta van bloed voorzien (en dus zuurstof en voedingsstoffen voor de foetus, het ongeboren kind). Deze aandoeningen zijn samengebracht onder één overkoepelende term 'placentair syndroom'. Hieronder vallen de volgende aandoeningen: zwangerschapshypertensie (verhoogde bloeddruk), zwangerschapsvergiftiging (gedefinieerd door een verhoogde bloeddruk in combinatie met eiwitverlies in de urine) en HELLP syndroom (acroniem van Hemolysis Elevated Liver enzymes and Low Platelets, wat staat voor een afbraak van rode bloedcellen, gestoorde leverfunctie en tekort aan bloedplaatjes), intra-uteriene groeiretardatie (IUGR, een te klein kind voor de zwangerschapsduur), spontane vroeggeboorte waarvoor geen andere oorzaak aan te wijzen is, foetale sterfte en loslating van de placenta. Dit proefschrift richt zich op de rol van de placentaire bloedvatontwikkeling (angiogenese) in de ziekteleer (pathofysiologie) van placentair syndroom. Hierin wordt een belangrijke rol gespeeld door endotheel, een bedekkend laagje cellen van o.a. bloedvaten. De resultaten van dit proefschrift tonen aan dat niet alleen biomarkers voor een slecht functionerend endotheel (endotheeldysfunctie), maar ook angiogene factoren gemeten in de moederlijke bloedsomloop al in de vroege zwangerschap afwijken, zelfs vóór het ontstaan van klinische symptomen van placentair syndroom. De resultaten zijn zelfs meer uitgesproken bij het zogenaamde 'vroege' placentair syndroom, waarbij de ziekte optreedt voor de 34e week van de zwangerschap. Daarnaast illustreren we de verschillen in angiogenese met behulp van onderzoek van de placentaire vlok- en bloedvatontwikkeling, waardoor we een stap dichter bij de ontrafeling van de pathofysiologie van placentair syndroom komen. De belangrijkste bevindingen van elk hoofdstuk worden hieronder samengevat.

Deel 1: Circulerende biomarkers in vroege zwangerschap en recidief placentair syndroom

Het eerste deel van dit proefschrift onderzoekt mogelijke biomarkers in de moederlijke bloedsomloop tijdens de vroege zwangerschap bij vrouwen die een recidief placentair syndroom ontwikkelen en vergelijkt deze vrouwen met vrouwen die geen recidief ontwikkelen. **Hoofdstuk 2** beschrijft de vroege aanpassing van het cholesterolprofiel bij vrouwen die recidiverende zwangerschapsvergiftiging ontwikkelden en vrouwen die een

normale bloeddruk hebben in hun volgende zwangerschap. Bij een normale zwangerschap neemt het lage-dichtheids-lipoproteïne (LDL) cholesterol (het 'slechte' cholesterol) tijdelijk af in het eerste trimester, terwijl bij vrouwen die later in de zwangerschap een recidief zwangerschapsvergiftiging ontwikkelen deze afname ontbreekt. Deze waarneming wijst op een abnormale aanpassing van het cholesterol metabolisme in de vroege zwangerschap, voorafgaande aan de klinische manifestatie van zwangerschapsvergiftiging. Van LDL is ook aangetoond dat het belangrijke angiogene receptoren vermindert, wat impliceert dat placentaire bloedvatontwikkeling kan afnemen of toenemen afhankelijk van welke receptor de overhand heeft in placenta's van vrouwen met of zonder zwangerschapsvergiftiging. Bovendien nemen we een stijging van hoge-dichtheids-lipoproteïne (HDL) cholesterol (het 'goede' cholesterol) in vroege normale zwangerschap waar, welke stagneert in vrouwen die een recidief zwangerschapsvergiftiging ontwikkelen. Dit wijst erop dat deze laatste groep vrouwen mogelijk een ongunstig cholesterolprofiel hebben wat gerelateerd is aan endotheeldysfunctie. In **Hoofdstuk 3** wordt de rol van vroege zwangerschapsadaptatie naar endotheel huishouding onderzocht door het meten van asymmetrisch dimethylarginine (ADMA) en de gerelateerde metabolieten. ADMA is een remmer van een van de belangrijkste endotheel-afgeleide vaatverwijdende stoffen, namelijk stikstofmonoxide (NO). ADMA en de gerelateerde metabolieten symmetrisch dimethylarginine (SDMA), L-citrulline en L-arginine zijn onderzocht bij vrouwen met een recidief zwangerschap met hoge bloeddruk en bij vrouwen met een normale bloeddruk. Zelfs vóór de zwangerschap worden lagere SDMA en L-citrulline spiegels gevonden bij de vrouwen met een hoge bloeddruk. De lagere L-citrulline spiegel suggereert dat er een lagere NO-productie is, wat niet alleen geassocieerd is met een verhoogd risico op endotheeldysfunctie, maar ook de placentaire bloedvatontwikkeling kan beïnvloeden. Bovendien lijkt L-arginine (een voorloper van NO) halverwege de zwangerschap lager te zijn bij de vrouwen met een verhoogde bloeddruk. **Hoofdstuk 4** beschrijft vroege zwangerschapsadaptatie aan het fibrinolytische systeem met betrekken tot endotheel integriteit. Dit fibrinolytische systeem zorgt ervoor dat een bloedstolsel langzaam wordt afgebroken. Er worden wederom vrouwen vergeleken die een recidief verhoogde bloeddruk ontwikkelen met vrouwen die een normale bloeddruk hebben in hun volgende zwangerschap. Hiertoe worden de bloedspiegels van plasminogeen-activator-inhibitor-2 (PAI-2) en totaal fibronectine gemeten en wordt de relatie met het plasmavolume (onderdeel van bloed) onderzocht. PAI-2 wordt geproduceerd door de placenta en zorgt voor afbraak van een bloedstolsel. Fibronectine daarentegen bevordert de bloedstolling en wordt beschouwd als een marker voor endotheel stress. Fibronectine spiegels zijn consequent hoger tijdens de zwangerschap bij vrouwen met een recidief verhoogde bloeddruk. In de deelgroep van vrouwen die voor de zwangerschap ook lage plasmavolumes hebben, zijn de fibronectine spiegels zelfs al vóór de zwangerschap hoger. Daarentegen zijn de PAI-2 spiegels lager halverwege de zwangerschap bij deze vrouwen. Deze lagere spiegels worden met

