CIRCULATING FACTORS IN PREECLAMPSIA

FROM PREDICTION TO TREATMENT

RUGINA NEUMAN

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Rugina Ischbelle Neümañ

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Circulating Factors in Preeclampsia
From Prediction to Treatment

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Van predictie tot behandeling

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Promotiecommissie:

Promotoren:	Prof. dr. A.H.J Danser
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Overige leden:	Prof. dr. S.A. Karumanchi
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	Prof. dr. M.E.A Spaanderman
Copromotor:	Dr. W. Visser

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General introduction

PREECLAMPSIA

GENERAL INTRODUCTION

Preeclampsia (PE) is a hypertensive, multisystem syndrome, exclusive to human pregnancy.[1] Historically, the concept of 'eclampsia' was first recognized by the ancient Greeks, as they noticed headaches and convulsions in pregnant women and stated that 'such cases were perhaps liable to some sort of fits'.[2] Centuries later, hypertension and proteinuria were both described in association with eclampsia, and became known as the classical syndrome of PE.[2] However, over the past years, it became evident that PE is much more than *just* hypertension and proteinuria. A significant proportion of women with PE may develop systemic manifestations such as eclampsia, pulmonary edema, renal insufficiency, cerebral hemorrhage and HELLP syndrome (i.e. hemolysis, elevated liver enzymes and low platelet count), which can even occur when proteinuria is absent.[3, 4] Alongside these maternal complications, the fetus is at significant risk for stillbirth, intrauterine growth restriction, iatrogenic preterm birth and other perinatal morbidity.[1] These effects extend many years beyond pregnancy, as evidenced by an increased risk of long-term cardiovascular disease in the mother and offspring.[5, 6] This complex, heterogeneous nature of PE has led to the recent changing of its definition, which nowadays includes the presence of maternal organ and/or uteroplacental dysfunction, even in the absence of proteinuria.[7]

CIRCULATING PROTEINS IN PE

The presence of a placenta is key to the development of PE, but the exact causes underlying this syndrome remain unknown.[3] One of the most recognized hypotheses is that abnormal placentation induces intermittent placental hypoxia and oxidative stress, driving an excessive release of inflammatory and anti-angiogenic proteins from the placenta into the maternal circulation. The concomitant increase of these circulating factors is thought to underlie the maternal systemic endothelial dysfunction typically associated with this syndrome.[1, 3, 8] In the next paragraphs, we provide a brief description of the circulating factors discussed within this thesis, and their roles in PE.

VEGF, PlGF and sFlt-1

The vascular endothelial growth factors (VEGF) are a family of proteins involved in both angiogenesis and vasculogenesis. VEGF-A (often referred to as VEGF) plays an important role in endothelial cell proliferation, migration and vascular permeability.[9] VEGF induces the release of nitric oxide and prostacyclin from endothelial cells, eliciting its actions upon binding to tyrosine kinase receptors, of which VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) are the main functional receptors. Placental growth factor (PlGF), another member of the VEGF family and highly homologous to VEGF, is considered equally important in the regulation of angiogenesis. PlGF binds exclusively

to VEGFR-1, thereby competing with VEGF and causing it to bind VEGFR-2. The anti-angiogenic soluble Fms-like tyrosine kinase (sFlt-1) is an alternatively spliced, soluble form of VEGFR-1, synthesized within the trophoblast cells of the placenta.[9] During PE, abnormally high sFlt-1 levels are released into the maternal circulation, thereby entrapping free circulating VEGF and PlGF and impairing their mitogenic and homeostatic effects on endothelial cells.[10, 11] As a result, the large increases of sFlt-1 contribute substantially to the clinical manifestations of PE, such as hypertension and proteinuria.[10, 11]

Endothelin-1

Endothelin-1 (ET-1) belongs to a family of three 21-amino acid peptides (ET-1, ET-2, ET-3) and is one of the most powerful vasoconstrictors known.[12] It is secreted by endothelial and trophoblast cells and functions as an autocrine or paracrine peptide.[13] Derived from the precursor peptide preproET-1, big-ET-1 is cleaved predominantly by endothelin converting enzymes into biologically active ET-1. ET-1 interacts with either ET_A receptors located on the vascular smooth muscle cells or ET_B receptors located on both endothelial and vascular smooth muscle cells. Both ET_A and ET_B receptors induce vasoconstriction, while the endothelial ET_B receptors elicit vasodilation.[14, 15] Plasma ET-1 levels are two- to three fold increased in women with PE compared to healthy pregnant controls,[14] while some studies have noticed a positive association between this factor and the severity of the syndrome.[16-18] In patients with a high anti-angiogenic state (sFlt-1/PlGF ratio$\geq$85), a strong relationship between sFlt-1 and ET-1 is witnessed.[19] This observation together with the finding that ET-1 is elevated in cancer patients treated with VEGF inhibitors[20] has led to the idea that, in PE, the ET-1 rise is a direct consequence of VEGF antagonism by sFlt-1.

PAPP-A2 and Inhibin A

Pregnancy-associated plasma protein-A2 (PAPP-A2) is a protease predominantly expressed in the placenta, that cleaves insulin-like growth factor (IGF)-binding proteins (IGFBP-5 and IGFBP-3). By increasing IGF-1 bioavailability, PAPP-A2 is believed to be essential in the regulation of fetoplacental growth and extravillous trophoblast invasion.[21-23] In placental tissue, mainly in syncytiotrophoblasts, and in maternal blood of women with PE, PAPP-A2 levels are markedly elevated when compared to normotensive controls.[23, 24]

Inhibins (inhibin A and B) are heterodimeric glycoprotein hormones, belonging to the transforming growth factor-ß superfamily. Classically, inhibin A is produced by the corpus luteum and inhibits follicle-stimulating hormone during the menstrual cycle. During pregnancy, inhibin A concentrations rise, as it is predominantly secreted by the placenta, although its exact function remains unclear.[25] Circulating inhibin A levels are 10-fold lower in women that remain normotensive throughout their pregnancy compared to those that develop PE.[25, 26]

Copeptin and MR-proANP

Arginine vasopressin (AVP), also known as antidiuretic hormone, is a small peptide hormone important for the regulation of body water homeostasis.[27] Synthesized in the hypothalamus and later stored in the posterior lobe of the pituitary gland, its release is stimulated by an increase in plasma osmolality, reductions in circulating plasma volume, and stress. Copeptin, also known as CT-proAVP, is the C-terminal portion of the AVP precursor ("preprovasopressin") and can be reliably measured as it is released at 1:1 ratio with AVP but displays higher stability in plasma. Elevated copeptin measured before the 16th week of gestation is associated with an increased risk of PE.[28] At the time of PE itself, copeptin values are two- to threefold increased in comparison to normotensive pregnancies.[29] Atrial natriuretic peptide (ANP) is a vasodilatory cardiac hormone with diuretic and natriuretic effects released from the cardiac atria in response to distension. There are conflicting data whether ANP concentrations are up- or downregulated[30-33] in PE due to its instable measurement, while the stable mid-regional part of its prohormone, MR-proANP, has been shown to be consistently upregulated in the heart and plasma of preeclamptic women.[34]

PREDICTION

The aim of every obstetrician is to be able to early detect PE and its associated complications. Particularly in women already suspected of PE, the clinical utility of the classical manifestations (i.e., hypertension and proteinuria) to detect serious adverse outcomes remains extremely poor.[35] The angiogenic markers sFlt-1/PlGF ratio or PlGF alone, when measured in the second or third trimester, have shown promising prognostic value to diagnose PE. In a large clinical trial evaluating 1273 women with suspected PE, a sFlt-1/PlGF ratio of 38 or lower was able to accurately exclude PE within 1 week, with a negative predictive value of 99.3%.[36] Another trial had shown that a triage PlGF value of $\leq$100 pg/mL was highly sensitive to identify women who were likely to develop PE necessitating delivery within 14 days.[37] In a few European countries including Germany, the sFlt-1/PlGF ratio has already been implemented as routine obstetric care, but only for its use in PE detection. For the prediction of severe PE-related complications, an sFlt-1/PlGF ratio$<$85 in women suspected of PE was found to accurately predict the absence of adverse outcomes within 14 days.[38] When comparing continuous *vs.* the cutoff values for this purpose, applying the sFlt-1/PlGF ratio as a continuous variable showed higher utility to predict adverse outcomes, especially when combined with clinical characteristics.[39]

Overview of circulating factors discussed within this thesis and their roles in PE.

Factor	Main Function	Main source in PE	Changes in Maternal Blood in PE
VEGF	Pro-angiogenesis, vasodilation	Placental trophoblasts	↓
PlGF	Pro-angiogenesis, vasodilation	Placental trophoblasts	↓
sFlt-1	Anti-angiogenesis; binding to VEGF, PlGF	Placental trophoblasts	↑
PAPP-A2	Cleavage of insulin-like growth factor binding protein	Placental trophoblasts	↑
Inhibin A	Negative feedback control of FSH; function in pregnancy unknown	Placental trophoblasts	↑
(CT-pro) ET-1	Vasoconstriction	Placental trophoblasts, endothelial cells	↑
(CT-pro) AVP	Osmotic homeostasis, vasoconstriction	Hypothalamus	↑
(MR-pro) ANP	Natriuresis, diuresis, vasodilation	Atrial myocytes	↑

TREATMENT

There are currently no drugs that can effectively treat PE. Delivery is the only cure, and is therefore recommended in case the syndrome occurs near term, to minimize maternal complication risk. In early pregnancy however, it is beneficial to prolong gestation to improve neonatal outcome as long as the maternal condition is stable. Clinical management is thus aimed at providing symptomatic relief, initiating antihypertensive drugs to stabilize maternal blood pressure and magnesium sulfate for either eclampsia prevention or neonatal neuroprotection. In patients with a gestational age <34 weeks, antenatal steroids are administered to improve fetal lung maturation.[40]

When considering novel therapeutic strategies, improving the maternal angiogenic profile by diminishing sFlt-1 and ET-1, or enhancing PlGF would form an ideal approach to treat PE.[41] Among several drugs investigated for this purpose, proton pump inhibitors (PPIs) were shown to dose-dependently reduce sFlt-1 production in placental explants and cytotrophoblast cells.[42] In a retrospective analysis evaluating the use of PPIs in women with suspected or confirmed PE, it was indeed reported that women using these drugs had lower circulating sFlt-1 and ET-1 compared to women not treated with PPIs.[43] The use of statins, pravastatin in particular, has also gained attention as a potential therapeutic or preventative drug, since it has shown beneficial effects on sFlt-1 levels in preclinical models and small human studies.[44-46] Similarly, sulfasalazine and metformin have shown significant ability to decrease sFlt-1 and upregulate PlGF in vitro, and are therefore currently under investigation in clinical trials.[47, 48]

ACUTE FATTY LIVER OF PREGNANCY

What is now known as acute fatty liver of pregnancy (AFLP), was first described more than 150 years ago, when sporadic cases of maternal deaths due to liver failure were attributed to widespread microvesicular fat infiltration on their liver biopsies.[49] AFLP is a relatively rare complication of pregnancy, with an incidence ranging from of 1 in 7,000 to 15,000 pregnancies, usually occurring in the third trimester.[50] Although maternal and perinatal mortality rates have significantly declined over the past years due to advances in supportive care, AFLP remains an obstetric emergency for both mothers and their newborn. The pathogenic mechanisms underlying AFLP are not fully understood, but it is assumed that defects in fatty acid oxidation enzymes in the placenta or fetus lead to the accumulation of fatty acids which are toxic to maternal hepatocytes. While some children born to mothers with AFLP (15-20%) are found to be deficient of the well-known fetal long-chain 3-hydroxyacyl-coA dehydrogenase (LCHAD), mutations in several other proteins have also been associated with AFLP in the literature.[50, 51]

A unique feature of AFLP is the presence of true hepatic dysfunction, which does not occur in other liver disorders of pregnancy, such as HELLP syndrome. Despite this difference, distinguishing these two entities remains difficult, as they partially share similar clinical and laboratory characteristics. Hypertension and proteinuria may also occur in conjunction with AFLP, further complicating the differentiation between these two disorders.[52] In a small study evaluating angiogenesis-related factors in one woman with AFLP, it was found that the serum levels of sFlt-1 and sFlt-1/PlGF ratio were higher compared to 12 women with HELLP syndrome, suggesting a potential role for these factors in the pathophysiology of AFLP.[53]

AIMS AND SCOPE OF THIS THESIS

The aims of this thesis are 1) to identify novel biomarkers and improve prediction of PE-related complications and 2) to explore novel strategies for the treatment of PE.

Part I: Towards better prediction of PE-related adverse outcomes

In **Chapter 2**, we developed a clinical prediction tool to identify women at risk for PE-related complications, using the data of a previously conducted prospective cohort study in pregnant women with suspected or confirmed preeclampsia, in which the biomarker value of sFlt-1 and PlGF was assessed. Making use of the same cohort, we analyzed the prognostic importance of PAPP-A2, inhibin A, copeptin and MR-proANP in comparison to the angiogenic markers for the prediction of PE-related adverse outcome in **Chapters 3-4**. **In Chapter 5**, we explored whether the degree of sFlt-1 and PlGF alterations during PE could be associated with the occurrence of hypertension 1 year after delivery.

Part II: Potential therapeutic strategies in PE

In **Chapter 6** we examined the effects and transfer of the endothelin receptor antagonists (macitentan, ambrisentan, sitaxentan) in the human placenta. In **Chapter 7** we made use of the Pregnancy-induced Amelioration of Rheumatoid Arthritis (PARA) cohort to examine whether inflammation and sulfasalazine could affect the levels of sFlt-1 and PlGF in pregnancies with RA. To investigate whether proton pump inhibitor (omeprazole) administration to preterm preeclamptic women could effectively lower sFlt-1 or increase PlGF levels in their maternal blood, we performed a prospective, randomized controlled trial in 50 women, which we will discuss in **Chapter 8.**

Part III: Angiogenesis-related factors in AFLP

We assessed in **Chapter 9** whether circulating levels of sFlt-1 and PlGF are altered in women with AFLP compared to women with PE or HELLP syndrome. In **Chapter 10** we established a method to accurately calculate total PlGF from already available free PlGF and sFlt-1 levels. Using this method, we compared total PlGF values in women with AFLP in relation to women without PE, PE or HELLP syndrome.

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CHAPTER 02

Prediction of pre-eclampsia-related complications in women with suspected or confirmed pre-eclampsia: development and internal validation of clinical prediction model

Langeza Saleh, Maaike M. Alblas, Daan Nieboer, **Rugina I. Neuman**, Yvonne Vergouwe, Ingrid A. Brussé, Johannes J. Duvekot, Ewout W. Steyerberg, Hans J. Versendaal, A.H Jan Danser, Anton H. van den Meiracker, Koen Verdonk, Willy Visser

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ABSTRACT

Objective: A clinical prediction model that can predict reliably the risk of preeclampsia (PE)-related pregnancy complications does not exist. The aim of this study was to develop and validate internally a clinical prediction model to predict the risk of a composite outcome of PE-related maternal and fetal complications within 7, 14 and 30 days in women with suspected or confirmed PE.

