Angiogenic biomarkers and their role in cardiovascular risk in women with a history of hypertensive disorders of pregnancy or spontaneous preterm birth
Summary

Background: Hypertensive disorders of pregnancy (HDP) and spontaneous preterm delivery (sPTD) are independent cardiovascular (CV) risk factors for the mother. Mechanisms linking HDP and sPTD with subsequent CV disease remain unclear. Potential roles have been suggested for endothelial dysfunction and anti-angiogenic biomarkers. The aim of this study was to assess levels of circulating angiogenic and endothelial markers and associations of these markers with CV parameters in women with previous HDP and sPTD, 5-12 years postpartum.

Methods: Concentrations of plasma soluble endoglin (sEng), soluble fms-like tyrosine kinase-1, vascular endothelial growth factor, and serum soluble (s)E-Selectin, tumor necrosis factor-a and interleukin-8 were determined by Enzyme Linked Immunoassays on a total of 268 women (n=164 HDP, n=29 sPTD). Concentrations were compared across pregnancy characteristics, and associations between angiogenic markers and CV parameters were assessed.

Results: Women with a history of HDP had higher sE-Selectin concentrations than women with a previous normotensive pregnancy. sE-Selectin was positively associated to mean arterial pressure, diastolic blood pressure, and negatively associated with HDL cholesterol. Women with a history of sPTD had higher sEng levels when compared to women who delivered term.

Conclusion: Women with a previous HDP exhibited endothelial activation years after pregnancy. Women with a history of HDP and higher sE-Selectin levels were more likely to have cardiac concentric remodeling, higher blood pressure and adverse metabolic changes. Women with a previous sPTD exhibited an enhanced anti-angiogenic state.

Samenvatting

Achtergrond: Vrouwen met een hypertensieve aandoening in de zwangerschap (HDP) of met een spontane premature bevalling (sPTD) hebben een verhoogd risico op het krijgen van cardiovasculaire ziekten (CVZ). Het doel van dit onderzoek was om te bepalen of endotheel activatie en angiogenese een rol spelen in de ontwikkeling van CVZ in vrouwen met een voorgeschiedenis van HDP of sPTD.

Methoden: 268 vrouwen, waarvan 164 met HDP en 29 met sPTD zijn geïncludeerd in de studie. Met behulp van ELISA zijn de concentraties van soluble endoglin, soluble fms-like tyrosine kinase-1, vascular endothelial growth factor bepaald in plasma, van soluble (s)E-Selectin, tumor necrosis factor-a in interleukin-8 in serum. Concentraties van deze stoffen zijn vergeleken op basis van zwangerschapskarakteristieken, en associaties tussen angiogene markers en CV parameters zijn vastgesteld.

Resultaten: Vrouwen met een normotensieve zwangerschap, hebben vrouwen met een voorgeschiedenis van HDP een significant hogere concentratie sE-Selectin. sE-Selectin is, in vrouwen met HDP, positief met bloeddruk geassocieerd, en negatief met HDL cholesterol. Concentraties van soluble endoglin zijn significant hoger na een doorgemaakt sPTD in vergelijking tot een voldragen zwangerschap.

Conclusie: Endotheel activatie is jaren na een door HDP gecompliceerde zwangerschap nog steeds aanwezig. Vrouwen met een voorgeschiedenis van HDP en hogere sE-Selectin waarden hebben meer kans op het hebben van concentrische hypertrofie van het hart, een hogere bloeddruk en lager HDL cholesterol dan vrouwen met lagere sE-Selectin waarden of een eerdere normotensieve zwangerschap. Een eerdere normotensieve premature bevalling geeft jaren na deze zwangerschap een aanhoudende imbalans in angiogenese.
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Introduction

Pregnancy, often considered as a cardiac stress test, is a time of increased maternal risk, most commonly of hypertensive disorders of pregnancy (HDP). HDP, which include gestational hypertension, preeclampsia (PE) and superimposed PE, occur in approximately 5-18% of pregnancies and are amongst the leading causes of maternal and perinatal morbidity (1, 2). A recent study conducted by Say et al. (3) demonstrated that HDP accounted for 14.0% of maternal deaths between 2003-2009. More specifically, approximately 76000 maternal deaths and 500000 deaths of infants are caused by HDP per year (4).

In view of the fact that there has never been a definite consensus on the classification and diagnostic criteria for HDP, differences in incidence and maternal outcome rates have been noted for the various HDP. Nowadays, classification of HDP is often done according to the diagnostic criteria recommended by the International Association for the Study of Hypertension in Pregnancy (ISSHP) (1, 2, 5). Following these diagnostic criteria, gestational hypertension is defined as the de novo onset of hypertension (≥ 140 mm Hg systolic blood pressure (SBP) and/or ≥ 90 mm Hg diastolic blood pressure (DBP)) after a pregnancy duration of 20 weeks. Chronic hypertension is characterized by hypertension with an onset prior to pregnancy, or before 20 weeks of gestation. Furthermore, preeclampsia (PE) is a syndrome characterized by the combination of gestational hypertension and proteinuria (≥ 300 mg/24 hours), with or without the presence of clinical symptoms, such as renal and/or hepatic dysfunction, neurological and/or hematological abnormalities. PE can be subdivided according to the moment of onset, with ‘early onset’ PE beginning before 34 weeks and ‘late onset’ PE after 34 weeks. Lastly, superimposed preeclampsia is described as the development of PE-related symptoms in women with chronic hypertension after 20 weeks of pregnancy.

Of the various HDP, PE is the most commonly researched. Despite extensive research, PE’s etiology is undefined and is therefore a disease of theories. Several theories on the pathophysiology of PE have been proposed, of which the most accepted theory is the ‘two-stage process’ (see Figure 1) (6). Central to this theory is poor placentation in the first half of pregnancy (stage one), which is theorized to result in a hypoxic and oxidatively stressed placenta (stage two). Poor placentation has been suggested to be the outcome of insufficient invasion of the maternal myometrium by spiral arteries, leading to increased resistance, reduced flow, and consequently placental ischemia (6-8). These ischemic effects lead to anti-angiogenic and pro-inflammatory factors being released into the maternal circulation (6, 9). The released anti-angiogenic factors, such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), and cytokines, including TNF-α and IL-6, are theorized to impair endothelial function (6).

Endothelial dysfunction, which is characterized by an imbalance in the vasodilating factor nitric oxide and the vasoconstricting substance Endothelin-1, is suggested to have adverse effects on renal function, and to result in the increased systemic vascular resistance and hypertension seen in the clinical syndrome of PE (6, 9, 10).
Hypertensive disorders of pregnancy and cardiovascular risk

In women, cardiovascular disease (CVD) is the biggest cause of death (11). Subtle sex differences in cardiovascular phenotype, treatment responsiveness, disease presentation and outcome have been reported (12-15). A history of HDP has been associated with increased lifetime cardiovascular (CV) risk (16). Indeed, the importance of PE as a pregnancy-related risk factor for CVD has now been recognized by the American Heart Association and has been included in CVD risk assessment guidelines (17, 18).

At time of pregnancy, women with PE demonstrate CV adaptations and changes in autonomic function (19-24). Changes in cardiac geometry and function, which include increased left ventricular mass and impaired systolic and diastolic function, have been reported in preeclamptic (PET) pregnancies (22, 24). More specifically, left ventricular mass is disproportionately increased in approximately 20% of women with early onset PE (22, 24). Reports focusing on vascular phenotype and function in women with HDP have described increased systemic vascular resistance (6) and arterial stiffness (23) in these women. Moreover, women with HDP have impaired autonomic function, including reduced diurnal variation in blood pressure (19) and reduced heart rate variability (20), which is closely associated with CV risk (21).

As mentioned before, HDP has been associated with long-term lasting cardiovascular risk (16). Meta-analyses have described considerably higher prevalences of lifetime CV complications in women with a history of PE when compared to women with previous normotensive pregnancies; formerly PET women are 12 times more likely to experience major cardiovascular events, such as myocardial infarction and stroke, and have a 6-fold higher incidence of overall death as compared to women who have had a normotensive pregnancy (25). Likewise, Bellamy and colleagues (16) examined the risk of future cardiovascular diseases, cancer, and overall mortality after PE, and reported increased relative risks for hypertension (3.70), ischemic heart disease (2.16), and mortality (1.49) in previous
Angiogenesis and endothelial function in postpartum CV risk

PET women (16). Besides, formerly PET women show an increased risk of stroke later in life (16), which may be attributed to the presence of cerebral white matter lesions years after pregnancy (26, 27).

Although PE is the most commonly researched of the various HDP, some studies have focused on associations between pregnancy induced hypertension (PIH) and later cardiovascular health. These studies provide evidence that PIH, independent of PE, is associated with endothelial dysfunction and an increased risk of developing hypertension and heart failure later in life (28-30).

Lifetime cardiovascular risk after HDP could potentially be explained by the persistence of cardiovascular changes in the early postpartum period. Cardiovascular changes that have been reported to remain in the early postpartum period are cardiac remodeling (31), cardiac dysfunction (31), arterial stiffness (32), carotid intima media thickness (32), capillary rarefaction (10), endothelial dysfunction (32), and higher daytime and nighttime ambulatory blood pressure (21).

Preterm delivery and cardiovascular risk

The most effective treatment option for PE at this moment is therapy delivery of the baby and placenta, hence 30.6% of PET episodes result in preterm (PT) delivery (33, 34). Recent publications have highlighted that delivering PT, independent of HDP, is associated with hypertension and a two to three fold increase in subsequent (ischemic) CVD risk (35-39). Elevations in pro-inflammatory markers and cholesterol have been reported in women at risk of spontaneous preterm delivery (sPTD) (40). Moreover, evidence for the presence of the metabolic syndrome, characterized by dyslipidemia and increased blood pressure, and increased intermedia thickness, a marker for subclinical atherosclerosis, ten years after sPTD has been provided in previous studies (38, 41).

Endothelial dysfunction and cardiovascular disease

Because the etiology behind the association between CVD and HDP or sPTD has not been fully elucidated, the search for predictive biomarkers and preventive measurements remains an unfulfilled goal.

Some of the identified risk factors for HDP, such as hypertension, oxidative stress and obesity, and pathophysiological features for HDP, like endothelial dysfunction, are similar of those in the pathogenesis of CVD (16, 28, 42-45).

The presence of endothelial dysfunction in women with previous HDP may, in part, explain the increased lifetime CV risk in these women (42, 46). It is well established through meta-analyses that flow mediated dilation, a specific test for endothelial function, is reduced prior to, and up to three years after, pregnancy (42, 47). In this study it will be examined if endothelial function is altered, and associated with CV risk, years after HDP or sPTD. Endothelial function will be assessed by the measurement of soluble E-Selectin.
Soluble E-Selectin

E-Selectin, otherwise known as endothelial cell leukocyte adhesion molecule-1, is a member of the Selectin family of cell adhesion molecules and is exclusively expressed by endothelial cells (48, 49). Cell adhesion molecules (CAMs) are crucial in the maintenance of endothelial stability, endothelial permeability and the facilitation of leucocyte-endothelial adhesion (48). Adhesion of leucocytes to the endothelium is an early step in the cascade of events leading to inflammation and, potentially, atherosclerosis (48-50).