name gezien bij de vrouwen die ook een laag plasmavolume hebben. Deze resultaten geven aan dat de mate van aanpassing aan de zwangerschap van het fibrinolytische systeem ten minste gedeeltelijk gerelateerd is aan een reeds bestaand verhoogd risico voor endotheeldysfunctie. Bovendien kan de placentaire bloedvatontwikkeling beïnvloed worden, aangezien zowel PAI-2 als fibronectine een rol spelen hierin. Een andere belangrijke rol in de bloedvatontwikkeling wordt ingevuld door oxidatieve stress. Oxidatieve stress is een stofwisselingstoestand waarbij er meer reactieve (tegenwerkende) zuurstofverbindingen vrijkomen dan gebruikelijk. Er komen bepaalde stoffen in het lichaam die een reactie aangaan met zuurstof. Deze stoffen heten vrije radicalen en worden weggevangen door antioxidanten. In **Hoofdstuk 5** wordt de totale antioxidantcapaciteit bestudeerd bij vrouwen met een recidief verhoogde bloeddruk of met de geboorte van een te klein kind (recidief placentair syndroom) en vrouwen zonder recidief. Vrouwen met recidief placentair syndroom vertonen een hogere antioxidantcapaciteit gemeten als TEACC (Trolox Equivalent Antioxidant Capacity gecorrigeerd voor urinezuur) vóór de zwangerschap en opnieuw bij 20 weken zwangerschap. Bovendien vertonen deze vrouwen een verminderde hemodynamische aanpassing aan de zwangerschap (hiermee worden de eigenschappen van de bloedstroom bedoeld). Deze bevindingen suggereren dat de hogere antioxidantcapaciteit in de vrouwen met recidief placentair syndroom een adaptieve respons is die wordt uitgelokt door langdurige vaatschade en laaggradige ontsteking. Dit doet vermoeden dat in recidief placentair syndroom het antioxidant systeem in eerste instantie wordt geactiveerd om deze vaatschade en ontsteking tegen te gaan, voordat het systeem uiteindelijk overmeesterd wordt en leidt tot het klinisch stadium van de ziekte.

Deel 2: Placentaire angiogenese in placentair syndroom

Het tweede deel van dit proefschrift onderzoekt de placentaire angiogenese en vlokken (villi, waaruit de placenta is opgebouwd en waarin zich de bloedvaten bevinden) om meer inzicht te geven in de pathofysiologie van het placentair syndroom. In **Hoofdstuk 6** bespreken we normale placentaire angiogenese en villeuze ontwikkeling en vergelijken we deze met veranderingen waargenomen in placentair syndroom. We veronderstellen dat placentaire angiogenese een cruciale rol speelt in de pathofysiologie van placentair syndroom en dat de villeuze bloedvatvoorziening wordt beïnvloed door de mate en timing van afwijkende spiraalarterie adaptatie, het type zuurstoftekort (hypoxie) en het type van reeds bestaande risicofactoren voor endotheeldysfunctie. Er zijn twee soorten hypoxie van belang bij placentair syndroom: zogenaamde uteroplacentaire hypoxie en postplacentaire hypoxie. Wij geloven dat ernstige spiraalarterie maladaptatie die optreedt tijdens de vroege zwangerschap uiteindelijk resulteert in postplacentaire hypoxie en leidt tot het