Methods: The data for this study were derived from a prospective, multicenter, observational cohort study on women with singleton pregnancy and suspected or confirmed PE at 20 to <37 weeks. For the development of the prediction model, the possible contribution of clinical and standard laboratory variables, as well as the biomarkers soluble Fms like tyrosine kinase-1 (sFlt-1), placental growth factor (PlGF) and their ratio, in the prediction of a composite outcome of PE-related complications, consisting of maternal and fetal adverse events, within 7, 14 and 30 days, was explored using multivariable competing risks regression analysis. We assessed the discriminative ability of the model using the concordance (c-) statistic. A bootstrap validation procedure with 500 replications was used to correct the estimate of the prediction model performance for optimism and to compute a shrinkage factor for the regression coefficients to correct for overfitting.

Results: Among 384 women with suspected or confirmed PE, 96 (25%) had an adverse PE-related outcome at any time after hospital admission. Important predictors of adverse PE-related outcome included sFlt-1/PlGF ratio, gestational age at the time of biomarker measurement and protein-to-creatinine ratio as continuous variables. The c-statistics (corrected for optimism) for developing a PE-related complication within 7, 14 and 30 days were 0.89, 0.88 and 0.87, respectively. There was limited overfitting, as indicated by a shrinkage factor of 0.91.

Conclusions: We propose a simple clinical prediction model with good discriminative performance to predict PE-related complications. Its usefulness in clinical practice awaits further investigation and external validation.

INTRODUCTION

Preeclampsia (PE), a pregnancy-specific syndrome traditionally characterized by elevated blood pressure and proteinuria, affecting approximately 5% of all pregnancies, is an important cause of death and complications for women and their babies.[1] The current management of patients with suspected or confirmed PE requires resource-intensive measures such as hospital admission for close monitoring of maternal blood pressure and laboratory trends due to the risk of eclampsia, liver, lung and kidney damage, intrauterine growth restriction, prematurity, and maternal and fetal demise.[2,3] Well-recognized risk factors for PE include a history of PE, nulliparity, advanced maternal age and pre-existing conditions such as hypertension, pre-gestational diabetes, obesity and chronic kidney disease. The precise cause of PE remains unclear. Ample evidence suggests a role for placental overproduction of soluble Fms-like tyrosine kinase-1 (sFlt-1), which results in an anti-angiogenic state due to the inhibition of vascular endothelial growth factors, including placental growth factor (PlGF).[4] Several models have been developed to calculate the predicted risk of PE, and some of these models include sFlt-1 and PlGF as predictors.[5,6] Despite the search for a prediction model that could estimate the risk of pregnancy complications rather than diagnosing the heterogeneous PE syndrome, such a model does not currently exist. We reported previously that incorporation of sFlt-1, PlGF and the sFlt-1/PlGF ratio in a model based on clinical and traditional laboratory variables has substantial incremental value in the prediction of both maternal and fetal complications as well as pregnancy prolongation in patients with suspected or confirmed PE.[7] We also demonstrated that incorporation of sFlt-1 and PlGF values separately was associated with superior prediction of complications as compared to proposed sFlt-1/PlGF ratio cut-offs.[7] As a follow-up of these findings, our aim was to develop and validate internally a clinical prediction model to predict the risk of developing a composite outcome of PE-related pregnancy complications within 7, 14 and 30 days in women with suspected or confirmed PE.

METHODS

Study design

Women with singleton pregnancy from varied ethnic backgrounds were recruited into a prospective multicenter cohort study at three Dutch hospitals (Erasmus MC and Maasstad Hospital in Rotterdam, Reinier de Graaf Hospital in Delft). The same study protocol and data collection forms were used at each center. All women provided written informed consent to participate in the study, which was approved by the local research ethics committee (MEC-2013-202).

Study population

The inclusion criteria for the original cohort were women ≥18 years of age with singleton pregnancy at a gestational age (GA) of ≥20 weeks who had PE, new onset of elevated blood pressure or aggravation of preexisting hypertension, new onset of proteinuria or aggravation of preexisting proteinuria, or decreased platelet levels, increased liver enzymes and one or more other reason(s) for clinical suspicion of PE such as epigastric pain, severe headache or excessive edema/severe swelling (face or extremities) (Figure S1). PE was defined according to the Dutch guideline of De Nederlandse Vereniging voor Obstetrie en Gynaecologie (NVOG) as new onset of hypertension (systolic blood pressure (SBP) of ≥140 and/or a diastolic blood pressure (DBP) of ≥90 mmHg) and new onset of proteinuria (protein-to-creatinine ratio (PCR) ≥30 mg/mmol, 24-hours urine ≥0.3g/day or dipstick ≥+2) at or after 20 weeks of gestation.[8] At the Erasmus MC and Maasstad Hospital, PCR was initially determined, and, when increased (≥30 mg/mmol), 24-hour urine was then collected. In Reinier de Graaf Hospital, PCR was determined after a positive dipstick test (++).

HELLP syndrome was defined as hemolysis (haptoglobin <0.1 g/L), elevated liver enzymes (ALAT >41 U/L or LDH >321 U/L) and low platelet count (<100*10^9/L), with or without PE. Partial HELLP syndrome was defined as the presence of two of the HELLP criteria. Superimposed PE was diagnosed in women with chronic hypertension who had new onset of proteinuria, a sudden increase in blood pressure, appearance of thrombocytopenia and increased liver enzymes, or a sudden increase in proteinuria in patients with pre-existing proteinuria. Isolated new-onset hypertension at ≥20 weeks was defined as gestational hypertension (GH). Patients with suspicion of PE but without GH were defined as having no hypertensive disease of pregnancy.[8]

Eligible patients for this sub-analysis were women with suspected or confirmed PE at a gestational age of 20 to <37 weeks. Women with (partial) HELLP syndrome or fetal death at study entry were excluded.

Outcome measures

A composite outcome of PE-related pregnancy complications, consisting of maternal and fetal adverse events, was established and defined as occurrence of at least one maternal and/or fetal complication. Maternal complications were defined as: acute renal failure (absolute increase in serum creatinine concentration of ≥0.3 mg/dL [26.4 μmol/L] from baseline, a ≥50% increase in serum creatinine or oliguria with <0.5 mL/kg/hour over a period of 6 hours), cerebral hemorrhage/edema or infarction, death, eclampsia, development of (partial) HELLP syndrome, pulmonary edema, placental abruption, visual disturbances and subcapsular liver hematoma. Fetal complications were defined as fetal death or fetal distress requiring immediate delivery. The duration until occurrence of a complication was defined as the number of days from study inclusion and blood sampling until occurrence of the complication. The occurrence of the composite outcome within the subsequent 7, 14 and 30 days was assessed. As agreed with the

obstetricians of the project group, patients with a score <5% have a very low risk of developing a complication within the forthcoming 7 days and therefore do not require hospitalization but can be followed at the outpatient clinic. Patients with risks of 5.0% or higher will be admitted to the hospital or remain in the hospital.

The treating obstetricians filled in forms in which they had to answer the following questions, taking into account available traditional variables: 'What is the chance of developing PE within this pregnancy? (0 -100%)'; 'What is the severity of the disease? (0 – 10)' and 'For how long can this pregnancy be temporized? (0, <2, 2-7, 8-14, >14 days)'. Clinical findings, physical examination, laboratory test results, maternal and fetal complications (diagnosed by the treating physicians) and background data of the patients were obtained from patient's electronic medical records.

Serum samples

For the measurement of sFlt-1 and PlGF, serum was prepared from venous whole blood, and assays were performed at the clinical laboratory of the Erasmus MC using an automated biochemistry analyzer (Cobas 6000, e module; Roche Diagnostics, Mannheim, Germany). Samples were stored at -80°C until analysis after completion of the study.

Statistical analysis

Patient characteristics were described as median and inter-quartile range for continuous variables and as n (%) for categorical variables. Missing data on candidate predictors were imputed using multivariate imputation by chained equations. Results from statistical analyses were pooled using Rubin's rules.[9]

Non-linearity of associations between continuous candidate predictors and the risk of developing complications was assessed using logarithmic transformations.

A Fine & Gray semi-parametric proportional hazards model was used to evaluate the effect of predictors on time to develop complications, adjusting for the competing risk of delivery. The candidate predictors considered were sFlt-1, PlGF, sFlt-1/PlGF ratio, gestational age, proteinuria (PCR ≥30 mg/mmol or ≥300 mg/24 hours or 2+ dipstick), DBP (systolic blood pressure was not considered because of its strong correlation with DBP, and a model with systolic blood pressure rather than DBP gave a similar fit), lactate dehydrogenase (LDH), uric acid and platelet count. The relationship between each predictor variable and the composite outcome was assessed using univariable logistic regression. The effects were quantified using subdistribution hazard ratios (sHRs). Subsequently, we developed a model to estimate the absolute risk of developing a pregnancy complication (i.e. the composite outcome of PE-related pregnancy complications) using the cumulative incidence function.

To ensure an accurate prediction model, candidate predictors were evaluated when at least 10 complications were registered. Subsequently, backward selection was applied to limit the number of predictors in the final prediction model. Model specification was based on the Akaike Information Criterion (AIC) in a backward selection procedure. This is equivalent to exclusion of candidate predictors with $p>0.157$. The discriminative ability of the developed model was assessed using the concordance (c)-statistic. Discrimination refers to how well the model distinguishes between those with and those without PE-related complications at specific time points. The c-statistic ranges from 0.5 for a model equivalent to a coin toss to 1.0 for a model with perfect discrimination.

The developed model was validated internally using bootstrap resampling. Prediction models were developed in bootstrap samples, with selection and coefficient estimation repeated in each. Such bootstrap validation maximizes statistical efficiency and validates directly the final model. A shrinkage factor was obtained for correction of calibration of predictions, and an optimism-corrected c-statistic was calculated. A prediction model was constructed in Excel to ease the implementation of the final model in clinical practice. We used SPSS Statistics 21 (IBM Corporation) and R Software version 3.3.2 (R foundation for statistical computing, Vienna, Austria; cmprsk and riskRegression libraries) for statistical analysis.

RESULTS

Of the 620 women with suspected or confirmed PE from the original cohort, 422 had a gestational age between 20 and <37 weeks and were eligible for inclusion in the current study, of whom 38 were excluded due to the presence of (partial) HELLP syndrome or fetal death at study entry. We therefore included 384 singleton pregnancies with suspected or confirmed PE at 20 to <37 weeks' gestation, of which 220 (57%) had a gestational age below 34 weeks (Table 1). Among these, 68 (18%) women had gestational hypertension, 130 (34%) had (superimposed) PE and 186 (48%) had no hypertensive disease of pregnancy at study entry (Table 1). The median time from inclusion to delivery was 21 days (IQR 8-41 days). In total 96 (25%) of pregnancies developed composite adverse PE-related maternal/fetal outcome at any time after hospital admission (Table 2). The percentage of missing values was low (1-3%), except in the case of the PCR (34%). In patients without a PCR measurement, either 24-hour urine protein or the dipstick test was assessed, which was taken into account in the statistical imputation procedure.

Transformation with the natural logarithm was the best fit for PlGF, sFlt-1/PlGF ratio, proteinuria, PCR, DBP, platelet count and the composite outcome, while a linear relationship between sFlt-1, gestational age, LD, uric acid and the outcome was a reasonable approximation. The final multivariable model included sFlt-1/PlGF ratio, gestational age at the time of biomarker measurement and PCR as continuous variables. The sFlt-1/PlGF ratio and gestational age at

the time of biomarker measurement were the strongest predictors for PE-related complications ($p<0.001$ for both), and PCR was also a significant predictor ($p= 0.03$) (Table 3).

Table 1. Characteristics of 384 pregnancies with suspected or confirmed pre-eclampsia from the PRE-RATIO study[7]

Characteristic	Value
Age (years)	31 (27 – 36)
Gestational age <34 weeks	220 (57.3)
Nulliparous	200 (52.1)
Current smoker	39 (10.2)
Race	
White	246 (64.1)
Black	73 (19.0)
Other	65 (16.9)
History of PE	74 (19.3)
Preexisting hypertension	101 (26.3)
Preexisting proteinuria	23 (6.0)
Clinical findings at time of admission	
Systolic blood pressure (mmHg)	138 (126 – 150)
Diastolic blood pressure (mmHg)	86 (80 – 95)
Protein to creatinine ratio (mg/mmol)	30 (14 – 82)
Lactate dehydrogenase (U/L)	184 (160 – 217)
Alanine transaminase (U/L)	15 (11 – 23)
Creatinine (μmol/L)	56 (49 – 65)
Uric Acid (mmol/L)	0.28 (0.23 – 0.34)
Platelet Count (10^9/L)	233 (186 – 279)
sFlt-1 (pg/mL)	3137 (1721 – 6843)
PlGF (pg/mL)	139 (62 – 354)
sFlt-1/PlGF ratio	23 (5 – 90)
Diagnosis at inclusion	
No hypertensive disease of pregnancy	186 (48.4)
Gestational hypertension	68 (17.7)
Preeclampsia	86 (22.4)
Superimposed preeclampsia	44 (11.5)

Data are given as median (interquartile range) or n (%)

As illustrated in Figure S2, a patient with suspected PE at a gestational age of 35+1 weeks with a PCR of 10 mg/mmol and a sFlt-1/PlGF ratio of 10 has a predicted probability of 2.6% for a complication within 7 days. This risk increases to 4.0% and 4.9% in the subsequent 7 and 23 days, respectively (Figure S2b). If the same patient had an increased PCR of 38 mg/mmol, the predicted probability for a complication within 7 days would increase to 3.3% (Figure S2c), while a solely elevated sFlt-1/PlGF ratio of 38 in the same patient increases the risk to 5.2% (Figure S2d).

Table 2. Pregnancy outcome and maternal and fetal complications in 384 pregnancies with suspected or confirmed pre-eclampsia

Outcome	Value
Gestational age at birth (weeks)	37.1 (34.2 – 38.2)
Preterm delivery <34 weeks	85 (22.1)
Preterm delivery 34 – 37 weeks	73 (19.0)
Female neonatal sex	186 (48.4)
Birth weight (gram)	2813 (1965 – 3329)
Duration until delivery (days)	21 (8 – 41)
Duration of hospitalization (days)	6 (3 – 13)
Final diagnosis	
No hypertensive disease of pregnancy	143 (37.2)
Gestational hypertension	64 (16.7)
Preeclampsia	102 (26.6)
Superimposed preeclampsia	53 (13.8)
(partial) HELLP syndrome	22 (5.7)
Maternal complications	
(partial) HELLP syndrome	22 (5.7)
Placental abruption	1 (0.3)
Pulmonary edema	7 (1.8)
Renal insufficiency	2 (0.5)
Visual disturbances	3 (0.8)
Fetal complications	
Fetal distress requiring elective CS	55 (14.3)
Fetal death	9 (2.3)
Composite adverse maternal/fetal outcome	96 (25.0)

Data are given as median (interquartile range) or n (%).
Note that cerebral hemorrhage/edema or infarction, maternal death, eclampsia, and subcapsular liver hematoma are not listed in the current table because these PE-related complications did not occur in the current study population. CS, Cesarean section.