Expression of E-Selectin is induced by the presence of activated cytokines, endotoxins, hyperthermia or hypoxia (48, 51). When expressed, E-Selectin is considered to be a marker of endothelial activation (49, 51). A soluble form of E-Selectin (sE-Selectin) exists, of which increased levels may also be considered to be a reflection of endothelial activation, dysfunction or damage (38, 51, 52).

Levels of adhesion molecules have been studied during pregnancy and were found to remain stable all through normotensive pregnancies (48). In contrast, sE-Selectin concentrations are elevated in PET pregnancies (53). Elevated levels of circulating sE-selectin may, in part, be responsible for the onset, as well as the progression, of PE (48).

To the best of my knowledge, only one study has assessed maternal sE-Selectin concentrations in sPTD, in which no significant associations between sE-Selectin and PTD were found (54). However, more research is needed to establish the role of sE-Selectin in preterm delivery.

Since higher levels of sE-Selectin have been reported in patients with acute MIs, coronary artery disease and hypertension (49, 53, 55, 56), it is hypothesized in this study that sE-Selectin will be associated with increased CV risk in women with a history of complicated pregnancies.

Angiogenesis in complicated pregnancies and cardiovascular disease

Associations between endothelial dysfunction and the angiogenic imbalance, which is fundamental in the pathogenesis of HDP, have been demonstrated (6). Hence, angiogenesis could be the missing link between HDP and the development of CVD. Of particular interest is the potential role of angiogenic factors in lifetime cardiovascular risk in women with complicated pregnancies.

With exception of the menstrual cycle and wound healing, angiogenesis—the formation of new vessels from pre-existing vasculature—is not necessary in adult vasculature (57). Angiogenesis is a complex process, in which the proliferation and migration of endothelial cells are required (57). Activation and inhibition of angiogenesis, and therefore the mitosis of endothelial cells, are regulated by a balance in pro and anti-angiogenic factors (the angiogenic balance) (57). Pro-angiogenic factors, such as vascular endothelium growth factors (VEGF), are stimulators of angiogenesis and endothelial cells, whereas anti-angiogenic factors inhibit the migration and proliferation of endothelial cells (57, 58).
**VEGF and sFlt-1**

VEGF is an important regulator of angiogenesis and endothelial cell survival (57, 59, 60). Under normal circumstances, VEGF is being released in response to hypoxia, in order to restore oxygen supply to hypoxic tissues (57). However, in PET pregnancies, in which placental hypoxia is a fundamental underlying abnormality, levels of VEGF are decreased (6, 61).

The pro-angiogenic factor exerts its effects via VEGF receptors (VEGFR) 1,2 or 3. Of these receptors, VEGFR-2 is located on the surface of vascular endothelial cells. Following VEGF-VEGFR binding, numerous intrinsic cascades are activated, of which four are important in angiogenesis and vascular function. These pathways include the PLC-DAG-PKC-MAPK-eNOS, the PLC-IP3-calcium-CM-eNOS, the PI3K-AKT-eNOS and the PI3K-AKT-mTOR pathway (59, 60).

The first three pathways result in the synthesis, and release, of endothelial nitric oxide synthase (eNOS). eNOS, which is expressed in the endothelium and in the myocardium, produces nitric oxide (NO), and contributes to cGMP-related vasodilation. The PI3K-AKT-mTOR pathway is important in endothelial cell survival and in the prevention against capillary rarefaction, which plays a role in hypertension. Lastly, cell proliferation has been attributed to the PLC-DAG-PKC-MAPK pathway (59, 60).

Soluble fms-like tyrosine kinase-1 (sFlt-1), a soluble VEGFR, is capable of binding and disrupting circulating VEGF (6, 61). Hence, sFlt-1 is an anti-angiogenic marker, which is able to inhibit VEGF-induced vasodilation, endothelial cell mitosis and survival (6).

In physiologic pregnancies, sFlt-1 levels increase, and levels of VEGF decrease during the last two months of pregnancy (9). These changes in concentration are more pronounced, and have an earlier onset, in PET pregnancies (9).

Beneficial effects on the cardiovascular system have been attributed to VEGF, i.e. VEGF stimulates the release of eNOS and is therefore able to contribute to vasodilation and a lower BP (60). Moreover, VEGF is important in the maintenance of endothelial function (59, 60). However, VEGF is able to induce vascular permeability, which attributes to inflammation and plaque formation. Indeed, a positive association between VEGF and atherosclerosis has been reported by a study of Sandhofer et al. (62).

The role of sFlt-1 in CVD has been examined in several in vivo studies, e.g., sFlt-1 has been reported to cause cardiomyopathy in mice that were unable to withstand anti-angiogenic insults (63). Furthermore, longterm increases in blood pressure have been described in the offspring of aforementioned mice, showing that sFlt-1 does not only affect maternal cardiovascular health, but may also have adverse affects on cardiovascular health of offspring born to HDP (63). Likewise, associations between circulating sFlt-1 levels and blood pressure have been described in PET pregnancies (6, 61, 63). Moreover, inhibition of VEGF expression has been associated with endothelial cell apoptosis and vascular rarefaction, which, in turn, may result in increased cardiac afterload (64, 65). Apart from in PE, elevated sFlt-1 levels have been reported in peripartum cardiomyopathy (63).
**Soluble Endoglin**

One of the soluble factors that is being released by the hypoxic placenta in PE, is Soluble Endoglin (sEng). sEng is an TGFβ antagonist, which attenuates TGFβ-eNOS mediated vasorelaxation (66). Significantly higher serum sEng levels have been reported in women with early PE when compared to women with normotensive pregnancies after approximately 14 weeks of gestation (67, 68). In PE, sEng is positively related to disease severity and onset (69). Moreover, it has been suggested that sEng contributes to the onset of endothelial and vascular damage seen in hypertension and HDP (66). Several studies have tested sEng concentrations in mothers who delivered preterm, showing that those with higher sEng levels are at increased for spontaneous PT delivery (70, 71). sEng may be of importance in CVD as the presence of this factor has previously been demonstrated in individuals with, or at risk for, CVD (24, 72-74).
Research Question and Hypotheses:

Currently, it is unclear which surrogate markers of cardiovascular disease are altered long term in women with a history of HDP or sPTD. It is also unknown whether angiogenesis, inflammation and/or endothelial function contribute to lifetime cardiovascular risk in these women. Clearer knowledge of the mechanisms driving the increase in cardiovascular risk may lead to improved understanding which biomarkers to test in this population.

The aim of this work is to examine potential differences in endothelial, angiogenic and inflammatory markers 5-12 years after HDP and sPTD. This study should lead to better insights into the role of angiogenic, inflammatory and endothelial markers in the pathogenesis of CVD in women with a history of HDP or sPTD. This aim resulted in the following research question: are there differences in angiogenic, inflammatory and endothelial biomarkers in pregnancies complicated by hypertensive disorders of pregnancy or preterm delivery, which may explain differences in cardiovascular phenotype 5-12 years postpartum?

Specifically, it has been hypothesized in this study that vascular endothelial growth factor (VEGF), Fms-like tyrosine kinase 1 (sFLT), soluble endoglin (sEng), TNF-α, IL-8 and soluble E-Selectin levels will be higher in women with a history of pregnancy complications when compared to women with previous uncomplicated pregnancies.

The secondary hypothesis is that, aforementioned factors will relate to impaired cardiovascular function in women with a history of hypertensive disorders of pregnancy and women with a history of preterm delivery, i.e.:

**Cardiac function:**

In literature, impaired systolic function has been reported in patients with HDP (22, 24). Inverse relations between left ventricular ejection fraction and sFlt-1 have been reported in patients with chronic kidney disease (75). Based on these findings in literature, the following hypothesis for systolic function was postulated for this study: anti-angiogenesis will be inversely associated with systolic function.

Women with HDP have been previously shown to have impaired diastolic function (22, 24). VEGF blockage has been suggested to result in reduced myocardial capillary density and cardiac dysfunction (76, 77). Therefore, it is hypothesized that angiogenesis will associate with diastolic function in women with previous HDP.

**Macrovascular stiffness:**

Positive correlations between endothelial derived vasoactive substances, such as NO, and aortic distensibility have been described (78). Endothelial dysfunction and anti-angiogenic biomarkers are able to decrease NO availability, whereas VEGF is known to increase NO bioavailability (79). Therefore, it is hypothesized that endothelial function and angiogenic factors will associate with aortic distensibility.
**Atherosclerosis**

Carotid intima media thickness (cIMT), a marker of subclinical atherosclerosis, can independently predict future cardiovascular risk, and has been demonstrated to be significantly higher in PE when compared to normotensive pregnancies (23). It has been suggested that angiogenesis is involved in the development, as well as the destabilization of atherosclerotic plaques (80). However, when angiogenesis is a response to plaque-induced hypoxia, it tends to preserve atherosclerotic plaque integrity, maintain endothelial integrity and increase eNOS, and could therefore be beneficial in plaque stability (80). Associations between VEGF, sFlt-1, sEng, sE-Selectin and cIMT have been described previously in different patient populations (62, 72, 81). Based on these findings in literature, it is hypothesized that cIMT will be higher in women with previous HDP and that angiogenesis and endothelial activation will associate with cIMT.

**Capillary density:**

The capillary system has been described to function on a ‘rota system’, in which several capillaries are perfused whereas others are not (10). Structural capillary density is the total of perfused (or functioning) and not perfused (i.e. closed) capillaries. A loss of structural capillary density per mm$^2$ of tissue may increase systemic vascular resistance up to 10-15% and is a systemic phenomenon that affects the brain and myocardium (10, 12, 82, 83). Indeed, a role of structural rarefaction has been implicated in the pathogenesis of stroke, hypertension and CV mortality (84). Capillary rarefaction has been reported in the development of hypertension and hypertensive disorders of pregnancy (12, 82, 84, 85). In these circumstances, capillary rarefaction was positively related to BP and anti-angiogenic factors (10, 84-86). A potential for endothelial dysfunction in capillary rarefaction has been suggested in literature (12).

The hypothesis of this study is that VEGF will be positively, whereas anti-angiogenic markers and sE-Selectin will be negatively associated with capillary density.

**Blood pressure**

As previously described in the introduction, angiogenesis and endothelial function are important regulators of vascular tone, hence it is hypothesized that angiogenesis and endothelial function will associate with blood pressure in women with previous HDP or preterm delivery.

**Cerebral brain volume and function:**

Literature has described inverse correlations between age and SBP with grey matter volumes (87). Since a role for angiogenic markers and endothelial function in blood pressure is hypothesized, it is also hypothesized that angiogenic and endothelial markers will associate with cerebral volumes and function.
Material and Methods

Line of research:
The Pregnancy Complications Vascular Study (PVS) assessed which, if any, of the cardiovascular changes, known to occur during pregnancies complicated by hypertension and/or preterm birth, persist 5-12 years postpartum. The results should help characterize cardiovascular phenotypes and explore if a common link between pregnancy complications and later cardiovascular disease exists. Participants were identified from John Radcliffe Hospital maternity records. The study protocol was ethically approved and informed consent was obtained at the initial visit. Study visits were scheduled 5-12 years post index pregnancy.