microscopisch beeld van distale villeuze hypoplasie (een beeld van ernstige versnelde uitrijping van de placenta), waarbij het klinische beeld afhankelijk is van het type moederlijk endotheeldysfunctie profiel: risicofactoren geassocieerd met metabool syndroom leiden tot vroeg optredende ernstige zwangerschapsvergiftiging met de geboorte van een te klein kind, terwijl andere risicofactoren voor endotheeldysfunctie (zoals reeds bestaande verhoogde bloeddruk, roken) alleen resulteren in vroeg optredende ernstige groeiachterstand van het kind (dus zonder zwangerschapsvergiftiging). Matige verstoring van de spiraalarterie adaptatie die wat later in de zwangerschap optreedt leidt tot uteroplacentaire hypoxie en het microscopisch beeld van ('reguliere') versnelde placentaire uitrijping. Ook hier is het klinische beeld (zwangerschapsvergiftiging of groeiachterstand van het kind) afhankelijk van het type moederlijk endotheeldysfunctie profiel. Wij denken dat onze hypothese zou kunnen worden onderzocht door de bloedstroom in de navelstrengslagader en de baarmoederslagader te bestuderen als een maat voor het type hypoxie en deze te correleren met microscopische placentaire bloedvatmetingen en klinische gegevens. Ten slotte testen we in **Hoofdstuk 7** onze hypothese dat placentaire bloedvatvoorziening is gecorreleerd met navelstreng- en baarmoederslagader snelheidsmetingen en klinische gegevens met behulp van een pilot-onderzoek. De hypoxische toestand is afgeleid van navelstrengslagers snelheidsmetingen, waarbij een afwezige of omgekeerde bloedstroom (ARED) geassocieerd is met postplacentaire hypoxie, terwijl een behouden bloedstroom (PED) gecombineerd met een verhoogde pulsatiliteitsindex van de baarmoederslagader geassocieerd is met uteroplacentaire hypoxie. De resultaten laten een geleidelijke schaal zien waarbij placentaire bloedvatparameters toenemen met een dalende ernst van navelstrengslagader snelheidsmetingen naar de niveaus in controle placenta's. Deze gegevens bieden ondersteuning voor onze hypothese dat de hypoxische toestand een belangrijke rol speelt in de pathofysiologie van placentair syndroom. Deze bevinding zou perspectieven kunnen bieden voor klinisch management, resulterend in een nog belangrijkere rol voor het verrichten van navelstrengslagader snelheidsmetingen.


V

Valorization

Valorization

This chapter describes the (future) valorization of this thesis. Valorization refers to the process of value creation from knowledge. This chapter depicts the potential impact of the research presented in this thesis and its societal and economic value.

Introduction

A healthy pregnancy is often taken for granted. Although most pregnancies indeed progress without complications and result in the delivery of a healthy child, a substantial number of women experience complications of pregnancy of varying clinical presentations and severity. There is a growing body of literature showing that many pregnancy complications are associated with disorders of the development of the placenta. The placenta is the temporary organ that joins the mother and fetus, transferring oxygen and nutrients to the fetus and permitting the release of waste products in the other direction. The different types of complications have been grouped together as 'placental syndrome'.

Placental syndrome is thought to result from defective development of placental villous and vascular structure, that is caused by maladaptation to pregnancy of certain maternal vessels, so-called spiral arteries. However, there still remains much to be elucidated regarding the developmental mechanisms of placental syndrome and how these are linked to the spectrum of clinical presentations.

Relevance

Placental syndrome accounts for roughly 15-25% of pregnancies and is an essential cause of both maternal and perinatal morbidity and mortality. Currently, treatment is targeted on control of maternal symptoms and monitoring fetal wellbeing in order to reduce the risk of obstetric complications associated with the syndrome by appropriate timing of the delivery. To date the only curative treatment is delivery of the placenta, which invariably implies iatrogenic, and frequently premature, delivery. Iatrogenic premature delivery constitutes a huge challenge to modern health economic systems and carries the additional burden of short-term neonatal morbidity and mortality and the long term cardiovascular and metabolic risks for both mother and offspring. By unravelling the pathophysiology of placental syndrome, doctors will be triggered towards earlier detection of symptoms and swifter initiation of treatment to minimize the rate of complications. Subsequently, the duration and scale of hospital admissions can be reduced resulting in decreased costs.

Furthermore, women developing placental syndrome during pregnancy have an increased risk for developing cardiovascular disease in later life. Pregnancy, in this respect, should be regarded as a 'stress test' for cardiovascular risk. When several clinical diseases within

the placental syndrome spectrum occur simultaneously (e.g. preterm birth, fetal growth restriction and preeclampsia) the risk of developing later ischemic heart disease is even seven times greater than in women with a normal pregnancy.

The results in this thesis show that women developing placental syndrome demonstrate increased circulating biomarkers for endothelial dysfunction already in the first half of pregnancy. Endothelial dysfunction represents an early stage of atherosclerosis and is therefore an important prognostic marker for cardiovascular disease. Thus, the results do not only provide directions to detect placental syndrome early in pregnancy, but also emphasize the connection between placental syndrome and (future) cardiovascular disease. In addition, this thesis provides an overview of the current knowledge of the etiology of placental syndrome which connects preexisting endothelial dysfunction to spiral artery maladaptation, and the latter to altered development of fetal vessels in the placenta.