The c-statistic (corrected for optimism) of the model for prediction of developing a PE-related complication within 7 days was 0.89, meaning that the model has an excellent ability to discriminate between patients who will develop a PE-related complication and those who will not. This was similarly found for prediction of developing a PE-related complication within 14 and 30 days, with c-statistics (corrected for optimism) of 0.88 and 0.87, respectively. The model was validated internally by performing a bootstrap validation procedure with 500 replications in which we obtained a shrinkage factor of 0.91, meaning there was limited overfitting. The regression coefficient for future patients should be multiplied by 0.91.

Only one out of the 170 (0.6%) pregnancies in the low risk group (<5% risk) developed composite adverse outcome within 7 days after study entry (Table 4). This 29 year old primigravida was included in the study at 34+6 weeks of gestation and delivered 3 days later by emergency

caesarean section because of suspicion of fetal distress due to suboptimal cardiotocography. Patients in the high-risk group, compared to the low-risk group, delivered earlier, had lower birth-weight centile and developed significantly more often composite adverse PE-related maternal/fetal outcome.

Table 3. Subdistribution hazard ratios of the multivariable model for prediction of composite adverse pre-eclampsia-related maternal/fetal outcome in women with suspected or confirmed pre-eclampsia.

Variable	Hazard ratio (95% CI)	P-value
GA at biomarker measurement (in weeks and days)	0.99 (0.98 – 0.99)	<0.001
ln PCR (in mg/mmol)	1.23 (1.02 – 1.49)	0.03
ln sFlt-1/PlGF ratio	1.79 (1.60 – 2.02)	<0.001

GA, gestational age; PCR, protein-to-creatinine ratio; sFlt-1/PlGF, soluble Fms-like tyrosine kinase-1/placental growth factor ratio.

Table 4. Characteristics and occurrence of adverse maternal and fetal outcomes in 384 pregnancies with suspected or confirmed pre-eclampsia, according to low (<5%) or high (≥5%) risk based on the prediction model

Variable	Low risk *n = 170*	High risk *n = 214*
Maternal age (years)	32 (27 – 35)	31 (27 – 36)
Nulliparous	76 (44.7)	124 (57.9)
Gestational age at entry (wks)	34.0 (30.2 – 35.3)	31.2 (27.0 – 35.0)*
Gestational age at entry <34 weeks	81 (47.6)	140 (65.4)*
Gestational age at birth (wks)	38.1 (37.0 – 39.0)	35.2 (30.2 – 37.0)*
Preterm delivery <34 weeks	4 (2.4)	81 (37.9)*
Preterm delivery 34 – 37 weeks	17 (10.0)	56 (26.2)*
Female neonatal sex	81 (47.6)	105 (49.1)
Birth centile <10th percentile	12 (7.1)	51 (23.8)*
Duration until delivery (days)	18 (8 – 39)	13 (4 – 26)*
Maternal complications within 7 days		
(Partial) HELLP syndrome	-	7 (3.3)
Placental abruption	-	1 (0.5)
Pulmonary edema	-	3 (1.4)
Renal insufficiency	-	1 (0.5)
Visual disturbances	-	1 (0.5)
Fetal complications within 7 days		
Fetal distress requiring elective cesarean section	1 (0.6)	53 (24.8)*
Fetal death	-	9 (4.2)*
Composite adverse maternal/fetal outcome within 7 days	1 (0.6)	53 (24.8)*

Data are given as median (interquartile range) or n (%). *P <0.05 compared with low-risk group.
Note that cerebral hemorrhage/edema or infarction, maternal death, eclampsia, and subcapsular liver hematoma are not listed in the current table because these pre-eclampsia-related complications did not occurred in the study population.

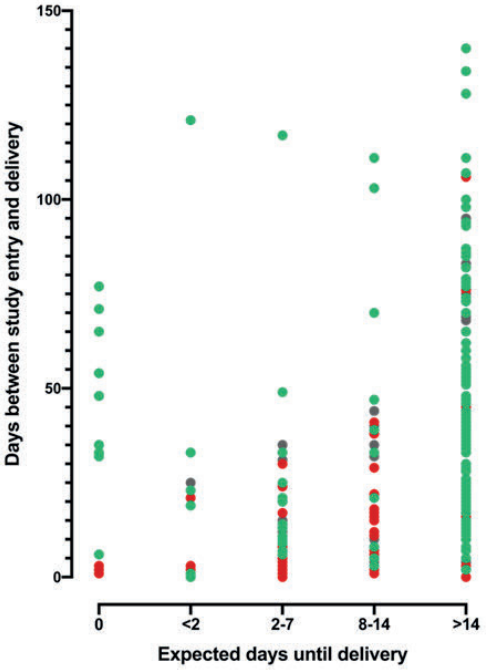

Figure 1. Expected number of days until delivery after inclusion predicted by physicians *vs* observed number of days from study entry until delivery in 323 women with suspected or confirmed pre-eclampsia, according to diagnosis at study entry. GH, gestational hypertension (grey circles); NHDP, no hypertensive disease of pregnancy (green circles); (S)PE, (superimposed) preeclampsia (red circles).

The predictions of the treating obstetricians for how long the pregnancy would continue were categorized as <2, 2-7, 8-14, >14 days (Figure 1, Table 5). The results show that obstetricians predict poorly the number of days until delivery. None of the obstetricians correctly predicted patients delivering immediately (0 days); all physicians thought that these patients would give birth at least one day later. In the group of patients who gave birth at 2-7 days, 8-14 days and >14 days after study entry, obstetricians were right in, respectively, 35% (18/51), 16% (10/61) and 69% (134/195) of cases.

Table 5. Expected number of days until delivery after inclusion predicted by obstetricians *vs* the observed number of days from study entry until delivery in women with suspected or confirmed pre-eclampsia.

Observed days until delivery	Expected days until delivery				
	0	**1**	**2 - 7**	**8 -14**	**>14**
0	0	4	1	0	1
1	2	3	4	1	
2 - 7	4	6	18	13	10
8 - 14	0	0	21	10	30
>14	9	6	20	26	134

Data of 323/384 women are shown in this table owing to missing forms.

DISCUSSION

We present the development and internal validation of a clinical model to predict a composite outcome of PE-related complications, consisting of maternal and fetal adverse events, within 7, 14 and 30 days, in women with suspected or confirmed PE. Continuous values of sFlt-1/PlGF ratio, PCR and gestational age at the time of blood sampling for biomarker measurements were strong predictors for the development of composite PE-related outcome. Internal validation, using bootstrap analysis, showed that the model provides excellent discrimination between pregnancies with a high or low risk of developing a complication. The resulting risk predictions for a complication within 7, 14 and 30 days after biomarker measurements can aid key in clinical decisions that are not addressed by existing single time point prognostic criteria.

The final model, consisting of gestational age, PCR and sFlt-1/PlGF ratio, for the prediction of a PE-related complication within 7 days, yielded a higher c-statistic than that achieved by other models investigated previously in this population. For clinical practice, we translated the final model into an easy to use prediction model (Figure S2). Our model is not limited to maternal surveillance but also addresses fetal risk associated with PE requiring delivery.

There has been a significant amount of research on the ability of the sFlt-1/PlGF ratio to predict the absence or presence of PE or PE-related complications. Different cut-off values of sFlt-1/PlGF ratio to rule in or to rule out PE and/or PE-related complications have been provided in previous studies.[10-13] We reported previously that the prediction of PE-related complications could be improved significantly by using continuous instead of dichotomous values of biomarkers.[7] Based on this finding, the sFlt-1/PlGF ratio is used as a continuous variable in our prediction model. This prediction model might be an aid for obstetricians to better predict which patients are at high risk of serious maternal and fetal PE-related complications, i.e. those who should be admitted to the obstetric ward, and which patients are at a low risk and can be followed at the outpatient clinic. This may reduce not only unnecessary preterm deliveries, antenatal admissions and fetal monitoring with false-positive diagnoses, but possibly also costs by limiting procedures performed due to diagnostic uncertainty. Of course, this should be balanced against the additional costs related to the measurement of the biomarkers.

For better assessment of its value, evaluation of our model in an external cohort would be worthwhile, but, until now, we have not been successful in doing so. If comparable c-statistics are achieved on external validation, we can conclude with more certainty that our model is generalizable to other populations of women with suspected or confirmed PE. Importantly, the patients on which our clinical prediction model is based are from one academic hospital and two general hospitals, providing a mix of more and less severe cases.

A crucial question is how our model can be used in clinical practice. After discussion with a group of obstetricians in our hospital, we concluded that patients with suspected or confirmed PE who have a calculated risk < 5% (sensitivity of 98% and thus a low false-negative rate), are at low risk of maternal and fetal complications, and can be followed safely at the out-patient clinic. We acknowledge that this threshold of <5% has been chosen arbitrarily. To establish that this threshold is valid and safe for use in clinical practice, we commenced a randomized intervention trial. In this trial, patients with suspected or confirmed PE are randomized to management according either to existing guidelines or to treatment based on the clinical prediction model. From this, it can be determined whether introduction of the prediction model is safe and a welcome aid for obstetricians in caring for patients with (suspected)PE and whether it results in fewer hospital admissions and additional investigations and measurements, with the potential of saving costs. As for its ease of use in a clinical setting, the prediction model in Excel is generally perceived to be convenient by the participating hospitals.

Strengths and limitations

A strength of this study is that we, for the first time, developed a model to predict a composite outcome of PE-related pregnancy complications that consists of maternal and fetal adverse outcomes, within 7, 14 and 30 days, in women with suspected or confirmed PE, rather than a model only for diagnosing PE. The current study also expands on former studies by the easy use of the prediction model in a clinical setting based on commercially available and fully automated immunoassays.

There are several limitations of this study, including the lack of external validation. External validation may ensure that predictions based on the developed clinical prediction model are generalizable to other populations. Second, our model is based on a relatively small sample size, which might have resulted in some degree of overfitting, despite our internal validation with bootstrap resampling. Furthermore, to be useful in clinical decision making for patients with suspected or confirmed PE presenting at the obstetric ward, it is important that the biomarkers can be measured instantaneously as a point of care measurement, which requires a dedicated clinical chemistry laboratory.

PERSPECTIVES

We present a multivariable prediction model with an additional tool to calculate the absolute risk of developing a composite outcome of PE-related pregnancy complications, consisting of maternal and fetal adverse events, in the subsequent 7, 14 and 30 days, in women with suspected or confirmed PE. This information can help physicians in identifying low risk patients who can be offered expectant management, leading to a decrease in the number and duration of admissions to the obstetric ward and potentially a reduction in costs, while simultaneously not compromising maternal and fetal health outcomes. Furthermore, we expect that implementation of the prediction model will improve timing of delivery, resulting in improved maternal and neonatal health.

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SUPPLEMENTARY DATA

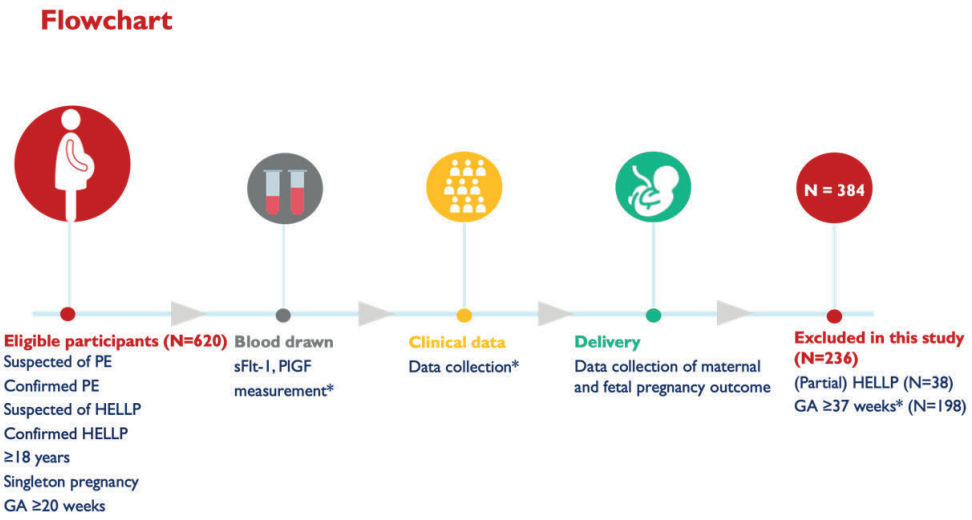

Figure S1. Flowchart of the study design. *Blood was drawn at study entry, but sFlt-1 and PlGF were measured after delivery to prevent any influence of this information on decision making. PE; preeclampsia, HELLP; Hemolysis Elevated Liver enzymes and Low Platelets syndrome, GA: gestational age at study entry.

Risk of pregnancy complications

Predictors in model	Value	Coef - 7 days	Coef - 14 days	Coef - 30 days
Intercept		0,07145825	0,1111437	0,1372515
Gestational age (weeks, days)	246	-1,18E-02	-1,18E-02	-1,18E-02
Protein-to-creatinine ratio	10	2,07E-01	2,07E-01	2,07E-01
sFlt-1/PlGF ratio	10,00	5,84E-01	5,84E-01	5,84E-01
Linear predictor multiplication		4,99623936	4,99623936	4,99623936
Shrink LP		4,546577818	4,546577818	4,546577818

Predicted probability of complication (7 days)	**2,6**
Predicted probability of complication (14 days)	**4**
Predicted probability of complication (30 days)	**4,9**

◂**Figure S2.** Details of the algorithm of the prediction model. (a) By entering the gestational age in weeks and days in the Excel calculator (which will automatically be converted to days in the algorithm), the protein-to-creatinine ratio (in mg/mmol) and the sFlt-1/PlGF ratio, a calculated percentage risk will be displayed for the development of PE-related maternal/fetal complications within 7, 14 and 30 days. (b–d) Example risk results for low protein-to-creatinine ratio of 10 and low sFlt-1/PlGF ratio of 10 (b), elevated protein-to-creatinine ratio of 38 and low sFlt-1/PlGF ratio of 10 (c) and low protein-to-creatinine ratio of 10 and elevated sFlt-1/PlGF ratio of 38 (d).

CHAPTER 03

PAPP-A2 and inhibin A as novel predictors for pregnancy complications in women with suspected or confirmed preeclampsia

Rugina I. Neuman, Maaike M. Alblas van der Meer, Daan Nieboer, Langeza Saleh, Koen Verdonk, Bhanu Kalra, Ajay Kumar, Kannan Alpadi, Anton H. van den Meiracker, Willy Visser, A.H. Jan Danser

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ABSTRACT

Background: We aimed to evaluate the value of inhibin A and PAPP-A2 (Pregnancy Associated Plasma Protein-A2) as novel biomarkers in the prediction of preeclampsia-related complications and how they compare with angiogenic biomarkers.