Study design
This research project was designed to determine whether concentrations of endothelial and/or angiogenic biomarkers differ between women with previous HDP or sPTD and women with previous uncomplicated pregnancies. Furthermore, it was determined whether these biomarkers were related to cardiovascular and metabolic changes 5-12 years after pregnancy.

Inclusion and exclusion criteria PVS
Eligible women were aged 21 to 50 years, and able to give informed consent. Eligible controls were women with a normal obstetric history (previous and index pregnancy). A normal obstetric history was defined as having no evidence of still birth, neonatal death, birthweight below the 5% centile, placental abruption, ≥3 miscarriages, any previous pregnancy complicated by PET or hypertension, proteinuria and/or gestational diabetes. Furthermore, controls had a DBP consistently <90mmHg and SBP consistently <140mmHg. Gestational hypertension and preeclampsia were defined as a new onset hypertension after 20 weeks gestation in the index pregnancy and a DBP>90mmHg on two separate occasions within a 24 hour period. Preeclampsia and superimposed preeclampsia were defined as having a raised blood pressure (as above) with a new onset proteinuria of: 300mg/24h or more of protein in a 24 hour urine collection, or more than 30mg of protein/mmol of creatinine in a single urine sample, or at least 2+ protein at least twice on consecutive dipstick testing. Preterm delivery was defined as delivery before 37 weeks of completed gestation.

Women showing any doubt about taking part in the study, and/or those who had a serious mental illness were excluded. Furthermore, women who had pre-existing renal, cardiac or vascular disease and/or insulin dependent diabetics (Type I/II or gestational) at time of index pregnancy, were excluded from the study. Moreover, women who were pregnant, who had a significant medical history relevant to vascular measures, including malignancy, vasculitides, systemic infection, recent major surgery/trauma, polycystic ovarian syndrome, at time of vascular studies were excluded.
Sample storage and enzyme linked immunoassays of circulating biomarkers.

Venous blood was previously collected, centrifuged, and stored at −80°C until analysis for circulating biomarkers. Maximum storage duration was 3.5 years. In this study EDTA plasma concentrations of soluble endoglin (sEng), sVEGFR-1 (or sFlt-1) and VEGF-A were quantified with sandwich Enzyme Linked Immunosorbent Assay (ELISA), using respectively human Endoglin/CD105, human VEGFR-1 and human VEGF-A ELISA kits (Quantikine; R&D Systems Europe, Abingdon, UK).

Moreover, serum concentrations of sE-Selectin, high sensitivity TNF-α and CXCL-8 (IL-8) were quantified with ELISA, using, respectively, human sE-Selectin/CD62E, human TNF-α HS, human CXCL8/IL8 ELISA kits (Quantikine; R&D Systems Europe, Abingdon, UK).

All sample measurements were performed in duplicate. Manufacturer's instructions were followed. A standard curve was constructed using known standard concentrations of all analytes provided with the R&D ELISA kits. A curve-fitting software program (FLUORomega® microplate reader) was used for quantification of the biomarker concentrations.

Blinded analysis at time of the measurements was ensured by coding of the samples with anonymized study-specific IDs (e.g. PVS001).

As an example for the methods used for the ELISAs, the method for Human Endoglin/CD105 Quantikine ELISA (R&D) is described. For the methods and technical details for the other analytes, see Appendix 1.

**Human Endoglin/CD105 Quantikine ELISA (R&D)**

Before use, reagents were brought to room temperature. The human Endoglin Standard, which was provided in the kit, was reconstituted with 1.10 mL deionized water. By means of this reconstitution, a stock solution of 100 ng/mL was produced. The standard stock solution was vortexed to ensure complete reconstitution and was allowed to sit for 15 minutes prior to making dilutions. 900 µL of Calibrator Diluent RD5K was pipetted into the 10 ng/mL tube, and 500 µL of Calibrator Diluent RD5k was pipetted into the remaining 6 tubes. To produce dilution series, 100 µL of the standard stock solution was pipetted in the 10 ng/mL tube. After mixing, 500 µL of the 10 ng/mL tube was transferred into the 5 ng/mL tube. The mixing, and transferring steps, were repeated for the remaining tubes. Samples were placed on ice until thawed.

**Assay procedure:**

First, 100 µL of Assay Diluent RD15 was added to each well (96 wells in total). Once the samples were thawed, they were vortexed to ensure the removal of any remaining ice crystals. Secondly, 50 µL of standard, control, or sample was added per well. Each standard, control and sample was assayed in duplicate. Hereafter, the plate was covered and incubated at room temperature, on a horizontal microplate shaker (0.12 orbit) set at 500 ± 50 rpm for 2 hours. 20 mL of the provided wash buffer concentrate was diluted with deionized water to prepare 500 mL of wash buffer. Each well was aspirated and washed four times. The plate was washed using an automatic washer. In each well, 400 µL of Wash Buffer was dispensed and aspirated. After the wash, 200 µL of Human Endoglin Conjugate was added per well. The wells were covered and the plate was incubated for another two hours at room temperature on
the shaker (0.12 orbit) set at 500 ± 50 rpm. After the incubation period, the aspiration and washing steps were repeated.

To produce the Substrate Solution, Color reagents A and B were mixed in equal volumes within 15 minutes of use. 200 µL of this Substrate Solution was added to each well. Then, the plate was incubated in the dark for 30 minutes at room temperature. 50 µL of Stop Solution was added to each well. Mixing was ensured by putting the plate on the shaker for a mean of 10 minutes. Within 30 minutes (from the time the Stop solution was added) the optical density of each well was determined by a FLUORomega® microplate reader set to 450 nm. Wavelength correction was set to 540 nm. A standard curve, averages and coefficients of variation (CV) were created by FLUORomega®. CVs of ≥15% were retested and only CVs lower than 15% were used for data analyses in this study.

**Cardiovascular measurements**

Cardiovascular changes associated with cardiovascular disease and hypertension were previously measured using standardized and validated methods (see Appendix 2 for more detail)

**Statistical Analysis**

Statistical analyses were performed using IBM SPSS Statistics version 20. Visual assessment of histograms and Shapiro-Wilk tests were used to test whether or not values were parametrically distributed. Descriptive statistics for continuous data on interval or ratio level of measurement are presented using means and standard deviations, nominal data as percentages and frequencies. Differences between individual groups were assessed using Student t-tests when data was distributed parametrically, or with Mann-Witney tests when non-parametrically distributed. All of the analyses were performed 2-sided. Correlation analyses were performed using Pearson’s correlation or the Spearman’s method when appropriate. In an effort to investigate the impact of the tested angiogenic factors on determinants of maternal cardiovascular risk, linear regressions were performed using a forced entry method. Unstandardized regression coefficients ($B$), or standardized regression coefficients ($β$) were used respectively for univariate and multivariate regression models. Mediation analyses, as previously described by McKinnon (88), were performed to determine potential causal pathways. Post-hoc testing was performed using Bonferroni correction when appropriate. P-values less than 0.05 were considered statistically significant. Non-significant trends were noted for P-values <0.1 and >0.05.
**Schematic overview of the study**

Analyses were divided into two parts, i.e. the first part in which differences in endothelial and angiogenic markers were assessed between women with a previous HDP or normotensive pregnancy, and the second part in which differences in endothelial and angiogenic markers were assessed between women with a previous normotensive PTD or normotensive term delivery. If differences in endothelial and/or angiogenic marker concentrations were found between groups, associations with CV risk parameters were tested.

**Schematic overview of part 1 of this study:**

**Schematic overview of part 2 of this study:**
Blood was available for, and angiogenic and endothelial markers were tested on, a total of 268 women. Data analyses were carried out on a cohort of 268 women in total, of which 75 women with former normotensive pregnancies, 143 formerly preeclamptic (59 early preeclamptic, 84 late preeclamptic) women, 21 women with previous PIH and 29 with spontaneous preterm delivery.

Part 1: Hypertensive Disorders of Pregnancy vs Normotensive Pregnancies

Study population

In this part of the study, differences in cardiovascular and angiogenic biomarkers between women with a previous HDP and women with a previous NTP were examined. Women with a normotensive pregnancy (NTP) were those women with normotensive pregnancies or delivered normotensive preterm. Women with a hypertensive disorder of pregnancy (HDP) consisted of those women with a history of PE or PIH. Demographic and characteristics of this part of the study are presented in table 1.

No significant differences were found in age, proportion of smokers, anthropometrics, total cholesterol, LDL cholesterol, triglycerides (TG) and high sensitivity CRP (hsCRP) between women with a previous NTP and HDP (P-values >0.05).

Women with a history of HDP had significantly higher DBP, SBP and mean arterial pressure (MAP) when compared to women with a previous NTP. Furthermore, women with a previous HDP had significantly lower HDL cholesterol, higher glucose concentration and insulin resistance (HOMA ir) when compared to former NTP women.

Table 1 Characteristics of the Study Population

<table>
<thead>
<tr>
<th></th>
<th>NTP (n=164)</th>
<th>HDP (n=104)</th>
<th>P -value</th>
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<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>41.0±5.15</td>
<td>42.1±4.7</td>
<td>0.17</td>
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<tr>
<td>Smokers, n (%)</td>
<td>9 (13)</td>
<td>7 (7)</td>
<td>0.19</td>
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<tr>
<td><strong>Anthropometrics</strong></td>
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<td></td>
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<tr>
<td>Height (m)</td>
<td>1.65±0.06</td>
<td>1.64±0.06</td>
<td>0.23</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70.5±14.5</td>
<td>71.8±14.4</td>
<td>0.35</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>25.8±5.3</td>
<td>26.7±5.5</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Blood pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>115.9±14.6</td>
<td>123.3±14.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>74.2±8.7</td>
<td>80.6±10.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>88.1±10.2</td>
<td>94.8±11.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Biochemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>4.97±0.84</td>
<td>4.84±0.80</td>
<td>0.34</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.94±0.79</td>
<td>2.89±0.65</td>
<td>0.74</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.64±0.39</td>
<td>1.51±0.32</td>
<td>0.049</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>0.83±0.34</td>
<td>0.93±0.45</td>
<td>0.20</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.86±0.43</td>
<td>5.06±0.43</td>
<td>0.006</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>47.92 ± 24.46</td>
<td>65.55 ± 36.77</td>
<td>0.002</td>
</tr>
<tr>
<td>HOMA ir</td>
<td>1.69 ± 1.43</td>
<td>2.16 ± 1.13</td>
<td>0.005</td>
</tr>
<tr>
<td>HsCRP (mg/L)</td>
<td>2.28±3.94</td>
<td>2.15±3.52</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Values are given as mean ±SD. P values <0.05 are considered statistically significant.
Circulating Levels of Angiogenic and Endothelial Biomarkers

Levels of serum sE-Selectin were significantly higher in women with a previous HDP than in women with NTP (31.86±15.32 pg/mL vs. 25.64±9.08 pg/mL; P=0.005) (see Table 2 and Figure 2). No differences in sFlt-1 (P=0.99), sEng (P=0.28) or VEGF-A (P=0.20) levels were observed between groups. IL-8 and TNF-α levels were not detectable.