Target group

The target group of this research consists namely of pregnant women with a previous history of placental syndrome, since recurrence risk is up to threefold increased in women with a previous pregnancy complicated by placental syndrome. Yet, the results of this research are also relevant to women who are pregnant for the first time and who, based on their cardiovascular or metabolic risk profile, would be considered as a high-risk population to develop obstetric complications.

The past few years approximately 170,000 babies are born annually in the Netherlands. Of these pregnancies about 25,000-42,000 women (15-25%) develop placental syndrome. Although some of these pregnancy complications are recurrences, it can be estimated that annually at least 25,000 new women develop an increased cardiovascular risk. However, currently our health care does not act upon the increased risk in these women.

Implications for health care

The primary aim of this research is to gain more insight into the pathophysiology of placental syndrome, which remains largely elusive. Placental syndrome consists of many different clinical entities, and even within these separate entities recent studies have implicated different etiologies. In preeclampsia, for example, there is growing conviction among researchers that there are two types: an early-onset and a late-onset type, depending on the timing of clinical disease onset during pregnancy. It is believed that the early-onset type is associated with fetal growth restriction and is caused by spiral artery maladaptation and defective placentation, while the late-onset type runs a milder course and is associated with normal sized babies and no or mild placental dysfunction. In conjunction, we use the term 'early placental syndrome' in parts of this thesis to emphasize the difference between the two types.

We hypothesize that the difference in clinical entities can be largely explained by a combination of the severity of spiral artery maladaptation and subsequent defective placentation with pre-existing maternal cardiovascular risk factors. Interestingly, spiral artery maladaptation predisposes to a lesion called acute atherosis, which is histologically similar to early atherosclerotic lesions. After a pregnancy complicated by (early) placental syndrome, the cardiovascular risk increases and leads to earlier development of cardiovascular disease years after this pregnancy. In this way, pregnancy is a women-specific cardiovascular stress-test. Currently, more women than men die of cardiovascular disease. In fact, cardiovascular diseases are the leading cause of death for women, being responsible for more deaths than cancer (including breast cancer), chronic respiratory disease, Alzheimer disease, and accidents combined. The primary cause of cardiovascular disease in women is coronary artery disease, in which the formation of atherosclerotic plaques in the coronary system of the heart is the principal manifestation. It starts with asymptomatic plaques that progress over the years to form symptomatic plaques, eventually leading to coronary occlusion. Disturbingly, most women who die suddenly of coronary artery disease have no previous symptoms. We find it regretful that in current practice, obstetric history is generally not taken into consideration when assessing women's cardiovascular risk, and we hope that our research will encourage health professionals to do so in the future.

Future implications for health care: PEARLS study

The insight gained from our current research into the relationship between placental syndrome and cardiovascular risk has led to the conception of the "PEARLS" (**P**lac**E**ntal **A**cute atherosis **R**ef**L**ecting **S**ubclinical atherosclerosis) study. This study received medical ethical approval has already been provided (NL52556.068.15/METC152019) and is funded with an amount of €73,840 by means of crowd funding supported by the Dutch heart association ("hartstichting"). This study is scheduled to start end of 2018 and aims to recruit 246 women over a period of 36 months at Maastricht University Medical Center+ (MUMC+) to evaluate the possibilities of the placenta as an accurate women-specific cardiovascular screening tool.

We hypothesize that acute atherosis in women mirrors atherosclerosis in other vascular beds and therefore the placenta is a valuable organ to examine histologically to assess the clinical status of her cardiovascular system and her personalized risk for cardiovascular disease later in life. Women who develop preeclampsia will be prospectively included to the study and compared to healthy pregnant women. At delivery, the placenta will be collected and histologically examined for the presence of acute atherosis in the maternal vessels. One year after delivery, women will undergo vascular assessments to assess early stages of atherosclerosis. We will investigate whether the presence of acute atherosis is an accurate screening tool for the presence of early atherosclerosis. In this way, tailored screening and preventive strategies can be developed.

The novel aspect of this project is that examining the vasculature of the placenta to determine a women's risk for coronary artery disease later in life has, to our knowledge, not been carried out before. The placenta is a relatively easy to investigate organ, which is often discarded after delivery and its examination imposes no further burden for the woman. Therefore, the PEARLS study will enable us to make a critical step towards studying the link between clinical pregnancy outcome and cardiovascular risk, and will add significantly to the current cardiovascular screening programs in women.

If our study demonstrates positive results, the evidence will drastically change classical cardiovascular risk assessment in women. In case the placental histological findings correlate with systemic plaque formations as an early marker of atherosclerosis, this finding will not only affect individuals' awareness (both for patient and doctor), but also fortify individual's motivation towards healthier lifestyle. In women's health, shallow awareness has been proven to negatively affect cardiac disease prognosis. As such, this study will tremendously affect clinical practice if the hypothesized relation exists. Examining placentas histologically post-partum may become the next community-based screening method for women and make the large step in early detection and prevention in cardiovascular disease as has been made in the 90's for breast cancer. Furthermore, this study will be a solid base for future grant applications such as the Netherlands Organization for Scientific Research (NOW) or local initiatives of MUMC+.