Methods and Results: Making use of a secondary analysis of a prospective, multicenter, observational study, intended to evaluate the usefulness of sFlt-1/PlGF ratio, we measured inhibin A and PAPP-A2 levels in 524 women with suspected/confirmed preeclampsia. Women had a median gestational age of 35 weeks (range, 20 – 41 weeks) while preeclampsia occurred in 170 (32%) women. Levels of inhibin A and PAPP-A2 were significantly increased in women with preeclampsia and in maternal perfusate of preeclamptic placentas. Inhibin A and PAPP-A2 (C-index = 0.73 and 0.75) significantly improved the prediction of maternal complications when added on top of the traditional criteria; gestational age, parity, proteinuria and diastolic blood pressure (C-index = 0.60). PAPP-A2 was able to improve the C-index from 0.75 to 0.77 when added on top of the sFlt-1/PlGF ratio for the prediction of maternal complications. To discriminate fetal/neonatal complications on top of traditional criteria, inhibin A and PAPP-A2 showed additive value (C-index = 0.79 to 0.80 and 0.82, respectively) but their discriminative ability remained inferior to that of sFlt-1/PlGF ratio or PlGF. Interestingly, the PAPP-A2/PlGF ratio alone showed remarkable value to predict pregnancy complications, being superior to sFlt-1/PlGF ratio in the case of maternal complications.

Conclusions: Inhibin A and PAPP-A2 show significant potential to predict preeclampsia-related pregnancy complications and might prove beneficial on top of the angiogenic markers.

INTRODUCTION

Preeclampsia is a multisystem disorder unique to pregnancy, characterized by the new onset of hypertension and proteinuria, intrauterine growth restriction or evidence of other end-organ damage occurring after 20 weeks gestation.[1, 2] Affecting 5 to 7% of all pregnant women, preeclampsia poses a great threat to maternal and fetal wellbeing worldwide.[3] Because the clinical presentation and course of preeclampsia can vary considerably, it remains important to identify those women at risk for developing severe complications such as eclampsia, pulmonary edema, HELLP syndrome, liver and kidney damage along with iatrogenic preterm birth, perinatal morbidity and mortality.[4] Since hypertension and proteinuria, the classical hallmarks of the disorder, have shown poor value to predict adverse outcomes[5], several biochemical markers are emerging to improve diagnostic tools applied to women with a clinical suspicion or diagnosis of preeclampsia.

The pathogenesis underlying preeclampsia remains uncertain, although it is well-recognized that placental ischemia triggers the release of placental factors into the maternal circulation, leading to the clinical syndrome of preeclampsia.[3] While certain placental factors, including the soluble Fms-like tyrosine kinase-1 (sFlt-1), placental growth factor (PlGF) or their ratio have been established as good candidate biomarkers for the prediction of preeclampsia or adverse outcomes[6, 7], other biomarkers are still being uncovered. In recent years, the circulating factors inhibin A and pregnancy associated plasma protein-A2 (PAPP-A2) have emerged as novel biomarkers for preeclampsia.[8-10] Inhibin A is a glycoprotein hormone belonging to the transforming growth factor family,[11] while PAPP-A2 is an insulin growth factor (IGF) binding protein protease thought to be involved in the regulation of IGF bioavailability[8]. Both inhibin A and PAPP-A2 are abundantly expressed in the placenta, and have been reported to be significantly elevated in the maternal circulation as well as placentas of pregnancies complicated by preeclampsia.[8, 12-15] Yet, current evidence regarding the role of these factors as biomarkers, particularly for the development of preeclampsia-related adverse outcomes remains scarce.

We hypothesized that inhibin A and PAPP-A2 could be better discriminating markers for the prediction of adverse outcome in women with suspected or confirmed preeclampsia than the angiogenic markers. In this secondary analysis, we examined the value of inhibin A and PAPP-A2 as predictors and compared their value to that of sFlt-1, PlGF and sFlt-1/PlGF ratio. In addition, we explored their levels in placental perfusate of healthy pregnant women versus women with an established diagnosis of preeclampsia.

METHODS

Study design and participants

This was a secondary analysis of a prospective cohort study involving women with suspected or confirmed preeclampsia enrolled between December 2013 through April 2016 at 3 Dutch hospitals (Erasmus Medical Center, Maasstad Hospital in Rotterdam and Reinier de Graaf Hospital in Delft) with the aim of evaluating the sFlt-1/PlGF ratio for the prediction of preeclampsia-related complications. All subjects provided written informed consent to participate in the study, which was approved by the local research ethics committee (MEC-2013-202). In- and exclusion criteria were described previously by Saleh *et al.*[6] Women with singleton pregnancies who had a confirmed clinical diagnosis of preeclampsia, or had symptoms such as hypertension, proteinuria, right upper quadrant abdominal pain, severe headache, visual disturbances, elevated liver enzymes or decreased platelet count were included in the study. Preeclampsia was defined according to the definition of the International Society for the Study of Hypertension in Pregnancy (ISSHP) of 2001 which was de novo hypertension (systolic blood pressure [SBP] of $\geq$ 140 and diastolic blood pressure [DBP] of $\geq$90 mm Hg) and proteinuria (protein-to-creatinine ratio $\geq$ 30mg/mmol or $\geq$300mg / 24h or 2+ dipstick) at or after 20 weeks of pregnancy or superimposed preeclampsia defined as chronic hypertension with the new onset of proteinuria or sudden increase of blood pressure or appearance of thrombocytopenia and increased liver enzymes or a sudden increase of proteinuria in patients with a pre-existing proteinuria. HELLP syndrome was defined as hemolysis, elevated liver enzymes and low platelet count, in the presence or absence of hypertension. Women with only suspicion of preeclampsia but without gestational hypertension were defined as suspected preeclampsia.

Data collection

For the analysis of sFlt-1 and PlGF, blood was taken at study entry only, and after centrifugation, serum was stored at -80°C until analysis. All samples were measured postpartum to avoid influence on decision making of the treating physicians.

Outcome measures

Patients diagnosed with (partial) HELLP at initial inclusion (n = 37) were excluded from the calculation of maternal complications. Maternal complications were defined as the development of one of the following after inclusion into the study; (partial) HELLP syndrome, eclampsia, pulmonary edema, subcapsular liver hematoma, cerebral hemorrhage/edema or infarction, visual disturbances, placental abruption, postpartum hemorrhage (blood loss $\geq$ 1000mL after delivery), and acute renal failure (absolute increase in the serum creatinine concentration of

≥ 0.3mg/dL [26.4umol/L] from baseline; ≥ 50% increase in serum creatinine; or oliguria with <0.5mL/kg per hour for a period of 6 hours).

Fetal/neonatal complications were defined as admittance to the neonatal intensive care unit (NICU); neonatal birth weight <10th percentile according to Perinatal Registration, The Netherlands; endotracheal intubation; intraventricular or intracranial hemorrhage; other intracerebral abnormalities; development of sepsis; respiratory distress syndrome; bronchopulmonary dysplasia defined as chronic lung disease developing in preterm neonates treated with oxygen and positive-pressure ventilation, with radiographic signs of inflammation and scarring, in need of artificial ventilation 4 weeks post-partum and at 36 weeks postmenstrual age; posthemorrhagic ventricular dilatation; periventricular leukomalacia, necrotizing enterocolitis and fetal or neonatal death. All patients (n = 524) were used for the calculation of fetal/neonatal complications.

Patient demographics, physical examination, laboratory test results, maternal and fetal/neonatal complications (diagnosed by treating physicians) were obtained from patient's electronic medical records and ascertained by two independent researchers.

Perfusion studies

Placental perfusate samples were obtained from previously conducted placental perfusion experiments in which transplacental drug transfer was evaluated. These perfusion experiments were previously described by Hitzerd *et al*.[16, 17] Perfusion experiments were conducted in healthy placentas and preeclamptic placentas. In brief, maternal and fetal perfusion media consisted of Krebs-Henseleit buffer at 37°C, supplemented with heparin (final concentration; 2500 IU/L) and aerated with 95% O_2 - 5% CO_2. The fetal circulation (closed circuit; flow rate, 6 mL/minute) was established by cannulating the chorionic artery and corresponding vein of an intact cotyledon. Maternal circulation (closed circuit; flow rate 12 mL/minute) was created by placing four blunt cannulas in the intervillous space. At t=0, at a concentration of ~10× C_{max}, either endothelin receptor antagonists (ERA), PDE-5 inhibitor sildenafil or no drug as a control were added to the maternal circulation to verify transfer to the fetal circulation.[16, 17] These high concentrations were chosen to prevent underestimation of transfer. Samples of the maternal and fetal circulations were taken every 30 min until the end of the experiment (180 min) for the determination of biomarker concentrations, and stored immediately at −80°C.

Biochemical measurements

Serum levels of sFlt-1 and PlGF were measured in 524 samples, using an automated analyzer (Cobas 6000, e-module; Roche Diagnostics, Mannheim, Germany), as described previously[6]. Analysis of sFlt-1 and PlGF in placental perfusate was also performed by an automated

analyzer (Cobas 6000, e module; Roche Diagnostics, Rotterdam, the Netherlands). ELISAs (AL-123 and AL-109) from Ansh Labs, Texas, USA were used to determine the levels of inhibin A and PAPP-A2. Maternal perfusate was diluted 1:100 for PAPP-A2 and 1:5 for inhibin A, while fetal perfusate was run undiluted. Serum was diluted 1:80 for PAPP-A2 and 1:4 for inhibin A. Greater dilutions were applied in case levels were above the highest standard. The analytical characteristics of the ELISAs have been published elsewhere.[18-20] The coefficients of variation for inhibin A (determined at concentrations of 101 and 345 pg/mL) and PAPP-A2 (determined at concentrations of 1.03 and 3.13 ng/mL) were 4.7 and 3.4% and 4.3 and 3.7%, respectively. The samples were blinded to the personnel running the assays.

Statistical analysis

Data are reported as median with interquartile range for continuous variables and as number with percentage for categorical variables. The normality of continuous variables was assessed using the Shapiro-Wilk W test. For the comparison of continuous variables between more than two groups, one-way ANOVA or Kruskall-Wallis test in the case of non-normal distribution was applied with a Dunnet or Bonferroni correction for multiple testing. For the comparison of categorical variables between two or more groups, Fisher's exact and X^2 (Chi-square) were applied. Logistic regression analysis was used to study the association between the dichotomous outcomes (maternal and fetal/neonatal complications) and the novel biomarkers and traditional predictors. Traditional predictors concerned gestational age (GA) at biomarker measurement, parity, proteinuria (urinary protein-to-creatinine ratio) and DBP. Due to a high correlation between SBP and DBP, SBP was not included in the model. PAPP-A2 was divided by PlGF to generate the PAPP-A2 to PlGF ratio. The markers sFlt-1, PlGF, sFlt-1/PlGF ratio, inhibin A, PAPP-A2 and PAPP-A2/PlGF ratio were assessed either alone or added to the traditional predictors, and were assessed in all women or women with a GA <37 weeks.

To test the added value of new markers on top of the sFlt-1/PlGF ratio (or PlGF) we fitted a logistic regression model containing sFlt-1/PlGF ratio (or PlGF) and a logistic regression model containing both sFlt-1/PlGF ratio (or PlGF) and either PAPP-A2 or inhibin A. PAPP-A2 or inhibin A were considered to have additional value if the likelihood ratio test comparing both models was statistically significant.

To assess the discriminative ability of the prediction models we used the C-index, which is equivalent to the area under the ROC curve for dichotomous outcomes. SPSS Statistics 21 (IBM Corporations) and R Software were used for the statistical analysis.

Table 1. Patient Characteristics According to Clinical Diagnosis.

Parameter	Suspected PE (n = 249)	GH (n = 105)	Preeclampsia/HELLP (n = 170)
Age, years	31 (27 - 35)	30 (27 - 34)	32 (28 - 36)[b]
Gestational age, weeks	35 (31- 38)	36 (34 - 38)	33 (29 - 36)[a,b]
Nulliparous, n (%)	136 (55)	70 (67)	101 (59)
Current smoker, n (%)	16 (7)	6 (6)	7 (4)
Race, n (%)			
White	175 (70)	82 (78)	105 (62)[b]
Black	36 (15)	12 (11)	31 (18)
Other	38 (15)	11(11)	34 (20)
Antihypertensives use, n (%)	51 (21)	29 (28)	100 (59)[a,b]
History of PE, n (%)	36 (15)	9 (9)	27 (16)
Preexisting hypertension, n (%)	58 (23)	0 (0)[a]	39 (23)[b]
Preexisting proteinuria, n (%)	12 (5)	0 (0)	7 (4)
Clinical findings at time of admission			
SBP, mmHg	130 (120 - 138)	145 (140 - 150)[a]	143 (130 - 154)[a]
DBP, mmHg	82 (75 - 89)	91 (85 - 97)[a]	90 (85 - 98)[a]
uPCR, mg/mmol	17 (11 - 27)	16 (11 - 21)	57 (36 - 219)[a,b]
LD, U/L	178 (159 - 205)	189 (166 - 210)	216 (183 - 279)[a,b]
ALT, U/L	14 (10 - 19)	14 (11 - 18)	19 (12 - 46)[a,b]
Creatinine, µmol/L	55 (50 - 62)	59 (54 - 66)[a]	61 (54 -72)[a]
Uric acid, mmol/L	0.27 (0.23 - 0.32)	0.29 (0.24 - 0.34)	0.33 (0.27 - 0.39)[a,b]
Platelet count, 10^9/L	238 (188 - 279)	227 (180 - 276)	211 (160 - 254)[a,b]
sFlt-1, pg/mL	3140 (1834 - 5207)	4902 (2394 - 7226)[a]	5641 (1870 - 10382)[a]
PlGF, pg/mL	189 (112 - 361)	110 (71 - 211)[a]	73 (33 - 132)[a,b]
sFlt-1/PlGF ratio	18 (6 - 40)	41 (15 - 87)[a]	71 (22 - 272)[a,b]
Inhibin A, pg/mL	1165 (595 - 1965)	1484 (862 - 2606)[a]	2248 (1374 - 4071)[a,b]
PAPP-A2, ng/mL	151 (75 - 300)	281 (145 - 471)[a]	380 (171 - 555)[a]
Pregnancy Outcomes			
Sex (M/F), n (%)	129 / 120 (52 / 48)	49 / 56 (47 / 53)	91 / 79 (54 / 46)
Gestational age at birth, weeks	38 (37 - 40)	38 (37 - 39)	36 (30 - 37)[a,b]
Birth weight, grams	3275 (2832 - 3658)	3140 (2646 - 3541)	2218 (1158 - 3173)[a,b]
Birth weight percentile <10, n (%)	23 (9)	17 (16)	36 (21)[a]
Time until delivery, days	19 (9 - 41)	9 (3 - 21)[a]	3 (1 - 13)[a,b]

Values are median (interquartile range) or number (%). *a* indicates comparison with suspected PE at a significance level of p<0.05; *b* indicates comparison with GH at a significance level of p<0.05. PE indicates preeclampsia; GH, gestational hypertension; HELLP, hemolysis, elevated liver enzymes, low platelet count; SBP, systolic blood pressure; DBP, diastolic blood pressure; uPCR, urinary protein-to-creatinine ratio, LD, lactate dehydrogenase; ALT, alanine aminotransferase; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; M, male; F, female.