Table 2 Circulating biomarkers of angiogenesis and endothelial activation

<table>
<thead>
<tr>
<th></th>
<th>NTP (n=104)</th>
<th>HDP (n=164)</th>
<th>P- value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-angiogenic biomarkers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble FLT-1 (pg/ml)</td>
<td>76.60 (18.83)</td>
<td>77.38 (20.90)</td>
<td>0.99</td>
</tr>
<tr>
<td>Soluble Endoglin (ng/ml)</td>
<td>4.02 (0.92)</td>
<td>3.92 (0.89)</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>Pro-angiogenic biomarker</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>55.12 (27.63)</td>
<td>63.30 (38.51)</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Biomarker of endothelial activation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble E-Selectin (ng/ml)</td>
<td>25.64 (9.08)</td>
<td>31.85 (15.32)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Values are given as mean (SD). P values <0.05 are considered statistically significant.

Figure 2. sE-Selectin levels were significantly higher in women with previous HDP when compared to previous NTP (31.86±15.32 pg/mL vs. 25.64±9.08 pg/mL; P=0.005)
In the following section, it was determined whether levels of circulating sE-Selectin were associated with variations in CV risk factors, phenotype and function in women with a history of HDP.

The results are described as follows: first associations -if existing- between sE-Selectin and cardiac phenotype and function will be examined, then those between sE-Selectin and vascular stiffness, blood pressure, metabolic profile and cerebral phenotype.

**Cardiac phenotype**

Years after pregnancy, significant higher LV mass index (49.88±7.08 g/m$^2$ vs 46.02±6.51 g/m$^2$; P<0.001), cardiac wall thickness (5.5±0.9 g/m$^2$ vs. 5.2±0.7 g/m2, P=0.02) and LV mass EDV ratio (0.71±0.01 vs. 0.63 ± 0.01, P<0.001) were found in women with a previous HDP when compared to women with a previous NTP.

In order to determine if sE-Selectin was associated with these differences in cardiac phenotype, regression analyses were tested (see Table 3).

### Table 3 Associations between sE-Selectin and cardiac phenotype according pregnancy characteristics

<table>
<thead>
<tr>
<th></th>
<th>sE-Selectin across cohort</th>
<th>sE-Selectin in NTP</th>
<th>sE-Selectin in HDP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>P- Value</td>
<td>B</td>
</tr>
<tr>
<td>LV Mass index</td>
<td>0.05</td>
<td>0.29</td>
<td>0.19</td>
</tr>
<tr>
<td>Wall thickness</td>
<td>0.01</td>
<td><strong>0.01</strong></td>
<td>0.012</td>
</tr>
<tr>
<td>LV mass/EDV</td>
<td>0.002</td>
<td><strong>0.002</strong></td>
<td>0.003</td>
</tr>
</tbody>
</table>

Bold text indicates statistical significance at P<0.05. Univariate regression analyses with LV mass index, wall thickness or LV mass/EDV as dependent variables and sE-Selectin as independent variable.

Across the cohort significant associations between sE-Selectin and cardiac wall thickness ($B=0.01$ g/m$^2$ per pg/mL; P=0.01) and LV mass EDV ratio ($B=0.002$ unit per pg/mL; P=0.002) were found.

It was then determined if (differences in) associations existed between women with a previous HDP or NTP. As can be seen in table 3, sE- Selectin was associated with LV mass EDV ratio in women with a previous NT pregnancy ($B=0.003$ unit per pg/mL; P=0.04), with a non-significant trend for association in women with a previous HDP (P=0.09). Furthermore, a non-significant trend for association between sE-Selectin and cardiac wall thickness was found in women with a previous HDP (P=0.07).
To examine whether women with higher sE-Selectin concentrations had significantly higher LV mass EDV ratio when compared to women with lower sE-Selectin concentrations, differences in LV mass EDV were tested across tertiles of sE-Selectin.

Figure 3 illustrates the increase in LV mass EDV ratio across tertiles of sE-Selectin in women with a previous NTP (lowest tertile of sE-Selectin vs. highest tertile of sE-Selectin; 0.60±0.02 vs 0.67±0.02; P=0.01), and in women with a previous HDP (lowest tertile of sE-Selectin vs. highest tertile of sE-Selectin; 0.67±0.02 vs 0.73±0.02; P=0.03).

![Figure 3. LV mass EDV ratio across tertiles of sE-Selectin in women with a previous NTP (left panel) and HDP (right panel).](image)

The LV mass EDV ratio in women with a HDP in the third tertile of sE-Selectin was significantly higher than the LV mass EDV ratio in women with a previous normotensive pregnancy in the third tertile of sE-Selectin (0.73±0.02 vs 0.67±0.02; P=0.03).

To determine if the association between sE-Selectin and LV mass EDV ratio in women with a previous NTP was modulated by factors known to be associated with LV mass EDV ratio, a model of LV mass EDV ratio was tested with multivariate regression (see Table 4). BMI was not related to LV mass EDV ratio and therefore not included in this multivariate regression (P=0.98). As can be seen in table 4, the relation between sE-Selectin and LV mass EDV ratio in women with NT pregnancies was still significant after adjustment (β=0.28; P=0.03).

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>β</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sE-Selectin</td>
<td>0.28</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>SBP</td>
<td>0.27</td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td>Age</td>
<td>0.24</td>
<td>0.07</td>
</tr>
<tr>
<td>HOMA ir</td>
<td>0.29</td>
<td><strong>0.03</strong></td>
</tr>
</tbody>
</table>

*Bold text indicates statistical significance at P<0.05. Multivariate regression analyses with LV mass EDV ratio as dependent variable and sE-Selectin, SBP, age and HOMA ir as independent variables.*
**Cardiac function**

Women with a history of HDP had reduced parameters of diastolic function, i.e. E/A ratio (1.34±0.35 vs. 1.52±0.45, P=0.003), septal e’ (10.47±2.68 cm/sec vs. 11.36±2.20 cm/sec; P=0.03) and lateral e’ (13.74 ±3.25 cm/sec vs. 15.07±3.23 cm/sec; P=0.01), when compared to women with a previous NTP. Moreover, an increased ejection fraction was found in women with a history of HDP when compared to women with NTP (65.6±5.4% vs. 63.7% ±4.3%; P=0.03).

Possible relations between sE-Selectin and parameters of diastolic and systolic function were examined using linear regression. Bivariate regression analyses showed that sE-Selectin was neither related to diastolic nor systolic function, i.e., no association existed between sE-Selectin and LV EF in women with either a previous HDP (P=0.98) or NTP (P=0.34). Likewise, sE-Selectin was not related to parameters of diastolic function in women with previous HDP (P= 0.13 for E/A ratio; P=0.23 for lateral e’; P=0.25 for septal e’) or in women with a previous NT pregnancy (P=0.68 for E/A ratio, P=0.07 for lateral e’; P=0.35 for septal e’).

**Macrovascular phenotype and stiffness**

Women with a former HDP had significant higher cIMT than women with a previous NTP 5-12 years after pregnancy (0.55±0.01mm vs. 0.52±0.01mm; P<0.001). Moreover, previous HDP women had significantly decreased global aortic distensibility when compared to women with previous NTP (4.95±0.17 cm²m² vs 5.71±0.26 cm²m²; P=0.02).

It was tested if sE-Selectin levels were related to the observed changes in cIMT and global aortic distensibility in women with previous HDP. Results of regression analyses are given in table 5. No associations between sE-Selectin and cIMT or global aortic distensibility were found in women with either a previous HDP or NTP (P-values >0.05).

**Table 5  Associations between sE-Selectin and parameters of vascular function and stiffness according to pregnancy characteristics**

<table>
<thead>
<tr>
<th></th>
<th>sE-Selectin across cohort</th>
<th>sE-Selectin in NT</th>
<th>sE-Selectin in HDP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>P-value</td>
<td>B</td>
</tr>
<tr>
<td>cIMT</td>
<td>0.0001</td>
<td>0.30</td>
<td>-0.001</td>
</tr>
<tr>
<td>Global aortic distensibility</td>
<td>-0.01</td>
<td>0.65</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Bold text indicates statistical significance at P<0.05. Univariate regression analyses with cIMT or global aortic distensibility as dependent variables and sE-Selectin as independent variable.
**Microvascular stiffness and rarefaction**

Women with a previous HDP were found to have distinct microvascular stiffness, which was indicated by increased augmentation pressure (8.79±4.30 mmHg vs 6.63±5.30mmHg; P=0.002), when compared to formerly NTP women. Furthermore, decreased functional and structural capillary density were found in women with a previous HDP when compared to former NTP women (106.32±2.07 capillaries/mm$^2$ vs 116.29±2.33 capillaries/mm$^2$; P<0.001 and 114.87±2.22 capillaries/mm$^2$ vs 123.28±2.46 capillaries/mm$^2$; P=0.002, respectively).

To assess whether sE-Selectin related to any of the significant changes in microvascular stiffness or capillary density, regression analyses were tested (see Table 6). It is evident from the results that sE-Selectin was not related to microvascular stiffness or capillary density in women with a previous HDP (P>0.05).

**Table 6 Associations between sE-Selectin and parameters of microvascular function and stiffness according to pregnancy characteristics**

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>sE-Selectin across cohort</th>
<th>sE-Selectin in NTP</th>
<th>sE-Selectin in HDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Augmentation pressure</td>
<td>$B=0.04$ P=0.32</td>
<td>$B=0.02$ P=0.83</td>
<td>$B=0.05$ P=0.25</td>
</tr>
<tr>
<td>Functional capillary density</td>
<td>$B=0.03$ P=0.85</td>
<td>$B=-0.26$ P=0.38</td>
<td>$B=0.17$ P=0.26</td>
</tr>
<tr>
<td>Structural capillary density</td>
<td>$B=0.06$ P=0.67</td>
<td>$B=0.09$ P=0.77</td>
<td>$B=0.12$ P=0.45</td>
</tr>
</tbody>
</table>

Bold text indicates statistical significance at P<0.05. Univariate regression analyses with augmentation pressure, functional capillary density or structural capillary density as dependent variables and sE-Selectin as independent variable.

**Blood pressure at time of study visit**

Years after pregnancy BP was significantly higher in women with a previous HDP than in women with previous NTP (see table 1).

To investigate whether levels of circulating sE-Selectin were related to variations in BP in women with previous HDP, regression analyses were tested. Using before mentioned method, positive relationships between variances in sE-Selectin concentration and both DBP ($B=0.19$ mmHg per ng/mL; P=0.001) and MAP ($B=0.17$ mmHg per ng/mL; P=0.004), with a trend for association with SBP (P=0.09) were found in women with a history of HDP.

In women with a NTP, no association between sE-Selectin and BP was observed (P=0.59 for SBP; P=0.49 for DBP; P=0.48 for MAP).
Figure 4. **sE-Selectin was significantly associated with DBP in women with previous hypertensive disorder of pregnancy (B=0.19 mmHg per ng/mL; P=0.001), not in women with normotensive pregnancy (P=0.49).**

To examine whether women with HDP and higher sE-Selectin concentrations had significantly elevated DBP compared to women with lower levels of sE-Selectin, differences in DBP were tested across tertiles of sE-Selectin. Figure 5 illustrates the increase in DBP across tertiles of sE-Selectin in women with a previous HDP (lowest tertile of sE-Selectin vs. highest tertile of sE-Selectin; 75.17±1.25 mmHg vs 80.07±1.35 mmHg; P=0.01).