C

Curriculum Vitae

Curriculum Vitae

Carmen Severens-Rijvers is geboren op 20 februari 1984 in Brunssum, waar ze ook is opgegroeid. Ze behaalde haar vwo-diploma aan het Sint-Janscollege te Hoensbroek in 2002. Vervolgens is Carmen geneeskunde gaan studeren aan de faculteit Health Medicine and Life Sciences van de Universiteit Maastricht. Nadat ze haar artsexamen behaalde in 2008 begon zij haar onderzoek bij de afdeling Obstetrie en Gynaecologie onder begeleiding van dr. Louis Peeters. Zij heeft een deel van haar onderzoeksresultaten gepresenteerd op meerdere internationale congressen en won in 2011 de Pfizers President Presenter Award op het SGI-congres. Later dat jaar stopte de financiering van haar onderzoek en Carmen begon als basisarts bij de afdeling Obstetrie en Gynaecologie in Maastricht. Al snel ondervond zij echter dat haar hart toch niet bij gynaecologie lag. Na een korte periode als verzekeringsarts te hebben gewerkt startte zij de opleiding tot patholoog in 2012 in Maastricht, waarvan zij afstudeerde in 2017. Ze bleef aan haar onderzoek werken naast haar full time opleiding, nu onder begeleiding van prof. dr. Marc Spaanderman (afdeling Obstetrie en Gynaecologie), prof. dr. Axel zur Hausen (afdeling Pathologie) en dr. Salwan Al-Nasiry (afdeling Obstetrie en Gynaecologie, wie het stokje heeft overgenomen van Louis na zijn pensioen). Nadat zij afstudeerde als patholoog, begon ze haar huidige carrière in het Maastricht Universitair Medisch Centrum+.

Carmen specialiseert zich o.a. in de perinatale pathologie, waarmee een mooie brug wordt geslagen tussen haar onderzoek en haar huidige beroep als patholoog. Ze is coauteur van een hoofdstuk in het leerboek 'Pathology of the Placenta', wat naar verwachting eind dit jaar gepubliceerd zal worden. Daarnaast is zij gevraagd te participeren als expert in een Delphi procedure met als doel een consensus te vormen over de definitie van foetale groeivertraging na een intra-uteriene vruchtdood.

Carmen is getrouwd met haar jeugdliefde Jos Severens en samen zijn zij trotse ouders van Sem.