RESULTS

Patient demographics according to clinical diagnosis

In this secondary analysis, 524 women with a median age of 31 (27 - 35) were included (Table 1). Of these women, 249 (48%) had suspected preeclampsia, 105 (20%) had gestational hypertension and 170 (32%) met the clinical criteria for preeclampsia and/or HELLP syndrome. Median GA at inclusion was 35 weeks of which 205 (39%) women were below 34 weeks. Women with preeclampsia/HELLP syndrome displayed higher SBP and DBP, more proteinuria and higher levels of sFlt-1/PlGF ratio in comparison to women only suspected of PE. As expected, women with preeclampsia/HELLP syndrome delivered earlier, while their newborns were more premature (GA < 34 weeks) and had lower birth weight percentiles. In total, 68 maternal complications (in some cases; ≥ 1 complication) developed in 64 (13%) women after inclusion, while 206 (39%) of all pregnancies had one or more fetal / neonatal complications (Table S1).

Inhibin A and PAPP-A2 according to clinical diagnosis

Women with preeclampsia/HELLP syndrome displayed higher levels of inhibin A (2248 [1374 – 4071] versus 1165 [595 -1965] pg/mL) and PAPP-A2 (380 [171 – 555] versus 151 [75 – 300] ng/mL) when compared to women only suspected of preeclampsia (Table 1 and Figure 1).

Inhibin A and PAPP-A2 levels in placental perfusate

Eleven healthy and four women with preeclampsia were included in the placental studies. Their clinical characteristics are depicted in Table 2. All healthy women underwent elective caesarean section due to previous caesarean section. All preeclampsia patients underwent a caesarean section because of maternal illness and fetal distress. As expected, the placentas from preeclamptic pregnancies were born at an earlier GA (< 34 weeks) and were associated with higher maternal DBP, lower birth weight and lower placental weight. In total, 12 healthy cotyledons (one placenta yielded two cotyledons) were utilized and perfused with either endothelin receptor antagonists (n = 6), sildenafil (n = 3) or no drug (n = 3). The data on the maternal-to-fetal transfer of these drugs have been reported before.[16, 17] Since no difference in biomarker levels was observed between the different drugs in the healthy placentas, all results were combined. Two of the four experiments with preeclamptic placentas were stopped after 90 min of perfusion due to fetal-to-maternal leakage. As shown in Figure 2, the concentrations of inhibin A, PAPP-A2, sFlt-1 and PlGF gradually increased with time in the maternal perfusate. The biomarkers were not detectable in the fetal perfusate (data not shown). The biomarkers PAPP-A2, inhibin A and sFlt-1 showed increased levels in all 4 preeclamptic placental perfusates when compared with the healthy placentas. With regard to PlGF, biomarker levels were within the low range of levels in healthy placentas. Results remained unchanged after adjustment for cotyledon weight (data not shown).

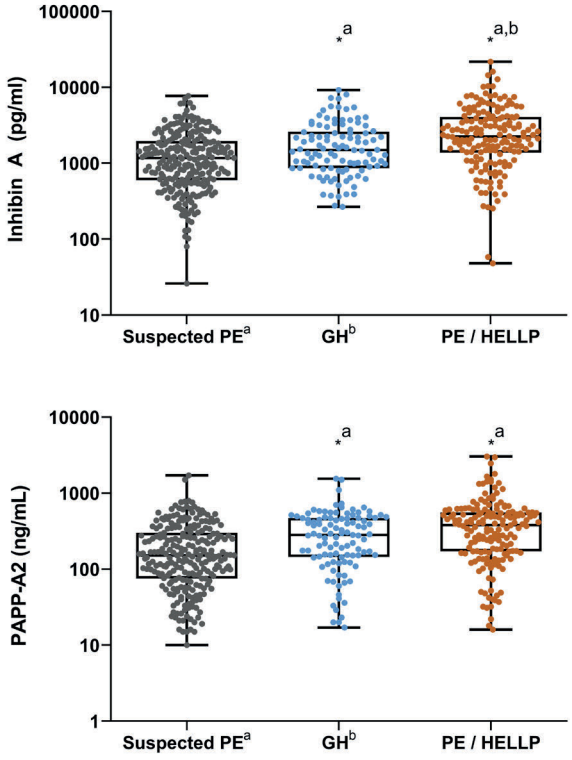

Figure 1. Inhibin A and PAPP-A2 levels in 524 women according to clinical diagnosis.
GH indicates gestational hypertension; HELLP; hemolysis, elevated liver enzymes and low platelets *indicates *P*<0.05 versus the other groups (a, versus suspected preeclampsia; b, versus GH); *a* indicates significance at *P*<0.05 level when compared with suspected preeclampsia; *b* indicates significance at *P*<0.05 level when compared with GH.

Table 2. Characteristics of healthy and preeclamptic placentas used for determination of biomarkers in placental perfusion studies.

	Healthy	PE
N	11	4
Maternal age (years)	34 (28 - 36)	31 (29 -33)
Gestational age (weeks, days)	39.0 (38.5 - 39.1)	31.4 (31.2 - 31.6)
Nulliparity, n	0	3
Current smoker, n	1	0
Caucasian ethnicity, n	6	3
Highest DBP (mmHg)	80 (73 - 80)	110 (107 - 113)
Fetal sex (M/F)	5/6	3/1
Birth weight (grams)	3580 (3338 - 3815)	1138 (1119 - 1228)
Birth weight (centile)	61 (53 - 85)	3 (0 - 6)
Placental weight (grams)	659 (622 - 753)	333 (318 - 341)

Values are median (interquartile range) or number. PE indicates preeclampsia; GA, gestational age: DBP, diastolic blood pressure, M, male; F, female.

Correlations between inhibin A and PAPP-A2 with the angiogenic markers

Inhibin A and PAPP-A2 were positively correlated with sFlt-1 (r = 0.51 and r = 0.59, respectively) and showed a negative correlation with PlGF (r = - 0.43 and r = - 0.51, respectively) (Table S2).

Prediction of maternal complications

In univariable analysis, PAPP-A2 showed the highest ability to discriminate between women with and without maternal complications when compared to inhibin A (C-index = 0.71 versus 0.69), but was inferior to PlGF and sFlt-1/PlGF ratio (Table 3). A combination of PAPP-A2 divided by PlGF (PAPP-A2 / PlGF ratio) showed the highest value in univariable analysis to predict maternal complications, even when compared to the sFlt-1/PlGF ratio. When inhibin A, PAPP-A2 and PAPP-A2/PlGF ratio were added to a model with traditional predictors (traditional model), the C-index improved from 0.60 for a model without biomarkers to 0.73, 0.75 and 0.76, respectively, in comparison to 0.72, 0.73 and 0.77 for sFlt-1, PlGF and sFlt-1/PlGF ratio. When restricting the univariable analysis to women with a GA below 37 weeks, the PAPP-A2/PlGF ratio showed the highest ability to predict maternal complications, followed by PlGF, sFlt-1/PlGF ratio and PAPP-A2 (Table 3). Multivariable analysis in this group was not performed, due to the limited number of maternal complications. When PAPP-A2 was added on top of the sFlt-1/PlGF ratio alone, the C-index significantly improved (Table S3).

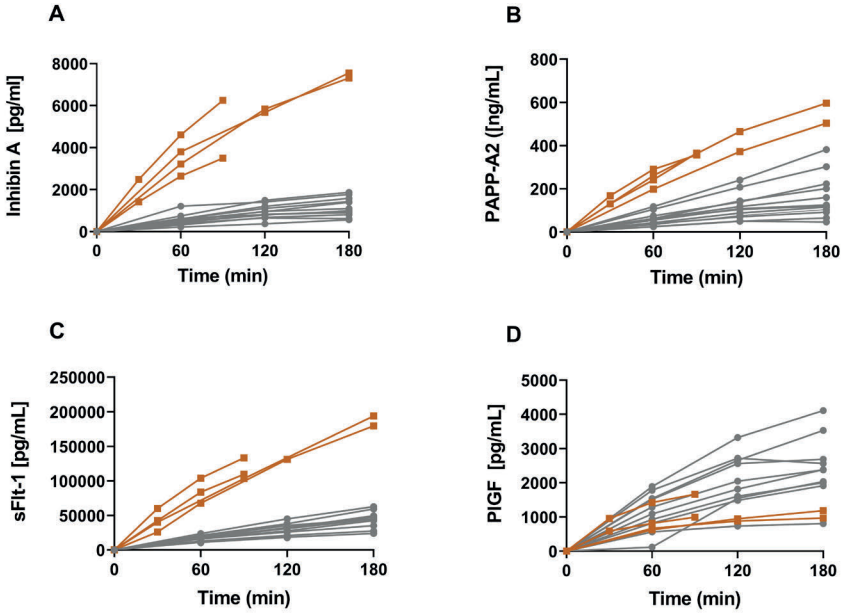

Figure 2. Placental perfusate levels of inhibin A (A), PAPP-A2 (pregnancy-associated plasma protein-A2) (B), sFlt-1 (C), and placental growth factor (D) on the maternal side in healthy placentas (grey circle) and preeclamptic placentas (orange squares). PAPP-A2 indicates pregnancy-associated plasma protein-A2; PlGF, placental growth factor; and sFlt-1, soluble Fms-like tyrosine kinase-1.

Table 3. Associations between Maternal Complications (n = 64) and Biomarkers in Women With Suspected or Confirmed Preeclampsia Without Hemolysis, Elevated Liver Enzymes, Low Platelet count Syndrome at Time of Inclusion (n = 487) and restricted to GA <37 weeks (n = 309)

Model/Biomarker	Univariable		Multivariable	
	Odds Ratio	C-Index	Odds Ratio	C-Index
Traditional model				0.60
sFlt-1	1.9 (1.4 - 2.5)	0.69	1.9 (1.5 - 2.5)	0.72
PlGF	0.2 (0.1 - 0.4)	0.73	0.2 (0.1 - 0.5)	0.73
sFlt-1/PlGF ratio	5.3 (2.9 - 9.9)	0.75	5.9 (3.1 - 11)	0.77
Inhibin A	3.9 (2.0 - 7.8)	0.69	6.3 (2.9 - 13)	0.73
PAPP-A2	4.5 (2.3 - 9.1)	0.71	7.8 (3.6 - 17)	0.75
PAPP-A2/PlGF ratio	5.9 (2.9 - 12)	0.76	6.4 (3.1 - 13)	0.76
GA at inclusion < 37 weeks (n =309)	Odds Ratio		C-index	
sFlt-1	1.9 (1.4 - 2.5)		0.70	
PlGF	0.1 (0.0 - 0.4)		0.80	
sFlt-1/PlGF Ratio	5.6 (2.9 - 11)		0.80	
Inhibin A	5.4 (2.2 - 13)		0.74	
PAPP-A2	10 (3.5 - 25)		0.79	
PAPP-A2/PlGF ratio	18 (7.0 - 48)		0.85	

Traditional model consists of gestational age at time of biomarker measurement, parity, diastolic blood pressure and proteinuria at inclusion. Multivariable includes traditional model with one of the biomarkers. Interquartile odds ratio and associated 95% confidence interval was calculated to aid interpretation of continuous predictors. It is defined as comparing the risk of maternal complications at the 75th percentile of the marker value versus the 25th percentile. GA indicates gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; PAPP-A2, pregnancy associated plasma protein-A2.

Prediction of fetal / neonatal complications

For the prediction of fetal/neonatal complications, inhibin A and PAPP-A2 showed poor discriminative ability with C-indices of 0.63 and 0.64, but the PAPP-A2 / PlGF ratio yielded a C-index of 0.74. When added on top of traditional predictors, the C-index increased from 0.79 for the traditional model only, to 0.80, 0.82 and 0.83 for inhibin A, PAPP-A2 and PAPP-A2/PlGF ratio, respectively. This implies that PAPP-A2 and PAPP-A2/PlGF ratio reached a value almost similar to that of PlGF and sFlt-1/PlGF ratio (Table 4). Restricting the calculations to women with GA < 37 weeks improved the predictive value of all biomarkers in multivariable analysis, with PAPP-A2 and PAPP-A2/PlGF ratio reaching the highest value when compared to inhibin A (C-index = 0.85 and 0.85 versus 0.81), but not when compared to sFlt-1/PlGF ratio (C-index = 0.86) or PlGF alone (C-index = 0.87) (Table 4). Inhibin A had added value beyond PlGF in predicting fetal/neonatal complications, however, the increase in discriminative ability was negligible (Table S4).

DISCUSSION

In this secondary analysis, we evaluated inhibin A and PAPP-A2 as novel biomarkers to predict adverse pregnancy outcome in women with suspected or confirmed preeclampsia, and compared their predictive value to that of the established angiogenic markers sFlt-1, PlGF and sFlt-1/PlGF ratio. We found that PAPP-A2 showed the highest value of the two biomarkers to predict maternal and fetal/neonatal complications, particularly when added to a model with traditional clinical predictors (GA at biomarker measurement, DBP, protein-to-creatinine ratio and parity). Conversely, inhibin A was a relatively weak predictor in univariable analysis, but showed additive value when added on top of traditional variables for the prediction of maternal complications. When compared to the angiogenic markers, PAPP-A2 performed nearly as well as the sFlt-1/PlGF ratio in multivariable analysis to predict maternal complications, while in the case of fetal/neonatal complications the two biomarkers showed a predictive value marginally inferior to that of sFlt-1/PlGF ratio or PlGF. Strikingly, when we incorporated the ratio of PAPP-A2/PlGF ratio in our prediction model, this model performed even better (univariable analysis), if not similar (multivariable analysis) to the sFlt-1/PlGF ratio to predict maternal complications, whereas for predicting fetal/neonatal complications, PAPP-A2/PlGF ratio showed similar value to that of PlGF or sFlt-1/PlGF ratio in multivariable analysis.

Few studies have been performed to investigate inhibin A and PAPP-A2 as biomarkers, while most of them focused on preeclampsia diagnosis rather than adverse pregnancy outcome. For inhibin A, its predictive value has mostly been studied in early pregnancy, around 11 to 18 weeks' gestation. Whereas some studies reported a weak value of inhibin A as a biomarker for preeclampsia diagnosis,[21-23] others have shown area under the curve (AUC) values ranging between 0.71 and 0.79, suggesting it was a relatively good predictor, either alone or in the presence of other maternal biomarkers.[10, 24] Yet, inhibin A remained inferior to sFlt-1 and PlGF, in agreement with our observations.[10, 21, 24] PAPP-A2 has been studied less extensively than inhibin A, although it has gained increasing interest over the past years. Early studies reported elevated PAPP-A2 levels both before and during the clinical onset of preeclampsia,[8, 25] which we were able to confirm in a larger cohort of women. Consequently, previous studies hypothesized that PAPP-A2 could be a potential biomarker in women with preeclampsia to predict adverse outcomes, and to our knowledge we are the first to investigate this concept.