Figure 5. **Diastolic blood pressure across tertiles of sE-Selectin in women with a previous HDP.**
BMI and age are known to be associated with BP. Therefore, a model of DBP, including sE-Selectin, BMI and age, was tested using multivariate regression (see table 7). If adjusted for before mentioned factors, sE-Selectin and DBP were still significantly associated ($\beta=0.20$; $P=0.02$).

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>$\beta$</th>
<th>$P$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sE-Selectin</td>
<td>0.20</td>
<td>0.02</td>
</tr>
<tr>
<td>BMI</td>
<td>0.26</td>
<td>0.003</td>
</tr>
<tr>
<td>Age</td>
<td>0.17</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Bold text indicates statistical significance at $P<0.05$. Multivariate regression analyses with DBP as dependent variable and sE-Selectin, BMI and age as independent variables.

**Metabolic profile**

Women with a history of HDP showed a more adverse metabolic profile with lower HDL cholesterol, increased insulin concentrations and insulin resistance when compared to women with a NT pregnancy, 5-12 years postpartum (see Table 1).

To determine whether levels of sE-Selectin related to the observed differences in metabolic parameters, associations between sE-Selectin and HDL cholesterol, insulin and insulin resistance were tested with linear regression. Variances in sE-Selectin levels were found to be negatively associated with HDL cholesterol ($B=-0.005$ mmol/L per ng/mL; $P=0.005$), with a non-significant trend for associations with insulin concentrations ($P=0.09$) and insulin resistance ($P=0.06$) in women with a history of HDP. In women with a NTP, no significant associations between sE-Selectin and before mentioned metabolic measures were observed ($P=0.29$ for HDL cholesterol, $P=0.24$ for insulin and $P=0.19$ for HOMA ir).

**Cerebral volumes and function**

Women with a history of PE had significant lower grey matter volumes (492 ±39 mL vs 475±30 mL; $P=0.04$) and increased mean diffusivity in the temporal lobes (7.30±0.19 $10^{-4}$ vs 7.20±0.18 $10^{-4}$; $P=0.046$).

To determine whether variances in sE-Selectin concentrations associated with the observed differences in cerebral volumes and function, associations between sE-Selectin with aforementioned cerebral measurements were tested using linear regression. sE-Selectin was not related to grey matter volumes ($P=0.77$) or mean diffusivity in the temporal lobe ($P=0.52$) across the cohort, in women with a NTP (respectively $P=0.90$ and $P=0.80$) or history of HDP (respectively $P=0.99$ and $P=0.64$).
**Summary of findings:**

A distinct increase in sE-Selectin was found in women with a previous HDP when compared to women with a NT pregnancy. Moreover, it was determined whether levels of circulating sE-Selectin associated with variations in cardiovascular phenotype and function in women with a previous HDP. Overall, sE-Selectin was found to be associated with MAP, DBP, HDL cholesterol, with a non-significant trend for association with LV mass EDV, wall thickness and HOMA ir, in women with a previous HDP. Moreover, women with HDP and higher sE-Selectin levels had significantly higher LV mass EDV ratio than women with HDP and lower sE-Selectin levels, and women with NTP and high sE-Selectin levels.
Part 2: Normotensive Preterm Delivery versus Normotensive Term Delivery

Study population
In this part of the study, differences in endothelial and angiogenic biomarkers between women with a spontaneous normotensive (NT) preterm delivery and women with a normotensive term delivery were examined. Women with a spontaneous normotensive preterm delivery were those women who delivered spontaneous preterm after a normotensive pregnancy (n=29). Women who delivered normotensive term were those women who delivered term after a normotensive pregnancy (n=75). Women with a previous HDP were excluded from the following analyses.

Demographic and characteristics of this part of the study are presented in table 8. No significant differences were found in proportion of smokers, anthropometrics, measurements of skin fold thickness, HDL, TG, glucose, insulin, insulin resistance or hsCRP between women with a previous NT term delivery or previous NT preterm delivery. Women with a previous spontaneous preterm delivery were older and had a significant higher SBP, MAP, total cholesterol and LDL than women with a previous NT term delivery.

<table>
<thead>
<tr>
<th>Table 8 Characteristics of the study cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Demographics</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
</tr>
<tr>
<td>Smokers, n (%)</td>
</tr>
<tr>
<td>Anthropometrics</td>
</tr>
<tr>
<td><strong>Height (m)</strong></td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
</tr>
<tr>
<td><strong>BMI (Kg/m²)</strong></td>
</tr>
<tr>
<td>Skinfold Thickness</td>
</tr>
<tr>
<td><strong>Mid-left-arm (cm)</strong></td>
</tr>
<tr>
<td><strong>Biceps (cm)</strong></td>
</tr>
<tr>
<td><strong>Triceps (cm)</strong></td>
</tr>
<tr>
<td><strong>Suprailiac (cm)</strong></td>
</tr>
<tr>
<td><strong>Subscapular (cm)</strong></td>
</tr>
<tr>
<td>Haemodynamics</td>
</tr>
<tr>
<td><strong>SBP (mmHg)</strong></td>
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<tr>
<td><strong>DBP (mmHg)</strong></td>
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<tr>
<td><strong>MAP (mmHg)</strong></td>
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<tr>
<td>Biochemistry</td>
</tr>
<tr>
<td><strong>Total Cholesterol (mmol/L)</strong></td>
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<tr>
<td><strong>Total Cholesterol HDL cholesterol ratio</strong></td>
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<tr>
<td><strong>LDL (mmol/L)</strong></td>
</tr>
<tr>
<td><strong>HDL (mmol/L)</strong></td>
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<tr>
<td><strong>Triglyceride (mmol/L)</strong></td>
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<tr>
<td><strong>Glucose (mmol/L)</strong></td>
</tr>
<tr>
<td><strong>Insulin (pmol/L)</strong></td>
</tr>
<tr>
<td><strong>HOMA ir</strong></td>
</tr>
<tr>
<td><strong>HsCRP (mg/L)</strong></td>
</tr>
</tbody>
</table>

Values are given as mean ±SD. P values <0.05 are considered statistically significant.
Circulating Levels of Angiogenic and Endothelial Biomarkers

Years after pregnancy, sEng concentrations were found to be higher in women who delivered normotensive preterm than in women who delivered normotensive term (4.50±1.22 ng/mL vs 3.85±0.71 ng/mL; P= 0.002) (see Table 9 and Figure 7).

No differences in sFlt-1 (P= 0.28), VEGF-A (P= 0.37) and sE-Selectin (P= 0.77) levels were observed between groups. IL-8 and TNF-alpha levels were not detectable.

Table 9 Circulating biomarkers of angiogenesis and endothelial activation in women who delivered preterm 5-12 years postpartum

<table>
<thead>
<tr>
<th></th>
<th>NT term delivery (n=75)</th>
<th>NT preterm delivery (n=29)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble FLT-1 (pg/ml)</td>
<td>75.35±18.98</td>
<td>80.03±18.32</td>
<td>0.28</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>55.64±26.74</td>
<td>53.75 ±30.43</td>
<td>0.37</td>
</tr>
<tr>
<td>Soluble Endoglin (ng/ml)</td>
<td>3.85±0.71</td>
<td>4.50 ±1.22</td>
<td>0.002</td>
</tr>
<tr>
<td>Soluble E-Selectin (ng/mL)</td>
<td>26.31±10.60</td>
<td>25.38 ±8.99</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Data are presented as mean ±SD. P-values < 0.05 are considered statistically significant.

Figure 7. Soluble Endoglin was significantly higher after spontaneous normotensive preterm delivery as compared with normotensive term delivery 5-12 years postpartum (4.50±1.22 vs 3.85±0.71; P=0.002)
Circulating sEng and Cardiovascular Risk Factors

In the following section, it was determined whether measurements of CV phenotype and function were different between women with a previous NT term or preterm delivery. If differences between CV parameters were observed, associations between circulating levels of sEng and the significant CV parameter were tested. The results are described as follows: first cardiac phenotype and function will be examined, then macrovascular phenotype and stiffness, microvascular stiffness, capillary density, blood pressure and, at last, metabolic profile.

Cardiac phenotype

LV mass index (46.31±7.28 g/m² vs. 45.82±6.01 g/m²; P=0.77), cardiac wall thickness (5.14±0.13 g/m² vs 5.15±0.11 g/m²; P=0.95) and LV mass EDV ratio (0.64±0.01 vs 0.61±0.01; P=0.21) were not significantly different between women with a spontaneous NT preterm delivery when compared to women with a NT term delivery.

Cardiac function

Years after pregnancy, LV ejection fraction was comparable between women with a previous normotensive term delivery and women with a previous normotensive preterm delivery (0.63±0.01% vs 0.64±0.01%; P=0.53). Likewise, no differences in septal S’ (9.11±0.25 cm/sec vs 9.76±0.31 cm/sec; P=0.14), lateral S’ (11.60±0.38 cm/sec vs 12.35±0.72 cm/sec; P=0.61), systolic velocity (2.49±0.07 cm/sec vs 2.32±0.08 cm/sec; P=0.12), systolic displacement (4.43±0.15 cm/sec vs 4.12±0.22 cm/sec; P=0.24), or strain rate (1.53±0.08 cm/sec vs 1.59±0.11 cm/sec; P=0.93) were observed between women with a previous normotensive term delivery and women with a previous normotensive preterm delivery.

To assess diastolic function, E/A ratio was used. This ratio was not significantly different between women with a normotensive term delivery and women with a normotensive preterm delivery (1.53±0.07 vs 1.50±0.08; P=0.87).

Macrovascular phenotype and stiffness

Women with a former NT preterm delivery had significant higher cIMT than women with a previous NT term delivery (0.54±0.01 mm vs. 0.51±0.01 mm; P=0.02), 5-12 years after pregnancy. Moreover, women with a previous NT preterm delivery had significant decreased global aortic distensibility when compared to women with previous NT term delivery (5.01±0.34 cm²/m² vs 6.23±0.35 cm²/m²; P=0.02).