Curriculum Vitae

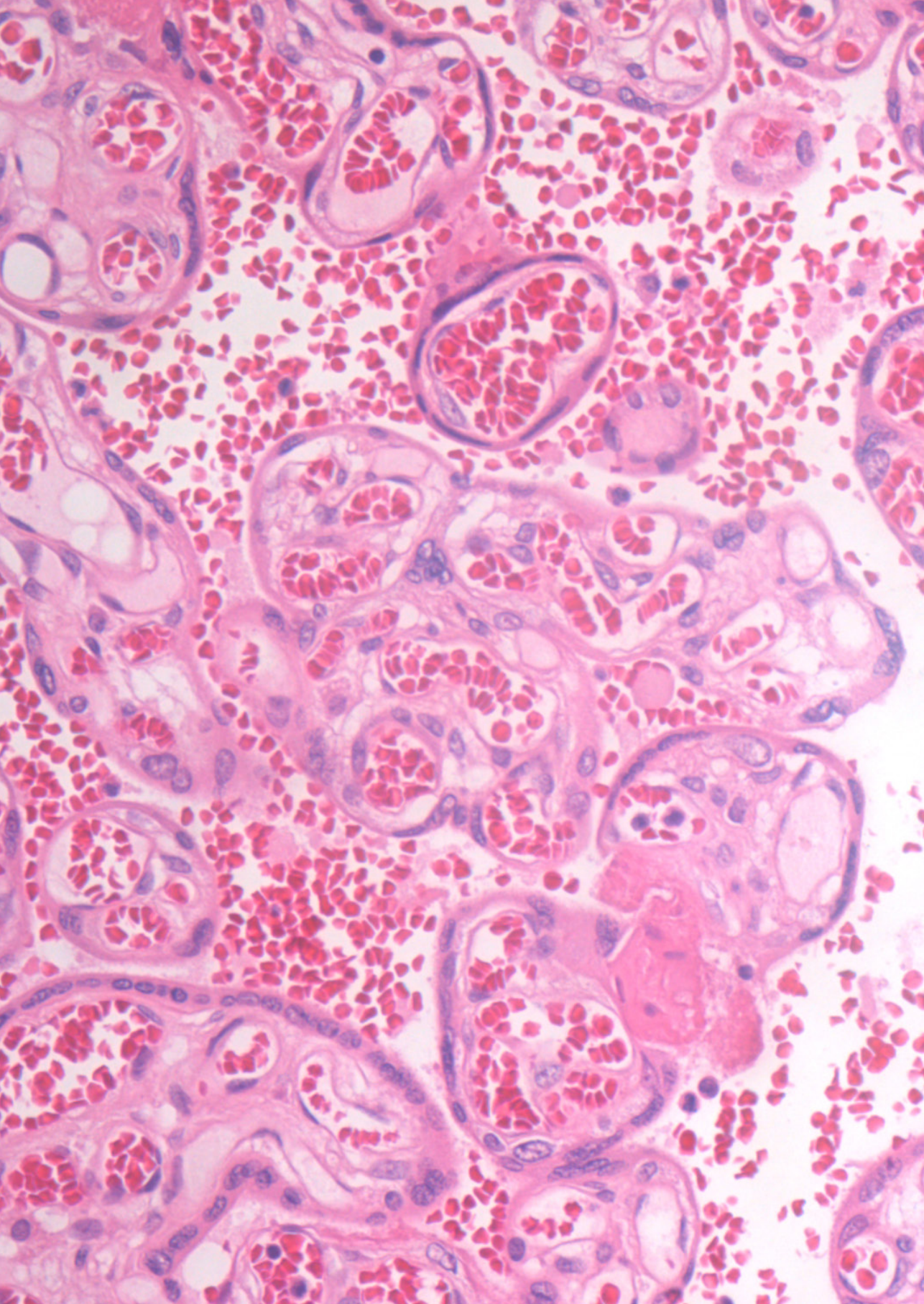
Carmen Severens-Rijvers was born on February 20th 1984 in Brunssum, The Netherlands, where she also grew up. She completed secondary school at Sint-Janscollege in Hoensbroek in 2002. Subsequently, Carmen started her study at the Faculty of Health Medicine and Life Sciences at the University of Maastricht. After obtaining her medical degree in 2008 she started her research at the department of Obstetrics and Gynecology under supervision of dr. Louis Peeters. She presented some of her research findings on several international conferences and won the Pfizers President Presenter Award at the SGI conference in 2011. Later that year financing stopped and Carmen started as a resident at the department of Obstetrics and Gynecology in Maastricht. Soon she discovered her heart was not in gynecology after all. After a short period of working as an insurance doctor, she started her pathology residency in 2012 from which she graduated in 2017. She continued working on her PhD research next to her full-time residency, now under supervision of prof. dr. Marc Spaanderman (department of Obstetrics and Gynecology), prof. dr. Axel zur Hausen (department of pathology) and dr. Salwan Al-Nasiry (department of Obstetrics and Gynecology, who replaced dr. Louis Peeters after his retirement). After graduating as a pathologist, she began her current career at the Maastricht University Medical Center+.

Carmen specializes in perinatal pathology among other subspecialties, connecting her research to her current profession as a pathologist. She is co-author of a chapter in the textbook 'Pathology of the Placenta', which is expected to be published by the end of this year. Furthermore, she was invited as an expert for a Delphi procedure to reach consensus on the definition of fetal growth restriction after intra-uterine fetal death.

Carmen is married to Jos Severens and together they are proud parents of Sem.


D

Dankwoord



Dankwoord

Dan is nu eindelijk het dankwoord aangebroken. Het feit dat dit vaak het eerst en het meest gelezen hoofdstuk is maakt het er ook niet makkelijker op. Met enige mate van ongeloof en met veel trots kijk ik terug op mijn onderzoeksperiode. Ik had het allemaal niet alleen kunnen doen en wil dan ook graag een aantal mensen in het bijzonder bedanken. Hiervoor wil ik gebruik maken van een muzikale metafoor, want – zoals velen van jullie weten – speelt muziek een grote rol in mijn leven.

De metafoor die ik wil schetsen is die van een rockster. Niet dat ik me nu zo voel, maar in mijn vrije tijd heb ik heel wat uurtjes besteed bij de bands van mijn grote liefde Jos. Samen zijn we ook naar veel concerten en festivals geweest waar we naar echte rocksterren stonden te kijken en luisteren, onder het genot van een koel biertje. Straks mag ik als rockster in de aula aan de Minderbroedersberg staan om mijn geschreven nummers ten gehore te brengen aan een zeer gemengd publiek, bestaande uit familie, vrienden en collega's.

Laat ik chronologisch beginnen, namelijk met dr. L. Peeters. Beste Louis, door jou ben ik begonnen met mijn onderzoek. Ik stond bij een concert van jou te luisteren en werd meteen verliefd op het vak. Door jou kon ik mijn eigen band oprichten en jij nam de rol van gitarist op je. Samen hebben we heel wat gejamd. Of om jouw woorden te gebruiken, hebben we samen heel veel 'fishing expeditions' meegemaakt. Deze zal ik nooit vergeten. Hoewel ik in de tijd dat ik bij jou mijn onderzoek deed nog dacht dat ik gynaecoloog wilde worden, heb jij altijd tegen mij gezegd dat ik uiteindelijk misschien wel iets heel anders zou gaan doen. Alsof je in de toekomst kon kijken! Door mijn onderzoeksperiode bij jou ben ik ook als persoon ontzettend gegroeid.