When we limited our analysis to women with GA <37 weeks, the predictive value of the novel biomarkers substantially increased, with PAPP-A2 and PAPP-A2/PlGF ratio showing the highest increase in C-index, suggesting that PAPP-A2 might be a better predictive biomarker when measured earlier in pregnancy. In a study by Kramer *et al*.,[13] placental expression of PAPP-A2 was downregulated in the second trimester of healthy pregnancy, which might explain the higher predictive value when PAPP-A2 is elevated. Likewise, circulating inhibin A

levels have shown to remain relatively low in the second trimester of a healthy pregnancy.[26] Due to the limited number of maternal complications, we were unable to investigate whether this was also true for women with a GA less than 34 weeks.

Table 4. Associations between fetal / neonatal complications and biomarkers in all women (n = 524) and restricted for GA< 37 weeks (n = 343).

Model/Biomarker	Univariable		Multivariable	
	Odds Ratio	C-Index	Odds Ratio	C-index
Traditional model				0.79
sFlt-1	1.9 (1.5 - 2.4)	0.65	1.9 (1.5 - 2.6)	0.81
PlGF	0.1 (0.0 - 0.2)	0.77	0.2 (0.0 - 0.3)	0.83
sFlt-1/PlGF Ratio	2.2 (1.8 - 2.8)	0.74	2.2 (1.6 - 2.9)	0.83
Inhibin-A	1.6 (1.3 - 1.9)	0.63	1.6 (1.2 - 2.0)	0.80
PAPP-A2	2.2 (1.5 - 3.2)	0.64	4.9 (2.9 - 8.3)	0.82
PAPP-A2 / PlGF ratio	2.8 (2.1 - 3.5)	0.74	2.3 (1.8 - 3.1)	0.83
GA at inclusion <37 weeks (n = 343)				
Traditional model				0.78
sFlt-1	2.5 (1.8 - 3.5)	0.70	2.7 (1.8 - 4.2)	0.83
PlGF	0.0 (0.0 - 0.1)	0.83	0.1 (0.0 - 0.1)	0.87
sFlt-1/PlGF Ratio	3.9 (2.5 - 6.4)	0.80	4.3 (2.4 - 7.6)	0.86
Inhibin A	2.1 (1.5 - 2.8)	0.68	2.1 (1.5 - 3.1)	0.81
PAPP-A2	4.2 (2.6 - 6.8)	0.70	10 (5 - 21)	0.85
PAPP-A2 / PlGF ratio	5.6 (3.5- 9.1)	0.80	4.6 (2.7 - 7.9)	0.85

Traditional model consists of gestational age at time of biomarker measurement, parity, diastolic blood pressure and proteinuria at inclusion. Multivariable includes traditional model with one of the biomarkers. Interquartile odds ratio and associated 95% confidence interval was calculated to aid interpretation of continuous predictors. It is defined as comparing the risk of fetal/neonatal complications at the 75th percentile of the marker value versus the 25th percentile. GA indicates gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; PAPP-A2, pregnancy associated plasma protein-A2.

To further establish the etiology of these biomarkers, we measured their levels along with those of sFlt-1 and PlGF in maternal and fetal placental perfusate. While none of the biomarkers were detectable in fetal perfusate, the biomarkers sFlt-1, inhibin A and PAPP-A2 were all elevated in the maternal perfusate of preeclamptic placentas in comparison to healthy placentas. This demonstrates that these biomarkers originate maternally, and that their synthesis is upregulated in preeclampsia. Median serum levels of these biomarkers during preeclampsia amount up to ~5641 (range 696 – 83967) pg/mL, 2248 (range 48– 21696) pg/mL and 380 (range 16 – 3024) ng/mL for sFlt-1, inhibin A and PAPP-A2, respectively (Table 1). When comparing our cotyledon effluent levels with these levels (Figure 2), it is likely that placental release of these three biomarkers has contributed to the increased biomarker levels in the maternal circulation during preeclampsia. The >99% drop of sFlt-1 after birth confirms this view.[27]

As expected, maternal perfusate levels of PlGF were not elevated nor markedly reduced in preeclamptic placentas when compared to healthy placentas, indicating its downregulation in preeclampsia is predominantly caused by additional factors such as the binding of excess circulating sFlt-1. Importantly, caution is granted when interpreting these findings, since the number of preeclamptic placentas remains relatively small. Moreover, it was not possible to match these placentas for gestational age, which might affect the effluent levels observed. However, this reflects the reality that successfully perfusing preterm placentas, particularly from preeclamptic patients, remains extremely difficult.

The fact that both inhibin A and PAPP-A2 were also positively correlated with sFlt-1 in the maternal circulation, raises the question whether similar mechanisms account for their upregulation during preeclampsia. Indeed, in a study by Macintire *et al.*[28], PAPP-A2 expression in placental explants was significantly upregulated during hypoxia, a well-known trigger for sFlt-1 synthesis and secretion[3], while another study has shown that inhibin A expression in differentiated cytotrophoblasts is upregulated by hypoxia inducible factor.[29] Nevertheless, PAPP-A2 did display added value on top of the sFlt-1/PlGF ratio to predict maternal complications, suggesting that there may still be additional regulatory mechanisms. Our observation that the PAPP-A2/PlGF ratio showed even better value than the sFlt-1/PlGF ratio alone to predict maternal complications, also agrees with this concept. Hence, these novel biomarkers, particularly PAPP-A2, might improve risk prediction models in which the sFlt-1/PlGF ratio is already included. Comparing the predictive value of PAPP-A2/PlGF ratio versus the sFlt-1/PlGF ratio is an interesting area for future research.

The present study has limitations. Since this is a secondary analysis, future prospective trials are necessary to validate our findings. Here, determining specific thresholds and estimating the sensitivity, specificity, negative and positive predictive values of these novel biomarkers might be of use to further estimate their predictive performance. Also, we were not able to assess the predictive value of these biomarkers in early pregnancy, since most women were already diagnosed with preeclampsia and in ~60% of women blood was taken at $\geq$34 weeks GA. Lastly, we did not perform repeated measurements of the biomarkers during pregnancy, which could have given more insight into their predictive value.

PERSPECTIVES

Our data illustrate that inhibin A and PAPP-A2 are not only good alternate biomarkers for the prediction of adverse pregnancy outcome, but could have additional value on top of the well-known angiogenic factors (sFlt-1, PlGF and their ratio). Moreover, combining PAPP-A2 with PlGF (by calculating the PAPP-A2/PlGF ratio) might further improve prediction beyond the sFlt-1/PlGF ratio. These findings emphasize the need to investigate their value prospectively alone and combined with the established angiogenic markers.

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SUPPLEMENTAL DATA

Table S1. Occurrence of adverse maternal outcomes in women without (partial) HELLP at time of inclusion (n = 487) and fetal / neonatal outcome in all pregnancies (n = 524).

Parameter	n (%)
Maternal Complication	
Eclampsia	0 (0)
(Partial) HELLP syndrome	25 (5)
Placental abruption	2 (0.4)
Pulmonary edema	3 (0.6)
Renal insufficiency	2 (0.4)
Visual disturbances	2 (0.4)
Postpartum hemorrhage	34 (7)
All women with 1 or more complication	64 (13)
Fetal / Neonatal Complication	
Admission to NICU	135 (26)
Endotracheal intubation	38 (7)
Birth weight percentile <10	76 (15)
Intraventricular hemorrhage	5 (1)
Intracranial hemorrhage	1 (0.2)
Other intracerebral abnormalities	8 (2)
Sepsis	38 (7)
Respiratory distress syndrome	64 (12)
Bronchopulmonary dysplasia	11 (2)
Posthemorrhagic ventricular dilatation	1 (0.2)
Periventricular leukomalacia	3 (0.6)
Necrotizing enterocolitis	1 (0.2)
Fetal or neonatal death	20 (4)
All pregnancies with 1 or more complication	206 (39)

Values are number (percentage). Other intracerebral complications include stroke, cysts, developmental anomalies, meningitis and vasculopathy. HELLP syndrome indicates hemolysis, elevated liver enzymes, low platelet count; NICU indicates neonatal intensive care unit.

Table S2. Correlations between inhibin A and PAPP-A2 with the angiogenic markers.

Biomarker	sFlt-1	PlGF	sFlt-1/PlGF ratio
Inhibin A	0.512*	- 0.426*	0.540*
PAPP-A2	0.590*	- 0.510*	0.633*

sFlt-1 indicates soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; PAPP-A2, pregnancy associated plasma protein-A2. *P<0.01.

Table S3. Value of inhibin A and PAPP-A2 on top of sFlt-1/PlGF ratio to predict maternal complications in all women without HELLP syndrome at time of inclusion (n = 487).

Biomarkers	Univariable Odds Ratio	C-index	Multivariable Odds Ratio	C-index	P-Value
sFlt-1/PlGF ratio	5.3 (2.9 - 9.9)	0.75	3.7 (1.9 - 7.4)		0.0001
Inhibin A	3.9 (2.0 - 7.8)	0.69	2.2 (1.0 - 4.8)	0.76	0.10
sFlt-1/PlGF ratio	5.3 (2.9 - 9.9)	0.75	3.4 (1.6 - 7.2)		0.0001
PAPP-A2	4.5 (2.3 - 9.1)	0.71	2.1 (0.9 - 4.9)	0.77	0.01

Multivariable includes sFlt-1/PlGF ratio with either inhibin A or PAPP-A2. Interquartile odds ratio and associated 95% confidence interval was calculated to aid interpretation of continuous predictors.
It is defined as comparing the risk of maternal complications at the 75th percentile of the marker value versus the 25th percentile. sFlt-1 indicates soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; PAPP-A2, pregnancy associated plasma protein-A2.

Table S4. Value of inhibin A and PAPP-A2 to predict fetal/neonatal complications on top of PlGF in in all women (n = 524)

Biomarkers	Univariable Odds Ratio	C-index	Multivariable Odds Ratio	C-index	P-Value
PlGF	0.1 (0 - 0.2)	0.77	0.1 (0.1 - 0.2)		0.0001
Inhibin A	1.6 (1.3 - 1.9)	0.63	1.2 (1.0 - 1.5)	0.77	0.04
PlGF	0.1 (0 - 0.2)	0.77	0.1 (0.1 - 0.2)		0.0001
PAPP-A2	2.2 (1.5 - 3.2)	0.64	0.9 (0.6 - 1.6)	0.77	0.12

Multivariable includes PlGF with either inhibin A or PAPP-A2. Interquartile odds ratio and associated 95% confidence interval was calculated to aid interpretation of continuous predictors.
It is defined as comparing the risk of fetal/neonatal complications at the 75th percentile of the marker value versus the 25th percentile. sFlt-1 indicates soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; PAPP-A2, pregnancy associated plasma protein-A2.

CHAPTER 04

Copeptin and Mid-Regional pro-ANP in Women with Suspected or Confirmed Preeclampsia: Comparison with the sFlt-1/PlGF ratio

Rugina I Neuman, Maaike Alblas van der Meer, Langeza Saleh, Sjoerd A.A van den Berg, Anton H. van den Meiracker, A.H. Jan Danser, Willy Visser

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ABSTRACT

Objectives: Arginine vasopressin (AVP) and atrial natriuretic peptide (ANP) may contribute to the pathogenesis of preeclampsia, but their role remains to be elucidated. We aimed to evaluate their role as biomarkers and whether they associate with angiogenic markers and/or clinical manifestations of preeclampsia.

Methods: We measured their surrogates C-terminal pro-AVP (Copeptin) and mid-regional pro-ANP (MR-proANP) making use of a retrospective analysis of a prospective cohort study involving 526 women with suspected or confirmed preeclampsia, originally aimed to evaluate the use of soluble Fms-like tyrosine kinase-1/Placental growth factor (sFlt-1/PlGF) ratio.

Results: Women with preeclampsia displayed elevated serum copeptin and MR-proANP in comparison with women suspected of preeclampsia. To discriminate maternal complications on top of traditional predictors (gestational age, parity, diastolic blood pressure and proteinuria), the sFlt-1/PlGF ratio displayed a higher C-index than copeptin and MR-proANP (0.76, 0.63 and 0.67, respectively, vs. 0.60 for the traditional predictors only), and the same was true for the discrimination of fetal/neonatal complications (C-index = 0.83, 0.79 and 0.80 vs. 0.79). When subdividing women according to sFlt-1/PlGF ratio ($\geq$ 85 versus $<$85), no differences in copeptin levels were observed, while MR-proANP levels was elevated in women with a ratio $\geq$ 85. Multiple regression analysis revealed that copeptin and MR-proANP were independent determinants of proteinuria.

Conclusion: Copeptin and MR-proANP have limited value to predict preeclampsia-related complications when compared with the sFlt-1/PlGF ratio. However, our data suggests that both copeptin and MR-proANP contribute to the occurrence of proteinuria, with copeptin exerting this effect independently of sFlt-1/PlGF ratio.

INTRODUCTION

Preeclampsia (PE) is a heterogeneous, pregnancy-specific disorder that is clinically characterized by new onset hypertension and proteinuria or other organ damage after 20 weeks' gestation.[1] Severe complications arise in a substantial proportion of women, leading to significant maternal and fetal morbidity and mortality worldwide.[1, 2] Because most pathogenic mechanisms underlying PE are not fully elucidated,[2] identifying biomarkers that allow early prediction of adverse pregnancy outcome remains challenging. In recent years, a complex interplay between placental and cardiovascular factors has been suggested to underlie the pathogenesis of PE,[3] and as such, increasing interest has emerged in a role for arginine vasopressin (AVP) and atrial natriuretic peptide (ANP) as cardiovascular biomarkers in PE. AVP, also known as antidiuretic hormone, is primarily involved in osmotic homeostasis.[4] Copeptin is the stable C-terminal part of the AVP precursor and as such better reflects AVP activity than the less stable AVP itself.[5] ANP is a vasodilatory peptide with diuretic and natriuretic effects, predominantly released after atrial distension. The stable prohormone fragment of ANP, mid-regional pro-ANP (MR-proANP), is released in equimolar amounts to ANP.[6] While evidence regarding their exact pathophysiological role is limited, elevated circulating copeptin and MR-proANP levels have been reported in women with PE, while some studies have shown promising results regarding their role in early PE prediction.[6-10] However, information about their value in the prediction of adverse maternal and fetal outcome is lacking.

A well-recognized hallmark of PE is the presence of an antiangiogenic state reflected by elevated soluble Fms-like tyrosine kinase-1 (sFlt-1) and reduced placental growth factor (PlGF) in the maternal circulation, leading to an increased sFlt-1/PlGF ratio.[1]

We recently reported that the determination of sFlt-1, PlGF or their ratio have substantial value to predict maternal and fetal/neonatal complications in women with suspected or confirmed PE on top of traditional predictors.[11] In the present study, we evaluated whether copeptin and MR-proANP are useful cardiovascular biomarkers to predict preeclampsia-related pregnancy complications and how their predictive value compares to that of the angiogenic markers. In addition, we explored whether the new biomarkers were related to blood pressure and proteinuria, two clinical hallmarks of PE, although according to new guidelines proteinuria is no longer required to diagnose PE.