To determine if sEng was associated with cIMT and global aortic distensibility, associations were tested. As can be seen in table 10, sEng was not associated with cIMT or global aortic distensibility in women with a previous NT term delivery, or in women with a previous NT preterm delivery (all P>0.05).
Angiogenesis and endothelial function in postpartum CV risk

Microvascular stiffness and rarefaction

Augmentation index and augmentation pressure were both significantly higher in women with a previous NT preterm delivery compared to women with a previous NT term delivery (respectively 21.93±1.47% vs. 17.85±1.10%; \( P = 0.047 \) and 7.86±1.02mmHg vs. 5.09±0.98 mmHg; \( P = 0.03 \)). Both functional and structural capillary density were not significantly different between women with a previous NT preterm delivery and women with previous NT term delivery (respectively 108.78 capillaries/mm\(^2\) vs 119.89±2.34 capillaries/mm\(^2\); \( P = 0.054 \) and 119.51±6.04 capillaries/mm\(^2\) vs 124.97±2.33 capillaries/mm\(^2\); \( P = 0.77 \)). To determine whether variances in circulating sEng concentrations were related to variations in augmentation index and augmentation pressure regression analyses were tested (see table 11). Using before mentioned method, no associations between variances in sEng levels with augmentation pressure or index were observed (all \( P > 0.05 \)).

| Tabel 10 Associations between sEng and macrovascular parameters |
|------------------|---|---|---|---|
|                  | sEng in NT term delivery | sEng in PT delivery |
|                  | \( B \) | P-value | \( B \) | P-value |
| cIMT             | -0.01 | 0.32 | -0.01 | 0.15 |
| Global aortic distensibility | 0.01 | 0.98 | -0.12 | 0.71 |

**Bold text indicates statistical significance at \( P < 0.05 \). Univariate regression analyses with cIMT or global aortic distensibility as dependent variables and sEng as independent variable.**


| Tabel 11 Associations between sEng and microvascular stiffness |
|------------------|---|---|---|---|
|                  | sEng in NT term delivery | sEng in PT delivery |
|                  | \( B \) | P-value | \( B \) | P-value |
| Augmentation pressure | -2.35 | 0.06 | -0.92 | 0.28 |
| Augmentation Index | -2.61 | 0.10 | 1.81 | 0.20 |

**Bold text indicates statistical significance at \( P < 0.05 \). Univariate regression analyses with augmentation pressure or augmentation index as dependent variables and sEng as independent variable.**
Blood pressure at time of study visit

Women who delivered NT preterm were found to have higher SBP (119.57±17.49 mmHg vs. 111.91±10.23 mmHg; P=0.02) and MAP (90.71±11.40 mmHg vs. 85.78±8.16 mmHg; P=0.03) when compared to women with NT term delivery. A non-significant trend for differences in DBP (72.72±0.91mmHg vs 76.31±1.67 mmHg; P=0.07) was observed between groups.

To test if levels of circulating sEng related to variations in BP in women with previous NT preterm delivery, regression was determined. Following this method, variances in sEng were not related to SBP or MAP in women who delivered NT preterm (respectively P=0.93 and P=0.94).

Metabolic profile

Women with a history of NT preterm delivery showed an adverse metabolic profile with higher total cholesterol and LDL cholesterol concentrations when compared to women with a previous NT term delivery (see table 12).

To determine potential associations in levels of sEng with the observed differences in metabolic parameters, linear regression was tested. As can be seen in table 12, sEng was not associated with total cholesterol or LDL cholesterol concentrations.

<table>
<thead>
<tr>
<th>Tabel 12 Associations between sEng and metabolic profile</th>
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<tbody>
<tr>
<td>sEng in NT term delivery</td>
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<td>--------------------------</td>
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<tr>
<td></td>
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<tr>
<td>Total cholesterol</td>
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<tr>
<td>LDL cholesterol</td>
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</table>

Bold text indicates statistical significance at P<0.05. Univariate regression analyses with total cholesterol or LDL cholesterol as dependent variables and sEng as independent variable.
Discussion and Conclusion

Part 1: sE-Selectin is elevated in women with previous HDP and may be associated with CV risk

In contrast to the hypothesis of this study, levels of angiogenic markers, including sFLT, VEGF and sEng, were not different in women with a previous hypertensive disorder of pregnancy when compared to women with a previous normotensive pregnancy. These findings suggest that the imbalance in anti-angiogenic and pro-angiogenic markers, which is seen during HDP, is resolved years after pregnancy. Moreover, the results disproved the hypothesis that an imbalance in angiogenic markers would be associated with ongoing cardiovascular changes after pregnancy. The findings in this study were in contrast with previously reported findings, in which higher sFlt-1 concentrations were reported in women five years after preeclamptic pregnancies when compared to women with normotensive pregnancies (42).

The results presented in this study provide evidence that circulating concentrations of sE-Selectin remain elevated in women years after pregnancies complicated by hypertensive disorders. This study compares well with other studies showing higher levels of sE-Selectin in women prior to the onset of PE (89), during PE (53), and up to three years after PET pregnancies (90). A small study with a total of 40 women studied 4.7 years postpartum showed no difference in sE-Selectin concentration was found between women with a history of preeclampsia and those with a history of normotensive pregnancy, however this study may well have been underpowered to detect differences (91).

The elevated levels of sE-Selectin imply that women with a previous HDP have endothelial activation and/or vascular inflammation years after pregnancy (92). Alternatively, the increased sE-Selectin levels may be a reflection of an ongoing regenerative process of endothelial cells in response to injury (93).

One of the main functions of the endothelium is the regulation of vascular tone. Endothelial activation or dysfunction, which is characterized by an imbalance in NO and Endothelin-1, has been associated with vasoconstriction, and may therefore be involved in hypertension and the pathogenesis of CVD (92). Indeed, endothelial dysfunction has been associated in literature with cardiovascular risk factors, such as hypertension and atherosclerosis, and has an independent and strong association with the onset of cardiovascular events (94). Moreover, increased sE-Selectin concentrations have been reported in patients with numerous cardiovascular diseases, such as acute myocardial infarction, coronary artery disease and hypertension (49, 56).

The role of sE-Selectin in subsequent CV risk in women with a previous HDP is unclear, hence this study assessed associations between sE-Selectin and cardiovascular risk factors in these women:
Cardiac phenotype

Women with a history of HDP were found to have greater LV mass, cardiac wall thickness and LV mass EDV ratio, which is indicative of cardiac concentric remodeling, when compared to women with a previous normotensive pregnancy, 5-12 years postpartum.

Research has shown that individuals with cardiac concentric remodeling have an increased cardiovascular risk when compared to individuals with a normal geometric pattern (95, 96). More specifically, the risk for the development of CVD increases with 33% or 59% for, respectively, any 10 g/m$^2$ augmentation in LV mass index or for every 0.1 unit increase in relative wall thickness (97). Furthermore, a large study with over 5000 participants has demonstrated that concentric remodeling is predictive of stroke development (98). Therefore, it may be suggested that women with a previous HDP have an increased lifetime CV risk as a result of concentric remodeling.

It was demonstrated in this study that women with a history of HDP and higher sE-Selectin levels were more likely to have a higher LV mass EDV ratio compared to women with a history of HDP with lower sE-Selectin levels. Moreover, women with a history of HDP and higher sE-Selectin levels had significantly higher LV mass EDV ratio when compared to former NTP women in the highest tertile of sE-Selectin. These results imply that women with a history of HDP and high postpartum sE-Selectin levels are more likely to have concentric changes of the heart, and therefore potentially more likely to develop CV disease or stroke.

Age, blood pressure and diabetes have previously been reported to correlate with concentric remodeling (98), which is why sE-Selectin was adjusted for these factors in multivariate regression. The association between sE-Selectin and LV mass EDV ratio were independent of aforementioned factors, which suggests that concentric remodeling and endothelial activation share pathophysiological mechanisms beyond those associated with conventional risk for concentric remodeling and pressure overload.

Findings of this study are in agreement with previous studies assessing associations between sE-Selectin and concentric remodeling (96, 99). Malmqvist et al. (96) have reported higher levels of E-Selectin in hypertensive patients with concentric LV hypertrophy when compared to hypertensive patients with a physiological cardiac geometry. Likewise, positive relations between sE-Selectin levels and LV mass have been described (99). To the best of my knowledge, this is the first study to show higher LV mass EDV ratios in HDP women with higher postpartum sE-Selectin levels years after pregnancy.

A possible confounder in the association between cardiac concentric remodeling and endothelial activation -and therefore sE-Selectin- is Endothelin-1 (100). This potent vasoconstrictor is characteristically expressed in endothelial dysfunction and has been shown to be an actor in pathological LV remodeling (100). Endothelin-1 was not tested in this study. Future research should assess the role of Endothelin-1 in the association between sE-Selectin and LV mass EDV ratio in former HDP women.
**Blood pressure**

Women with previous HDP and higher sE-Selectin levels were more likely to have elevated mean arterial and diastolic blood pressure. The association between sE-Selectin and DBP in women with previous HDP was independent of traditional risk factors for hypertension, such as age and BMI (101).

The direction of the association between sE-Selectin and blood pressure is unclear; i.e., sE-Selectin may be released into the circulation as a response to hypertension-induced shear stress, or may itself result in higher BP due to its contribution to the release of vasoconstricting substances (92).

The demonstrated associations between sE-Selectin and DBP and MAP are in agreement with results of previous studies, in which positive associations between sE-Selectin and diastolic blood pressure have been demonstrated in healthy Japanese men (102) and in patients with essential hypertension (103).

**sE-Selectin and metabolic profile**

Similar as previously has been reported during, and years after, HDP, an adverse metabolic profile, including insulin resistance and decreased HDL cholesterol concentrations, was observed years after HDP in this study. In these women, negative associations between sE-Selectin levels and HDL cholesterol concentrations were found, with a non-significant trend for association between sE-Selectin and insulin resistance.

The direction of the relationship between sE-Selectin and metabolic factors cannot be concluded from the results. However, classic cardiovascular risk factors, making diabetes and hypercholesterolemia, are etiologic for endothelial activation, hence it seems likely that the adverse metabolic profile contributed, at least partially, to the elevated sE-Selectin in HDP women (92).

Findings of this study compare well with other studies showing negative associations between sE-Selectin and HDL cholesterol in both patients with, or without, hyperlipidemia (104). Moreover, insulin resistance has been reported to increase E-Selectin (92).

**Macrovascular, microvascular and cerebral changes**

sE-Selectin has previously been described to promote endothelial-leukocyte adhesion, and to be a reflection of vascular inflammation (92). Inflammation of vessels and endothelial activation is thought to be crucial in the formation of atherosclerotic plaques, which is why it was hypothesized that sE-Selectin would be related to cIMT (105, 106). This hypothesis was disproved in this study, as no relation between sE-Selectin and cIMT was found. This finding is in agreement with another large study which did not find associations in variances of sE-Selectin levels with cIMT either (107).

Moreover, no associations between sE-Selectin and arterial stiffness, global aortic distensibility, microvascular stiffness, capillary density or cerebral volume and function were found. These negative findings may indicate that either cerebral, macro and microvascular changes precede the onset of HDP independent of sE-Selectin, or that endothelial activation is not important in the pathogenesis of cerebral volume and function loss and macro and microvascular stiffness and rarefaction.
Furthermore, no difference in flow mediated dilation (P=0.59), a specific test for endothelial function, with a lower FMD being indicative of endothelial dysfunction, was observed in this study between women with previous HDP or NTP. The fact that sE-Selectin was significantly increased, but flow mediation dilation was not decreased, in women with a history of HDP, could potentially indicate that endothelial activation and endothelial dysfunction are distinct entities, and can be altered, and measured, dependent from each other.