Prof. dr. M. Spaanderman en dr. S. Al-Nasiry, beste Marc en Salwan, jullie sloten na enkele jaren van jamsessies aan als drummer en bassist. Jullie gaven mij ontzettend veel vrijheid om aan mijn onderzoek te werken. Als ik eenmaal de basis had gelegd voor een nummer, konden jullie als geen ander het tempo en ritme aangeven. Jullie hebben samen de muziek gedragen. Zonder jullie waren de nummers niet hetzelfde geweest. Ik heb super veel geleerd van jullie en voelde me altijd begrepen en gewaardeerd. Bij elke tegenslag (en dat waren er veel...) wisten jullie me weer te motiveren.

Prof. dr. A. zur Hausen, beste Axel, waar is een band zonder tourmanager? Jij hebt met name veel achter de schermen gewerkt. Zo waren er altijd nieuwe snaren voor de gitaar, ofwel CD34 kleuringen voor de placenta's. Je was mijn pathologie opleider en hebt je vertrouwen in mij getoond door mij aan te nemen als patholoog binnen jouw afdeling. Ik kon altijd bij je terecht met vragen. Je was steevast enthousiast over mijn onderzoek, zelfs al ging het niet over virussen.

Dr. C. Peutz-Kootstra, beste Carine, jij zorgde voor de finishing touches met de gitaarsolo's die je speelde. Door jouw inbreng zijn we naar de microvasculatuur gaan kijken in de placenta. Jouw onophoudelijke enthousiasme werkte aanstekelijk en jouw kennis en kunde bracht de nummers werkelijk naar een hoger niveau. Ik vond het buitengewoon plezierig om met jou samen te werken. Je stond altijd voor me klaar met goede adviezen en nieuwe ideeën.

Een speciaal woord van dank aan dr. P. Nikkels. Beste Peter, ik ben vereerd dat jij samen met mij wilde schrijven aan een nummer. Je bent mijn grote idool en samen hebben we een mooi duet gemaakt. Ik mocht drie maanden in jouw studio meekijken in het kader van mijn opleiding tot patholoog en specialisatie in de perinatale pathologie. Ik heb onwijs veel van jou geleerd en ben een grote fan van jou. Super gaaf dat ik nog steeds maandelijks naar je toe mag komen om casuïstiek uit te wisselen en de liefde voor het vak kan delen.

Daarnaast heb ik met meerdere gastmuzikanten mogen samenwerken, namelijk alle coauteurs. Bedankt voor alle input en dynamiek die jullie in de nummers hebben gestopt. Jullie zorgden allemaal voor een eigen klankkleur, waardoor elk nummer een ander karakter kreeg. Ik wil graag twee personen in het bijzonder bedanken. Bjorn Winkens, jij was een vaak terugkerende gastmuzikant en ik had een fijne klik met jou in de studio. Je hielp me elke keer weer met de statistische vraagstukken. Dan keek ik weer met een beduusde blik naar de formules die je uitschreef, maar gelukkig wist je die altijd weer goed te vertalen zodat ik de analyses zelf kon uitvoeren in SPSS. Zelfs buiten onze repetities kwamen we elkaar tegen tijdens optredens van Jos als hij in de buurt speelde. De bidon prijkt nog steeds op je fiets. Super gaaf! Jack Cleutjens, waar was ik geweest zonder jouw programma om alle vaatjes te tellen. Je zag me al aankomen als ik weer iets wilde veranderen. Je dacht steeds goed mee en herschreef het programma keer op keer voor mij zonder ook maar te mopperen.

En dan mijn vaste crew, Lianne Brancatisano-Geerdink en Lara Heij, mijn paranimfen, wat heb ik toch veel steun gehad aan jullie. Lianne, we leerden elkaar kennen tijdens de eerste onderwijsgroep van de geneeskunde opleiding in 2002 en we hadden meteen een klik. We hebben lief en leed gedeeld! Wij zijn allebei onderzoek gaan doen naast onze opleiding tot specialist en daarna jij als kinderarts en ik als patholoog. Jij begrijpt mij als geen ander als het aankomt op onderzoeksgerelateerde frustraties. Wat heb ik een bewondering voor jou hoe jij alles voor elkaar krijgt: een dubbele superspecialisatie en een prachtige dochter Isabella. Je bent mijn grote voorbeeld, ik vind je een echte powervrouw! Wat ben ik blij dat ik jou als vriendin mag hebben. Lara, jou heb ik leren kennen toen je bij ons in de opleiding pathologie kwam. Wie had toen gedacht dat er zo'n goede vriendschap zou ontstaan. Je bent me al voorgegaan met promoveren, wat was dat een bijzondere dag om

mee te mogen maken! Ook jouw zwangerschapsverlof stond in het teken van het afronden van je promotie. Samen hebben we heel wat frustraties kunnen uitwisselen, liefst onder het genot van ontelbare koppen koffie. Ik zal ze nooit vergeten. Op naar nog vele koppen meer! Binnenkort ga je ook je opleiding afronden en je bent nu al hard op weg naar een geweldige (academische) carrière. Ongelofelijk, ik ben trots op je!