METHODS

Study design and participants

This is a secondary retrospective analysis of a prospective cohort study that enrolled women with either suspected or confirmed PE between December 2013 through April 2016 at three Dutch hospitals (Erasmus Medical Center and Maasstad Hospital in Rotterdam and Reinier de Graaf Hospital in Delft) originally aimed to evaluate the sFlt-1/PlGF ratio for the prediction of PE-related complications. All subjects provided written informed consent to participate in the study, which was approved by the local research ethics committee (MEC-2013-202). The inclusion criteria were described previously by Saleh *et al*.[11] In short, women with singleton pregnancies who had a confirmed diagnosis of PE, or had PE symptoms such as hypertension, proteinuria, right upper quadrant abdominal pain, severe headache, visual disturbances, elevated liver enzymes or decreased platelet count were included. PE was defined according to the definition of the International Society for the Study of Hypertension in Pregnancy (ISSHP) of 2001 which was de novo hypertension (systolic blood pressure [SBP] of $\geq$ 140 and/or diastolic blood pressure [DBP] of $\geq$ 90 mm Hg) and proteinuria (protein-to-creatinine ratio $\geq$ 30mg/mmol or $\geq$300mg / 24h or 2+ dipstick) at or after 20 weeks of pregnancy. Superimposed PE was diagnosed in women with chronic hypertension with new onset of proteinuria or a sudden increase of blood pressure or appearance of thrombocytopenia and increased liver enzymes or a sudden increase of proteinuria in patients with a pre-existing proteinuria. HELLP syndrome was defined as hemolysis, elevated liver enzymes and low platelet count, in the presence or absence of hypertension. Partial HELLP was defined as having at least two HELLP criteria. The group with only suspicion of PE, but without gestational hypertension, was defined as no hypertensive disease of pregnancy. Women who had a fetus with chromosomal or congenital anomalies were excluded from the study.

Data collection

Blood was taken at study entry only, and after centrifugation, serum was stored at -80°C to be analyzed later. Measurement of sFlt-1 and PlGF was originally performed in 620 samples, using an automated analyzer (Cobas 6000, e module; Roche Diagnostics, Mannheim, Germany) as described previously.[11] Serum copeptin and MR-proANP were measured in 526 of the 620 samples and were analyzed on a BRAHMS Kryptor instrument (Thermo Fisher Scientific, BRAHMS GmbH.,Henningsdorf, Germany).

Clinical findings, physical examination, laboratory test results, maternal, fetal and neonatal complications (diagnosed by treating physicians), and background data were obtained from electronical medical records and ascertained by two independent researchers.

Because the development of (partial) HELLP was considered a maternal complication, all patients diagnosed with (partial) HELLP at initial inclusion (n = 37) were excluded from the calculation of maternal complications. Other maternal complications were defined as eclampsia, pulmonary edema, subcapsular liver hematoma, cerebral hemorrhage/edema or infarction, postpartum hemorrhage (blood loss ≥ 1000 mL after delivery), and acute renal failure (absolute increase in the serum creatinine concentration of ≥ 0.3 mg/dL [26.4 μmol/L] from baseline; ≥ 50% increase in serum creatinine; or oliguria with <0.5 mL/kg per hour for a period of 6 hours).

Fetal/neonatal complications were defined as fetal death, neonatal (birth) weight <10th percentile according to Perinatal Registration, The Netherlands; development of sepsis; admittance to the neonatal intensive care unit (NICU); artificial ventilation and its duration; bronchopulmonary dysplasia defined as chronic lung disease developing in preterm neonates treated with oxygen and positive-pressure ventilation, with radiographic signs at 36 weeks postmenstrual age; respiratory distress syndrome (RDS); necrotizing enterocolitis; intraventricular hemorrhage; periventricular leukomalacia and posthemorrhagic ventricular dilation. All patients (n = 526) were used for the calculation of fetal/neonatal complications.

Statistical analysis

Women were subdivided according to copeptin levels (≤ 12 vs. >12 pmol/L), based on a previous study specifying that the 97.5th percentile of copeptin levels in the third trimester of healthy pregnancy was 12.1 pmol/L.[12] As the increase in copeptin in pregnancies with PE seems to fall within trimester-specific reference intervals, a single measurement of copeptin may be challenging to interpret. Therefore, to further elucidate the role of copeptin in PE, patient characteristics were compared in women with relatively high copeptin levels (>12 pmol/L) versus the normal range of copeptin (≤12 pmol/L). Copeptin and MR-proANP levels were further assessed according to sFlt-1/PlGF ratio based on a previously suggested cutoff value of ≥85 vs <85.[13]

Data are reported as median (range) for continuous variables and as number (percentage) for categorical variables. The normality of continuous variables was assessed using the Shapiro-Wilk W test. Differences between continuous variables were tested with the Student's t-test and Mann-Whitney U test in case of non-normally distributed data. Fisher's exact and X^2 were used to compare categorical variables between groups. For the comparison of continuous variables between more than two groups, one-way ANOVA or Kruskall-Wallis test in case of non-normally distributed data, was applied. Spearman rank order correlation was applied to calculate correlation coefficients. Multiple linear regression was applied to determine the variables affecting mean arterial blood pressure (MAP; calculated as ((2xDBP) + SBP)/3) and

urinary protein-to-creatinine ratio (uPCR). For the analysis of the latter, non-parametrically distributed data were log-transformed before correlation was performed. Logistic regression analysis was used to study the association between the dichotomous outcomes (maternal and fetal/neonatal complications) and the novel biomarkers and traditional predictors. Traditional predictors concerned gestational age, parity, proteinuria (uPCR) and DBP. SBP was not included as it correlated highly with DBP and a model with SBP instead of DBP gave a comparable fit. The markers sFlt-1, PlGF, sFlt-1/PlGF ratio, copeptin and MR-proANP were assessed either alone or added to the traditional predictors. Models were compared using concordance C-statistic for the dichotomous outcomes. We used SPSS Statistics 21 (IBM Corporations) and R Software for the statistical analysis.

RESULTS

A total of 526 women (age 18 – 47 years) were evaluated in the study. Median gestational age at inclusion was 35 weeks; 206 (39%) women had a gestational age below 34 weeks and 138 (26%) between 34 and 37 weeks. At time of enrollment, 134 (25%) women met the clinical criteria for (superimposed) PE and 37 (7%) had HELLP syndrome; 250 (48%) women were suspected of PE but had no hypertensive disease of pregnancy and 105 (20%) women were diagnosed with gestational hypertension (Table 1). Maternal complications occurred in 65 (13%) of the 489 women without (partial) HELLP syndrome at inclusion, while 207 (39%) of all 526 pregnancies had fetal/neonatal complications, often >1 complication per patient.

Copeptin and MR-proANP according to clinical diagnosis and sFlt-1/PlGF ratio

Women with confirmed (superimposed) PE displayed higher median copeptin (6 (range, 1 – 61) *vs* 4 (range (1 – 42) pmol/L) and MR-proANP (52 (range, 2 – 342) *vs* 31 (range, 2 – 299) pmol/L) levels than women suspected of PE but no HDP. Similarly, women with (partial) HELLP syndrome had higher copeptin (6 (range, 1 – 204) vs (4 (range, 1 – 42) pmol/L) and MR-proANP (69 (range, 14 – 282) pmol/L) vs 31 (range, 2 – 299) pmol/L) levels than those with suspected PE but no HDP (Figure 1). When subdividing the women according to sFlt-1/PlGF ratio of ≥85 (n=125) vs <85 (n = 401), serum copeptin level did not differ between the two groups, (5 (range, 1 – 204) *vs* 5 (range, 1 – 122) pmol/L; $P = 0.509$), but MR-proANP level was significantly elevated in women with sFlt-1/PlGF ratio ≥ 85 compared to those with ratio < 85 (64 (range, 2 – 342) *vs* 32 (range, 2 – 282) pmol/L; $P < 0.0001$).

Table 1. Characteristics, clinical findings and diagnosis at time of study inclusion of 526 women with suspected or confirmed preeclampsia.

Parameter	
n (%)	526
Maternal age (years)	31 (18 - 47)
Gestational age	
< 34 weeks	206 (39)
≥ 34 to <37 weeks	138 (26)
≥ 37 weeks	182 (35)
Nulliparous	308 (59)
Current smoker	29 (6)
Race	
White	364 (69)
Black	79 (15)
Other	83 (16)
History of preeclampsia	72 (14)
Preexisting hypertension	98 (19)
Preexisting proteinuria	19 (4)
Clinical findings	
SBP, mmHg	138 (95 - 210)
DBP, mmHg	86 (45 - 135)
MAP, mmHg	103 (61 - 160)
uPCR, mg/mmol	27 (1 - 2564)
LD, U/L	189 (88 - 1519)
ALT, U/L	15 (5 - 1533)
Creatinine, µmol/L	58 (26 -153)
Uric acid, mmol/L	0.29 (0.12 - 0.84)
Platelet count, 10^9/L	226 (36 - 499)
sFlt-1, pg/mL	3876 (79 - 83967)
PlGF, pg/mL	128 (3 - 9499)
sFlt-1/PlGF ratio	31 (1 - 1899)
Copeptin, pmol/L	5 (1 - 204)
MR-proANP, pmol/L	37 (2 - 342)
Clinical diagnosis at inclusion, n (%)	
No HDP	250 (48)
Gestational hypertension	105 (20)
(Superimposed) preeclampsia	134 (25)
(Partial) HELLP syndrome	37 (7)

Data are given as median (range) or n (%). ALT, alanine transaminase; DBP, diastolic blood pressure; HDP, hypertensive disease of pregnancy; HELLP, hemolysis, elevated liver enzymes and low platelets; LD, lactate dehydrogenase; MAP, mean arterial pressure; MR-proANP, mid-regional pro-atrial natriuretic peptide; PCR, protein-to-creatinine ratio; PlGF, placental growth factor; SBP, systolic blood pressure; and sFlt-1 soluble fms-like tyrosine kinase; uPCR, urinary protein-to-creatinine ratio.

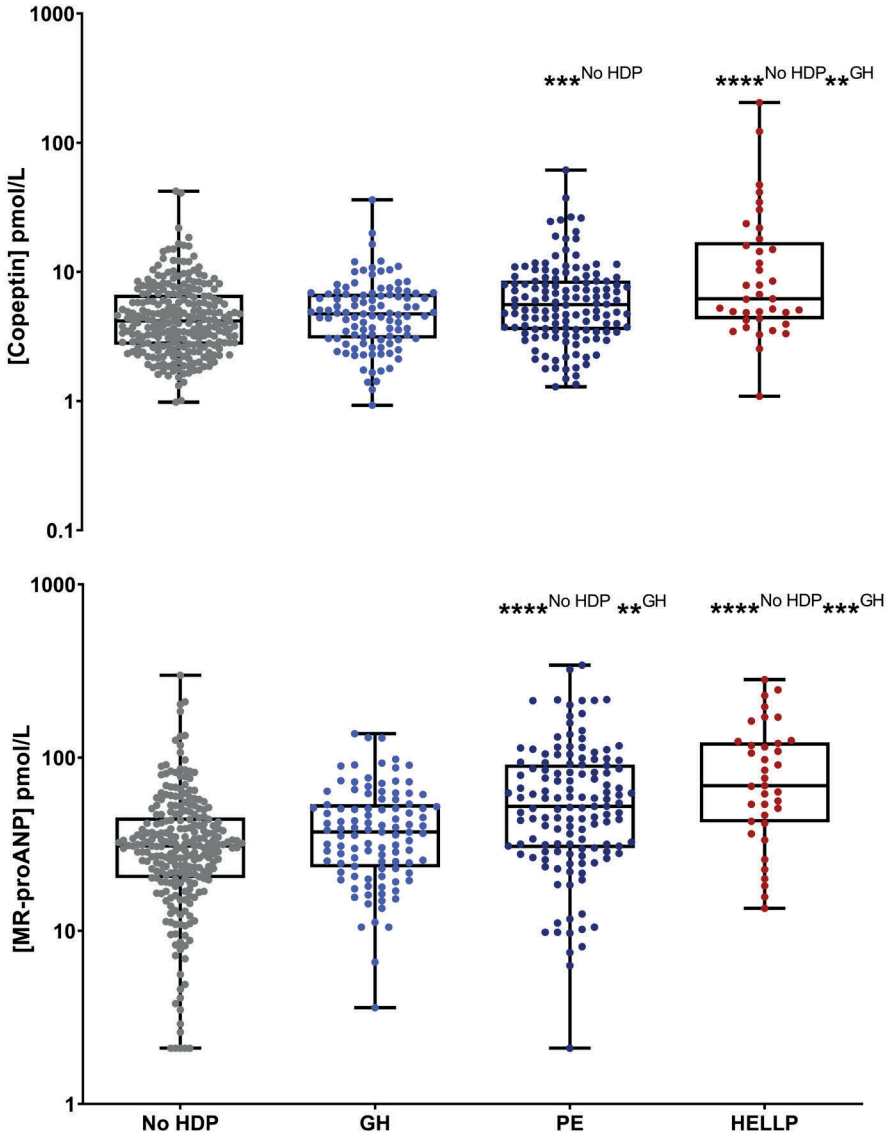

Figure 1. Box-and-whiskers plots showing levels of copeptin and mid-regional pro-atrial natriuretic peptide (MR-proANP) in 526 pregnant women, according to whether clinical diagnosis at inclusion was: suspected pre-eclampsia but no hypertensive disease of pregnancy (No HDP); gestational hypertension (GH); (superimposed)
pre-eclampsia (PE); or (partial) hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome. Boxes are median and interquartile range and whiskers are range; **P<0.01; ***P <0.001; ****P<0.0001.

Patient characteristics according to copeptin levels

Of the 526 women in whom copeptin was measured, 482 (92%) had copeptin ≤12 pmol/L and 44 (8%) had >12 pmol/L (Table S1). There were no differences in gestational age at time of inclusion. More cases of preexisting proteinuria were observed in women with high copeptin, while they had higher SBP and delivered on average 8 days earlier than women with copeptin ≤12 pmol/L. uPCR was also significantly higher in women with copeptin >12 pmol/L, whereas higher levels of alanine transaminase, creatinine, uric acid and lactate dehydrogenase (LD) were observed. The two groups did not differ in diagnosis at study entry, with the exception of higher rate of (partial) HELLP syndrome in women with high copeptin levels. Excluding women with preexisting proteinuria gave similar results with the exception of more nulliparous cases in women with high copeptin levels and no difference in SBP between the two groups (data not shown). The biomarkers sFlt-1, sFlt-1/PlGF ratio and MR-proANP were significantly elevated, while PlGF was significantly lower in women with copeptin >12 pmol/L. The significance for sFlt-1 and PlGF disappeared when excluding cases with (partial) HELLP syndrome from the analysis. Maternal complication rates were similar between groups, except for renal insufficiency, which developed more often in women with copeptin >12 pmol/L at inclusion (Table S2). Neonates born to mothers with high copeptin were often premature, which most likely underlies the lower birth weight, while neonatal complications such as admission to NICU, RDS and sepsis occurred more frequently in neonates born to mothers with copeptin >12 pmol/L (Table S2).