**Strengths and limitations of the study and suggestions for future research**

A strength of this study is the cohort size and the various CV parameters that were tested for associations with sE-Selectin. A limitation of the study is that hormonal effects of the menstrual cycle were not taken into account. Self reported data on time of the last menses was obtained during study visits, but was deemed unreliable for this study. Hormones, of importance in the menstrual cycle, were not tested in this study. In literature the importance of a regular menses on, and associations between sex hormones and, CVD and acute cardiac events have been described (108, 109). Moreover, positive influences on arterial stiffness, augmentation index, endothelial cells, flow mediated dilation, NO release, HDL cholesterol, systemic vascular resistance and cardiac geometry have been attributed to sex hormones (110-112). Furthermore, sex hormones are able to influence levels of angiogenic markers (113) and sE-Selectin (92).

It would be interesting to follow participants over a period of time, including prior, during and after pregnancy. This way, associations between endothelial activation and changes in CV phenotype and function over time could be assessed. Previous research has already shown increased sE-Selectin levels before PE onset (89), however, evidence on the implications of pre-pregnancy sE-Selectin on CVD is currently lacking. As described Endothelin-1, which was not tested in this study, may be a confounder in associations between sE-Selectin and BP and LV remodeling (92, 100). To learn more about the mechanisms behind the sE-Selectin and CV risk, it would be interesting to examine possible relations between Endothelin-1, endothelial activation and CV parameters. Moreover, future research should test concentrations of Von Willebrand Factor, which has been described in literature as a potential plasma gold standard marker of endothelial damage (90). If examined in relation with sE-Selectin, conclusions on the endothelial function years after hypertensive disorders can be drawn with more certainty. Lastly, it could be interesting to do an intervention study in women with a history of HDP with aspirin administration. In vitro studies have reported an inhibiting role for aspirin in E-Selectin expression, and beneficial role in CV outcome (114). A specific focus on cardiac concentric remodeling, blood pressure and metabolic profile would be advised.

**Conclusion**

This study demonstrated that women with a previous HDP have higher blood pressure than women with NTP, with signs of cardiac concentric remodeling and an adverse metabolic profile. Moreover, the results imply that women with a history of HDP and higher sE-Selectin levels are more likely to have an increased LV mass EDV ratio, elevated blood pressure and adverse metabolic changes when compared to former HDP women with lower levels of sE-Selectin or women with a normotensive pregnancy. Overall, sE-Selectin may be seen as a potential index to assess increased susceptibility for CVD in women with previous HDP.
Part 2: sEng is Elevated Years After Normotensive Preterm Delivery

To the best of my knowledge, this is the first study to show distinct elevations in sEng levels in women, years after normotensive preterm delivery. The results of this study add evidence to previous studies that have associated maternal sEng levels with subsequent spontaneous preterm birth (115, 116). A large study by Mc Donald et al. (71), in which 628 women were followed, demonstrated that women in the highest quartile of sEng had an increased risk of spontaneous PTD when compared to those in the lowest quartile. These findings were in agreement with a study by Mijal et al. (70) in which associations between increased levels of mid-pregnancy sEng and medically indicated PTD, or spontaneous PTD before 35 weeks, were elucidated. Moreover, Lewandowski et al. (117) have reported increased sEng in young adults born preterm, in whom levels of sEng were correlated to BP.

The pathophysiology of spontaneous PTD is not well known. However, PTD has been suggested to be the outcome of the synergistic activity of impaired placentation and vascular development, and imbalances in angiogenic factors and pro-inflammatory markers (71, 118). sEng is believed to be an anti-angiogenic factor and to be involved in immune regulation, vascular permeability and integrity (71), hence sEng could be of importance in the pathogenesis of sPTD. Indeed, it has been suggested that sEng-induced anti-angiogenesis has a disastrous impact on the growth, structure and function of the placental vasculature, which predisposes to spontaneous PTD (71).

Recently, it has been highlighted that delivering PT is associated with a 2.5 fold increase in subsequent CV risk (36). In this study higher SBP, MAP, total cholesterol, LDL cholesterol, cIMT, microvascular and aortic stiffness were found in women who delivered PT compared to women who delivered term. It was hypothesized that sEng would be related with these differences in CV parameters in women with sPTD. However, no associations between sEng and before described CV parameters were observed in women with spontaneous PTD. Although this could reflect the true situation, the analyses may have been underpowered by the small number of included women with normotensive preterm delivery.

Suggestions for future research

Spontaneous PTD has been associated with metabolic pathways in literature (71). Here we show that women with spontaneous PTD have increased total cholesterol and LDL cholesterol. Future studies are advised to focus on the role of factors excreted by adipocytes, in particular those of Leptin and Adiponectin, in spontaneous preterm birth and sEng expression. Reduced levels of Leptin have been reported in sPTD and have been associated with metabolic, inflammatory and angiogenic functions (112, 119). Moreover, a potential role for Leptin in hypertension, myocardial wall thickness, arterial wall thickness and PWV has been implicated (120-122). In literature negative correlations between sEng and leptin adiponectin ratio has been described (123). Therefore, reduced leptin may contribute to sEng’s role in preterm birth, metabolism and CV risk. Another interesting, by adipocytes excreted, marker is Adiponectin. Decreased levels of Adiponectin have been demonstrated in preterm labor (124), LV hypertrophy (125), hypertensive patients (125), arterial stiffness (126) and insulin resistance (127). Correlations between sEng and Adiponectin have been reported in preeclamptic patients (128), but have not been tested in PTD.
Conclusion

To date several studies have examined circulating maternal biomarkers of spontaneous preterm birth during pregnancy, but none have reported increased levels of sEng years postpartum. In conclusion, this study showed that sEng concentrations are higher years after pregnancy in women who delivered preterm compared to women who delivered term. sEng was not related to any of the observed CV changes in women with sPTD. However, the preterm delivery group was small (n=29), and may be underpowered to show significant relations between sEng and CV risk. The affect of elevated sEng on CV risk in women who delivered spontaneous preterm needs further investigation.
Appendices

Appendix 1: Technical details for Enzyme linked immunosorbent assays

According to the manufacturer, the mean minimum detectable dose (MMD) of sFlt-1 was 3.5 pg/mL, with intra-assay and inter assay median coefficients of variation (CVs) of 2.6-3.8% and 5.5-9.8% respectively. The MMD of sEng was reported as 0.007 ng/mL. Intra-assay and inter assay CVs were reported being 2.8-3.2 inter assay% and 6.3-6.7% respectively. The MMD of VEGF is reported as 9.0 pg/mL by R&D. The intra-assay and inter assay CVs were 4.5-6.7% and 6.2-8.8%, respectively. According to the R&D manufacturer, the MMD of CXCL8/IL8 was 3.5 pg/mL. The intra-assay and inter assay median CVs have been reported to be 4.4-4.7% and 5.2-8.2% respectively. sE-Selectin’s MMD was reported as being 0.009 ng/mL. The intra-assay and inter assay median CVs have been reported to be 5.1-6.9% and 7.3-8.6% respectively.

Methods for Human VEGF Quantikine ELISA (R&D systems)

Reagent and sample preparation:

Before use, the reagents were brought to room temperature. The VEGF Standard, which was provided in the kit, was reconstituted with 0.9mL Calibrator Diluent RD6U before use. By means of this reconstitution a stock solution of 2000 pg/mL was produced. The standard stock solution was vortexed to ensure complete reconstitution and was allowed to sit for 15 minutes prior to making dilutions. 500 µL of Calibrator Diluent RD6U was pipetted into 6 tubes. To produce a dilution series, 500 µL of the standard stock solution was pipetted in the 1000pg/mL tube. After thorough mixing, 500 µL of the 1000pg/mL tube was transferred into the 500pg/mL tube. The mixing and transferring steps were repeated for the remaining tubes to produce a dilution series. The samples were placed on ice until thawed.

Assay procedure:

First, 100 µL of Assay Diluent RD1W was added to each well (96 wells in total). Once the samples were thawed, they were vortexed to ensure the removal of any remaining ice crystals. Secondly, 100 µL of Standard, control, or sample was added per well. Each standard, control and sample was assayed in duplicate. Hereafter the plate was covered and incubated at room temperature on a horizontal microplate shaker (0.12 orbit) set at 500 ± 50 rpm for 2 hours. 20 mL of wash buffer concentrate, which was provided with the kit, was diluted with deionized water to prepare 500 mL of wash buffer. Each well was aspirated and washed, for a total of four washes. The plate was washed using an automatic washer; in each well, 400 µL of Wash Buffer was dispensed and aspirated. After the wash, 200 µL of VEGF Conjugate was pipetted to each well. The wells were covered and the plate was incubated for another 2 hours at room temperature on the shaker (0.12 orbit) set at 500 ± 50 rpm). After the two hours incubation period, the aspiration/wash steps were repeated.
To produce the Substrate Solution Color reagents A and B were mixed in equal volumes within 15 minutes of use. 200 µL of this Substrate Solution was added to each well. Then, the plate, which was protected from light, was incubated for 30 minutes at room temperature. 50 µL of Stop Solution was added to each well, thorough mixing was ensured by putting the plate on the shaker for a mean of 10 minutes. Within 30 minutes (from the time the Stop solution was added) the optical density of each well was determined, using a FLUORomega microplate reader set to 450 nm. Wavelength correction was set to 540 nm. A standard curve, averages and CVs was created by Omega, which is capable of generating a four parameter logistic curve-fit.

**Methods Human VEGF R1/Flt-1 Quantikine ELISA (R&D systems)**

**Reagent and sample preparation:**

Before use, the reagents were brought to room temperature. The Human VEGF R1 Standard, which was provided in the kit, was reconstituted with 0.8 mL deionized water before use. By means of this reconstitution a stock solution of 20,000 pg/mL was produced. The standard stock solution was vortexed to ensure complete reconstitution and was allowed to sit for 15 minutes prior to making dilutions. 900 µL of Calibrator Diluent RD6-10 was pipetted into the 200 pg/mL tube, and 500 µL of Calibrator Diluent RD6-10 was pipetted into the remaining 6 tubes. To produce a dilution series, 100 µL of the standard stock solution was pipetted into the 2000 pg/mL tube. After thorough mixing, 500 µL of the 200pg/mL tube was transferred into the 1000 pg/mL. The mixing and transferring steps were repeated with the remaining tubes to produce a dilution series. The samples were placed on ice until thawed.