Ik heb ook goede muziekpromotoren gehad, Roos Huijbrechts en evelienjagtman.com. Jullie hebben mij voorzien van geweldig promotiemateriaal. Roos, jij hebt helemaal naar mijn wens een prachtige tekening gemaakt die nu op de kaft en in de binnenkant van dit boek prijkt. Een robijnen hart met rozen. De robijn heb ik gekozen voor de ontgiftende werking die deze edelsteen heeft en het hart staat voor de moederliefde voor het (ongeboren) kind. Hoe passender wil je het hebben bij het thema zwangerschapsvergiftiging? De rozen staan ook voor liefde, maar ook voor vertrouwen. Vertrouwen die de zieke zwangere vrouw legt in de geneeskunde voor een optimale zorg voor haar en haar ongeboren kind. Evelien en Mariska hebben de tekening vervolgens prachtig verwerkt in dit boek en gezorgd voor een schitterende lay-out die helemaal bij mij past. Bedankt dames!

Een rockster is natuurlijk nergens zonder zijn fans..! Ik mag mezelf gelukkig prijzen met zo'n grote en diverse fangroep achter me. Als eerste wil ik alle AIOS pathologie bedanken voor alle steun en ook voor het uitsnijden van de PULSE placenta's (een ander onderzoek buiten dit proefschrift). Ik weet dat de meesten van jullie placenta's niet zo spannend vinden, maar ze werden toch met zorg en liefde uitgesneden door jullie.

Uiteraard wil ik ook alle pathologen van MUMC+, VieCuri en Zuyderland bedanken. In het bijzonder Myrurgia Abdul Hamid, hoe vaak heb ik wel niet op je kamer gezeten om te ventileren over weer een tegenslag? Elke keer wist je me weer tot inzicht te brengen. En Ruud Clarijs, door mijn gesprekken met jou heb ik een goed ritme kunnen vinden om naast de opleiding aan mijn onderzoek te werken.

De (ex)band- en crewleden van Up the Irons en System Pilot, nu zijn de rollen omgedraaid. Normaal sta ik zelf natuurlijk als fan (en crew) in de weekenden bij de optredens. Mijn hoofd zat vaak vol met mijn onderzoek als ik onderweg in de auto achter de laptop zat te werken, maar jullie konden mijn hoofd goed leeg maken! Andere keren zat ik backstage te werken aan mijn onderzoek en hadden jullie op dat moment weinig gezelligheid aan mij. Tijdens en na de show was er dan weer wel tijd om te genieten. Tom Heijnen en Paul Marcelis, jullie hebben met name mijn onderzoek gevolgd. Ik vond het heel fijn om er met jullie over te praten tussen de shows door. Tom, binnenkort zal ik weer dienst doen als je drumroadie.

Maar mijn grootste fans zijn toch wel mijn ouders. Ontelbare en lange telefoontjes en bezoekjes zijn er geweest als ik er weer eens doorheen zat. Jullie wisten me altijd weer opnieuw te motiveren en te helpen een positieve kant aan tegenslagen te vinden. Pap, hoe vaak heb jij me wel niet ergens afgezet of opgehaald, niets was te gek. En mam, ik wil niet weten hoeveel kaarsjes je voor mij gebrand hebt.

En natuurlijk last but definitely not least, Jos Severens, mijn jeugdliefde, mijn eigen groupie. Liefste Jos, normaal ben ik natuurlijk jouw groupie, maar nu ben jij de mijne. Dankjewel voor al je steun. Wat heb jij veel vrije avonden en weekenden aan je neus voorbij zien gaan. Als jij dan eens geen optreden had in het weekend, zat ik toch weer aan mijn onderzoek te werken. En zo ging het ook voor veel avonden. We zijn al meer dan ons halve leven samen en ik hou elke dag alleen maar meer van je. Jij bent er altijd voor me en hebt me zo goed ondersteund, natuurlijk ook tijdens mijn promotie. Je hebt zelfs nog tastbaar aan dit proefschrift bijdragen. Zien jullie allemaal de figuur op pagina 11? Die heeft Jos in gescand! ;) Maar de allergrootste bijdrage is toch wel onze zoon Sem geweest. Sem, jij bent mijn allernieuwste fan en ik die van jou! Jij lag lekker naast me terwijl ik dit proefschrift heb afgerond. Jij brengt zo veel vreugde in mijn leven, dat is onvoorstelbaar! Ik heb nu twee grote liefdes in mijn leven. En zie je het plaatje aan het begin van dit dankwoord? Dat is jouw placenta.

Al met al is het maar goed dat dit alles een metafoor is, want muzikaal ben ik absoluut niet. Als ik een concert zou geven, zou de zaal voor het einde leeg zijn. Hopelijk blijven jullie tijdens mijn verdediging allemaal zitten en kunnen we daarna backstage nagenieten op de receptie.