**Assay procedure:**

First, 100 µL of Assay Diluent RD1-68 was added to each well (96 wells in total). Once the samples were thawed, they were vortexed to ensure the removal of any remaining ice crystals. Secondly, 100 µL of Standard, control, or sample was added per well. Each standard, control and sample was assayed in duplicate. Hereafter the plate was covered and incubated at room temperature on a horizontal microplate shaker (0.12 orbit) set at 500 ± 50 rpm for 2 hours. 20 mL of wash buffer concentrate, which was provided with the kit, was diluted with deionized water to prepare 500 mL of wash buffer. Each well was aspirated and washed, for a total of four washes. The plate was washed using an automatic washer; in each well, 400 µL of Wash Buffer was dispensed and aspirated. After the wash, 200 µL of VEGF R1 Conjugate was pipetted to each well. The wells were covered and the plate was incubated for another 2 hours at room temperature on the shaker ((0.12 orbit) set at 500 ± 50 rpm ). After the two hours incubation period, the aspiration/wash steps were repeated. To produce the Substrate Solution Color reagents A and B were mixed in equal volumes within 15 minutes of use. 200 µL of this Substrate Solution was added to each well. Then, the plate, which was protected from light, was incubated for 30 minutes at room temperature. 50 µL of Stop Solution was added to each well, thorough mixing was ensured by putting the plate on the shaker for a mean of 10 minutes. Within 30 minutes (from the time the Stop solution was added) the optical density of each well was determined, using a FLUORomega microplate reader set to 450 nm. Wavelength correction was set to 540 nm. A standard curve, averages and CVs were created by Omega, which is capable of generating a four parameter logistic curve-fit.
Methods for Human CXCL8/IL-8 Quantikine ELISA Kit (R&D systems)

Reagent and sample preparation:

Before use, the reagents were brought to room temperature. The human IL-8 Standard, which was provided in the kit, was reconstituted with 500µL Calibrator Diluent RD6Z. By means of this reconstitution a stock solution of 2000 pg/mL was produced. The standard stock solution was vortexed to ensure complete reconstitution and was allowed to sit for 15 minutes prior to making dilutions. 500 µL of Calibrator Diluent RD6Z was pipetted into 6 Eppendorf tubes. To produce a dilution series, 500 µL of the standard stock solution was pipetted in the 1000pg/mL tube. After thorough mixing, 500 µL of the 1000pg/mL tube was transferred into the 500pg/mL. The mixing and transferring steps were repeated for the remaining tubes to produce a dilution series. The samples were placed on ice until thawed.

Assay procedure:

First, 100 µL of Assay Diluent RD1-85 was added to each well (96 wells in total). Once the samples were thawed, they were vortexed to ensure the removal of any remaining ice crystals. Secondly, 50 µL of Standard, control, or sample was added to each well. Each standard, control and sample was assayed in duplicate. Hereafter the plate was covered and incubated at room temperature. 20 mL of wash buffer concentrate, which was provided with the kit, was diluted with deionized water to prepare 500 mL of wash buffer. Each well was aspirated and washed, for a total of four washes. The plate was washed using an autowasher; in each well, 400 µL of Wash Buffer was dispensed and aspirated. After the wash, 100 µL of Human IL-8 Conjugate was pipetted into each well. The wells were covered, and the plate was incubated for another 2 hours at room temperature. After the two hours incubation period, the aspiration/wash steps were repeated. To produce the Substrate Solution Color reagents A and B were mixed in equal volumes within 15 minutes of use. 200 µL of this Substrate Solution was added to each well. Then, the plate, which was protected from light, was incubated for 30 minutes at room temperature. 50 µL of Stop Solution was added to each well, thorough mixing was ensured by putting the plate on the shaker for a mean of 10 minutes. Within 30 minutes (from the time the Stop solution was added) the optical density of each well was determined, using a FLUORomega microplate reader set to 450 nm. Wavelength correction was set to 540 nm. A standard curve, averages and CVS were created by Omega, which is capable of generating a four parameter logistic curve-fit.
**Methods for Human sE-Selectin/CD62E Quantikine ELISA Kit (R&D systems)**

**Reagent and sample preparation:**

Before use, the reagents were brought to room temperature. The sE-selectin standard, which was provided in the kit, was reconstituted with 1 mL deionized water before use. By means of this reconstitution a stock solution of 80 ng/mL was produced. The standard stock solution was vortexed to ensure complete reconstitution and was allowed to sit for 15 minutes prior to making dilutions. 900 µL of Calibrator Diluent RD6-11 was pipetted into the 8 ng/mL tube, 500 µL of Calibrator Diluent RD6-11 was pipetted into the remaining 6 tubes. To produce a dilution series, 100 µL of the standard stock solution was pipetted in the 8ng/mL tube. After thorough mixing, 500 µL of the 8ng/mL tube was transferred into the 4ng/mL tube. The mixing and transferring steps were repeated for the remaining tubes to produce a dilution series. The 8 ng/mL standard serves as the high standard. The Calibrator Diluent serves as the zero standard (0 ng/mL). The serum samples were placed on ice for thawing. Once the samples were thawed, they were vortexed to ensure the removal of any remaining ice crystals before dilution. The samples were diluted 10-fold before use. For this, 30 µL of the serum sample was diluted in 270 µL of Calibrator Diluent RD6-11.

**Assay procedure:**

First, 100 µL of Assay Diluent RD1W was pipetted into each well (n=96). Secondly, 100 µL of Standard, control, or (the 10-fold diluted) sample was added per well. Each standard, control and sample was assayed in duplicate. Hereafter the plate was covered and incubated at room temperature for two hours. 20 mL of wash buffer concentrate, which was provided with the kit, was diluted with deionized water to prepare 500 mL of wash buffer. Each well was aspirated and washed, for a total of four washes by an automated microplate washer. In each well, 400 µL of Wash Buffer was dispensed and aspirated. After the final aspiration steps, the plate was inverted and blotted against paper towels. After the wash, 200 µL of sE-selectin Conjugate was added to each well. The wells were covered and the plate was incubated for another 2 hours at room temperature. After this two hours incubation period, the aspiration/wash steps were repeated. To produce the Substrate Solution Color reagents A and B (provided in the kit) were mixed in equal volumes within 15 minutes of use. 200 µL of this Substrate Solution was dispensed into each well. After this the plate, was incubated at room temperature in the dark for 30 minutes. 50 µL of Stop Solution was added to each well, thorough mixing was ensured by putting the plate on the shaker (0.12 orbit) set at 500 ± 50 rpm for a mean duration of five minutes. Within 30 minutes (from the time the Stop solution was added) the optical density of each well was determined, using a FLUORomega® microplate reader set to 450 nm. Wavelength correction was set to 540 nm. A standard curve, averages and CVs were created by Omega, which is capable of generating a four parameter logistic curve-fit.
**Methods for Human TNF-alpha Quantikine HS ELISA Kit (R&D systems)**

**Reagent and sample preparation:**

Before use, the reagents were brought to room temperature. The human TNF-α HS standard, which was provided in the kit, was reconstituted with 4 mL Calibrator Diluent RD6-13 before use. By means of this reconstitution a stock solution of 32 pg/mL was produced. The standard stock solution was mixed to ensure complete reconstitution and was allowed to sit for 15 minutes prior to making of the dilution series. 500 µL of Calibrator Diluent RD6-13 was pipetted into each of the 6 polypropylene tubes. To produce a dilution series, 500 µL of the standard stock solution was pipetted in the 16 pg/mL tube. After thorough mixing, 500 µL of the 16 pg/mL tube was transferred into the 8 pg/mL tube. The mixing and transferring steps were repeated for the remaining tubes to produce a dilution series. The 32 pg/mL standard serves as the high standard. The Calibrator Diluent serves as the zero standard. The serum samples were placed on ice until thawed, after which they were vortexed to ensure the removal of any remaining ice crystals.

**Assay procedure:**

First, 50 µL of Assay Diluent RD1F was added to each well (n=96). The Assay diluent was mixed before and during use. Secondly, 200 µL of Standard, control, or sample was added to each well. Each standard, control and sample was assayed in duplicate. Hereafter, the wells were covered and incubated at room temperature for three hours. 100 mL of wash buffer concentrate, which was provided with the kit, was diluted with deionized water to prepare 1000 mL of wash buffer. Each well was aspirated and washed, for a total of six washes by an automated microplate washer. In each well, 400 µL of Wash Buffer was dispensed and aspirated. After the final aspiration steps, the plate was inverted and blotted against paper towels for ten times to remove excess wash buffer. After the wash, 200 µL of mixed Human TNF-α HS Conjugate was added to each well. Wells were covered and the plate was incubated for another 2 hours at room temperature. After this two hours incubation period, the aspiration/washing steps were repeated. To produce the Substrate Solution, the lyophilized substrate was reconstituted with 6.0 mL of substrate Diluent 10 minutes before use. 50 µL of this Substrate Solution was pipetted into each well. The plate was covered with a new adhesive strip and was incubated for 1 hour at room temperature. Amplifier Solution was produced by reconstituting the lyophilized Amplifier with 6.0 mL of amplifier Diluent ten minutes before use. 50 µL of Amplifier Solution was added to each well. The plate was then incubated for 30 minutes at room temperature in the dark. 50 µL of Stop Solution was added to each well. Within 30 minutes (from the time the Stop solution was added) the optical density of each well was determined, using a FLUORomega® microplate reader set to 490 nm. Wavelength correction was set to 650 nm. A standard curve, averages and CVs was created by Omega, which is capable of generating a four parameter logistic curve-fit.
Appendix 2: Cardiovascular imaging

Cardiovascular changes associated with cardiovascular disease and hypertension were previously measured using standardized and validated methods.

Cardiac measures:
Assessment of cardiac structure and function was carried out using a 1.5 Tesla clinical cardiovascular magnetic resonance scanner (Siemens Sonata, Germany). Echocardiography (Philips iE33) was used to measure left ventricle dimensions, including posterior wall thickness and left ventricular volumes during end diastole and end systole, in apical views. Ejection fraction was calculated from the following equation:

\[ \text{LV Ejection fraction (\%)} = \frac{\text{LV end diastolic volume} - \text{LV end-systolic volume}}{\text{LV end diastolic volume}} \times 100 \]

Ventricular volumes were indexed to body surface area (Montsellan equation). Assessment of strain by speckle tracking was previously done using TomTec (Munich, Germany).

Vascular measures:
Assessment of aortic structure and function at multiple levels was previously carried out by using a 1.5 Tesla clinical cardiovascular magnetic resonance scanner (CMR) (Siemens Sonata, Germany). Maximal and minimum aortic area was measured. Aortic distensibility was calculated by dividing aortic compliance by central pulse pressure measured by CMR scan. Blood pressure was determined from the average of three automated sphygmanometer measures after a ten minute seated rest. Aortic Pulse Wave Velocity was measured by an oscillometry device (Vicorder, Taunton, UK) as a measure of central arterial stiffness. Carotid intima media thickness was measured by ultrasound, as a measure of subclinical structural atherosclerosis. Endothelial function was quantified using the ultrasound technique of flow mediated dilatation (FMD) of the brachial artery. This technique enables comparison of brachial artery size, both pre and post maximal flow stimulation. Microvascular capillary density and structure was assessed using intravital video capillaroscopy.

Cerebral measures:
Assessment of cerebral structure was carried out by using a 1.5 Tesla clinical CMR scanner (Siemens Sonata, Germany). Image analysis was assessed by FMRIB’s automated segmentation tool software, which is a method for differentiating white and grey matter volumes. All the volumes were adjusted for skull volumes. White matter integrity was determined by diffusion tensor imaging.

Biochemistry:
Measurements of lipid profile, glucose, insulin and inflammatory indices such as high sensitivity CRP were performed by laboratories in the John Radcliffe Hospital, University of Oxford. Insulin resistance was quantified by the Homeostatic model assessment (further noted as HOMAir):

\[ \text{HOMA ir} = \frac{\text{Glucose} \times \text{Insulin}}{22.5} \]
References


Angiogenesis and endothelial function in postpartum CV risk


109. Schisterman EF, Mumford SL, Sjaarda LA. Failure to consider the menstrual cycle phase may cause misinterpretation of clinical and research findings of cardiometabolic biomarkers in premenopausal women. Epidemiologic reviews. 2014;36:71-82.