Associations of copeptin and MR-proANP with sFlt-1, PlGF, sFlt-1/PlGF ratio

Copeptin correlated marginally with sFlt-1 ($r = 0.18$; $P < 0.01$) and sFlt-1/PlGF ratio ($r = 0.14$; $P < 0.01$), while no correlation was found with PlGF ($r = - 0.07$, $P = 0.089$) (Table S3). MR-proANP correlated with sFlt-1 ($r = 0.30$; $P < 0.01$), PlGF ($r = - 0.29$; $P< 0.01$) and sFlt-1/PlGF ratio ($r = 0.35$; $P <0.01$). Copeptin and MR-proANP also correlated with each other ($r = 0.35$; $P <0.01$).

Determinants of MAP and uPCR

Serum sFlt-1, creatinine, copeptin, MR-proANP and mean arterial pressure (MAP) correlated positively with uPCR, while a negative correlation was observed with gestational age at time of measurement and PlGF (Table S3). Next, parameters displaying a significant correlation with uPCR were added into a multiple linear regression model. Under such conditions, copeptin, MR-proANP and PlGF remained as determinants of uPCR, explaining 33% of its variation (Table S4). Excluding cases of pre-existing proteinuria did not influence these results, with the same determinants explaining 32% of uPCR variation (data not shown).

Table 2. Association of incidence of maternal complications and incidence of fetal/neonatal complications with novel biomarkers, alone or in combination with traditional predictors.

	Maternal complications*				Fetal/neonatal complications†			
	Univariable		Multivariable		Univariable		Multivariable	
Model/Biomarker	Odds ratio (95% CI)	C-index	Odds ratio (95% CI)	C-index	Odds ratio (95% CI)	C-index	Odds ratio (95% CI)	C-index
Traditional model‡				0.60				0.79
sFlt-1	1.9 (1.5 - 2.5)	0.69	1.9 (1.5–2.5)	0.73	1.9 (1.5–2.4)	0.65	1.9 (1.5–2.6)	0.81
PlGF	0.2 (0.1 - 0.4)	0.72	0.3 (0.1–0.5)	0.73	0.1 (0.1–0.2)	0.76	0.2 (0.1–0.3)	0.83
sFlt-1/PlGF ratio	5.2 (2.8 - 9.7)	0.75	5.8 (3.0–11)	0.76	2.2 (1.8–2.8)	0.74	2.2 (1.6–2.9)	0.83
Copeptin	1.1 (0.7 - 1.9)	0.55	1.2 (0.7–2.0)	0.63	1.2 (1.1–1.3)	0.55	1.0 (0.9–1.2)	0.79
MR-proANP	2.2 (1.3–3.8)	0.66	2.1 (1.2–3.7)	0.67	2.2 (1.7–2.7)	0.69	1.7 (1.3–2.2)	0.80

To aid interpretation of continuous predictors, interquartile odds ratios and associated 95% CI were calculated; this is defined as comparing risk of complication at 75th percentile *vs* at 25th percentile of marker value. Multivariable analysis includes traditional model plus one biomarker. *Evaluated in 489 women with suspected or confirmed pre-eclampsia without (partial) HELLP (hemolysis, elevated liver enzymes and low platelet count) syndrome at time of inclusion. †Evaluated in all 526 women with suspected or confirmed pre-eclampsia at time of inclusion. ‡Traditional model consists of gestational age, parity, diastolic blood pressure and proteinuria. MR-proANP, mid-regional pro-atrial natriuretic peptide; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1.

Mean arterial pressure (MAP) correlated positively with serum sFlt-1, creatinine, MR-proANP and uPCR while it correlated negatively with PlGF. Using multivariate regression analysis, only PlGF remained as an independent determinant of MAP (Table S4).

Prediction of maternal and fetal/neonatal complications

Copeptin and MR-proANP were not associated with higher risk of developing maternal complications, with C-indices between 0.55 and 0.66 in univariable analysis (Table 2). When copeptin and MR-proANP were added to a model with traditional predictors (traditional model) the C-index increased only marginally from 0.60 for a model without biomarkers, to 0.63 and 0.67 in comparison with 0.73, 0.73 and 0.76 for sFlt-1, PlGF and their ratio, respectively. For the prediction of fetal/neonatal complications copeptin showed marginal discriminative ability with a C-index of 0.55, whereas MR-proANP performed better when compared with sFlt-1 (C-index = 0.69 versus 0.65) (Table 2). When added on top of the traditional model, the sFlt-1/PlGF ratio displayed a higher C-index than copeptin and MR-proANP (Table 2).

DISCUSSION

Both AVP and ANP have been reported to be elevated in women with the syndrome of PE.[14, 15] In this study, we examined AVP and ANP, measured as their stable surrogates copeptin and MR-proANP, as potential biomarkers for the prediction of adverse pregnancy outcome and their association with clinical characteristics and angiogenic markers, in a cohort of women with suspected or confirmed PE. In accord with previous studies[6-8, 16] we observed elevated circulating levels of copeptin and MR-proANP in women with PE or HELLP syndrome, when compared to women only suspected of PE without hypertensive disease of pregnancy. However, our findings indicate that copeptin and MR-proANP have limited value to predict maternal complications in uni- and multivariable analysis when compared with sFlt-1, PlGF or their ratio. In addition, copeptin did not show additive value for the prediction of fetal/neonatal complications, while the predictive value of MR-proANP was comparable to that of sFlt-1, but not better than PlGF and sFlt-1/PlGF ratio in univariable analysis, and inferior to all three angiogenic markers when added on top of the traditional variables. Few studies have evaluated the role of copeptin and MR-proANP as biomarkers for PE prediction. In a cohort involving 136 healthy pregnant women and 169 PE women, copeptin levels measured at 16 weeks' gestation were associated with an increased risk for PE (OR 1.61).[10] In another study comprising 50 PE, 54 healthy pregnant and 33 non-pregnant women, it was concluded that copeptin was a strong predictor for diagnosing PE when measured in all three trimesters, with areas under the curve (AUC) of 0.90, 0.90 and 0.78, respectively.[9] Conversely, two small studies were unable to demonstrate such findings.[8, 17] To discriminate between 77 normotensive and 107 preeclamptic pregnancies, Sugulle *et al.* measured MR-proANP at 24 to 42 weeks' gestation and showed an AUC of 0.85 which was

inferior to that of sFlt-1 (0.94).[5] In contrast to copeptin and MR-proANP, another cardiovascular biomarker, N-terminal pro-B natriuretic peptide (NT-proBNP), has shown incremental value to predict short-term delivery due to PE when added to the sFlt-1/PlGF ratio >38.[18] Similarly, a recent study demonstrated that adding NT-proBNP >174 to an sFlt-1/PlGF ratio >45 significantly increases the positive predictive value of sFlt-1/PlGF ratio.[19] In a study by Verlohren *et al.*, elevated NT-proBNP levels associated with imminent delivery but did not predict hypertensive disease of pregnancy. It should be noted that this cohort predominantly consisted of late-onset cases.[20] The abovementioned studies focused on the diagnosis of PE and did not investigate pregnancy outcome as an endpoint.

As far as we know, our study is the first to allow conclusions regarding the role of cardiovascular biomarkers copeptin and MR-proANP for the prediction of PE-related complications in relation with sFlt-1, PlGF and their ratio.

When we evaluated patient characteristics by classifying women according to copeptin (≤12 vs >12pmol/L) women with high copeptin displayed greater sFlt-1/PlGF ratio, but this is most likely related to the increased occurrence of HELLP syndrome in this group, since both sFlt-1 and PlGF were no longer different when patients with this condition were excluded. In fact, we and others[10] found a weak correlation between copeptin and sFlt-1 but not between copeptin and PlGF. In addition, copeptin levels did not differ between women with sFlt-1/PlGF ratio ≥85 versus <85, whereas MR-proANP levels were elevated in women with a ratio ≥85. Given these observations, we assume that the effects mediated by AVP, but not ANP, are independent of a high antiangiogenic state as reflected by an elevated sFlt-1/PlGF ratio. In support of this concept, a recent study showed that low-dose AVP infusion in pregnant mice induced a clinical PE phenotype which was indeed independent of placental hypoxia.[21] Moreover, despite the occurrence of hypertension, renal glomerular endotheliosis, IUGR and decreased placental PlGF in this mouse model, placental sFlt-1 was not elevated, in agreement with our findings.[21] Our observation that copeptin was not associated with blood pressure, a clinical manifestation classically determined by PlGF as observed by us and others,[22] again implies that copeptin and sFlt-1 / PlGF act independently of each other. Interestingly, both copeptin and MR-proANP correlated positively with serum creatinine (Table S3) and when added in multiple linear regression analysis, both remained independently associated with proteinuria, even after excluding women with preexisting proteinuria. As proteinuria is no longer required to establish the diagnosis of PE, one should keep in mind that copeptin and MR-proANP merely contribute to a specific phenotype of PE. As such, our data illustrates the importance of viewing PE as a complex disorder with distinct patterns of cardiovascular and angiogenic markers contributing to different phenotypes of the condition.

A few limitations of our study must be acknowledged. First, we did not perform repeated measurements of copeptin and MR-proANP during pregnancy. Longitudinal data of these markers may give more insight into their association with proteinuria and renal function. Second, our cohort mostly consisted of women already diagnosed with (suspicion) PE and in over 60% of women blood was sampled at a gestational age of 34 weeks or later. Particularly when considering the hypothesis that alterations in the cardiovascular system precede placental dysfunction[3], the predictive value of these biomarkers potentially improves when measured in early pregnancy. Our data gives new insight into the role as biomarkers of copeptin and MR-proANP in women with suspected or confirmed PE. We observed limited value of copeptin and MR-proANP as predictors of adverse pregnancy outcome, especially when compared with the angiogenic markers sFlt-1, PlGF and sFlt-1/PlGF ratio. However, interestingly, we found that copeptin and MR-proANP were associated with proteinuria but not blood pressure, with copeptin acting independently of the (anti)angiogenic state, suggesting that copeptin could be contributing to a specific phenotype of PE.

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SUPPLEMENTAL DATA

Table S1. Patient Characteristics according to Copeptin Levels (n = 526)

Parameter	Copeptin ≤12	Copeptin >12	*P* value
n (%)	482	44	…
Age, y	31 (18 - 47)	30 (19 - 44)	0.465
Gestational age (wks), n (%)	35 (20 - 41)	35 (21 - 40)	0.495
Nulliparous, n (%)	277 (58)	31 (70)	0.094
Current smoker, n (%)	25 (5)	4 (9)	0.268
Race, n (%)			
White	339 (70)	25 (57)	0.063
Black	69 (14)	10 (23)	0.135
Other	74 (16)	9 (20)	0.374
History of preeclampsia, n (%)	68 (14)	4 (9)	0.492
Preexisting hypertension, n (%)	87 (18)	11 (25)	0.257
Preexisting proteinuria, n (%)	14 (3)	5 (11)	0.004
Clinical findings at time of admission			
SBP, mmHg	137 (95 - 210)	143 (115 - 178)	0.035
DBP, mmHg	86 (45 - 135)	90 (65 - 113)	0.595
MAP, mmHg	103 (62 - 160)	106 (81 - 133)	0.237
PCR, mg/mmol	24 (5 - 1657)	145 (1 - 2564)	0.000
LD, U/L	185 (88 - 159)	247 (135 - 1376)	0.000
ALT, U/L	14 (5 - 862)	25 (7 - 1533)	0.000
Creatinine, µmol/L	57 (26 -111)	87 (43 - 153)	0.000
Uric acid, mmol/L	0.29 (0.12 - 0.54)	0.44 (0.21 - 0.84)	0.000
Platelet count, 10^9/L	227 (36 - 499)	218 (44 - 424)	0.295
Additional Biomarkers			
sFlt-1, pg/mL	3792 (79 - 32061)	5716 (1013 - 83967)	0.022
PlGF, pg/mL	134 (3 - 9499)	97 (11 - 410)	0.002
sFlt-1/PlGF ratio	29 (1 - 1899)	64 (6 - 1824)	0.000
Copeptin, pmol/L	4 (1 -12)	18 (12 - 204)	0.000
MR-proANP, pmol/L	34 (3 - 342)	75 (8 - 282)	0.000
Diagnosis at inclusion (%)			
No HDP	235 (49)	15 (34)	0.062
Gestational hypertension	101 (21)	4 (9)	0.074
(superimposed) preeclampsia	121 (25)	13 (30)	0.517
(partial) HELLP syndrome	25 (5)	12 (27)	0.000

Values are median (range) or n (%). ALT indicates alanine transaminase; DBP, diastolic blood pressure; HDP; hypertensive disease of pregnancy; HELLP; hemolysis, elevated liver enzymes and low platelet; LD, lactate dehydrogenase; MAP, mean arterial pressure; MR-proANP, mid-regional pro-atrial natriuretic peptide; PCR, protein-to-creatinine ratio; PlGF, placental growth factor; SBP, systolic blood pressure; and sFlt-1 soluble Fms-like tyrosine kinase.

Table S2. Pregnancy Outcome according to Copeptin Levels

Parameter	Copeptin ≤ 12	Copeptin > 12	*P* value
n (%)	482	44	
Gestational age at birth, wks	38 (21 - 42)	36 (26 - 41)	0.001
Prematurity, wks			
<34	76 (16)	17 (39)	0.000
34 - 37	54 (11)	7 (16)	0.351
Gender M/F	248 / 234	23 / 21	0.917
Birth weight, g	3095 (170 - 4915)	2403 (530 - 3880)	0.001
Days until delivery	12 (0 - 140)	4 (0 - 70)	0.000
Maternal complications, n %			
Placental abruption	2 (0)	0 (0)	1.000
Renal insufficiency	0 (0)	3 (7)	0.001
Pulmonary edema	4 (1)	1 (2)	0.356
Postpartum hemorrhage	32 (7)	6 (14)	0.087
HELLP syndrome	21 (4)	4 (9)	0.072
Fetal complications, n (%)			
Admission to NICU	113 (23)	22 (50)	0.000
Endotracheal tube	33 (7)	5 (11)	0.274
Birth weight percentile < 10	69 (14)	8 (18)	0.499
Respiratory distress syndrome	53 (11)	11 (25)	0.007
Sepsis	30 (6)	8 (18)	0.004
Fetal death	19 (4)	1 (2)	0.578

Values are median (range) or n (%). NICU indicates neonatal intensive care unit.

Table S3. Univariable regression to assess correlations between different biomarkers.

Variable	Copeptin pmol/L	MR-proANP pmol/L	MAP, mm Hg	uPCR mg/mmol
G.A measurement, wks	0.089*	-0.116**	0.014	-0.198*
MAP, mm Hg	0.081	0.173**	…	0.174**
uPCR, mg/mmol	0.261**	0.419**	0.174**	…
sFlt-1, pg/mL	0.176**	0.296**	0.201**	0.172**
PlGF, pg/mL	-0.074	-0.292**	-0.293**	-0.331**
sFlt-1/PlGF ratio	0.138**	0.352**		
Creatinine, umol/L	0.321**	0.405**	0.176**	0.15*
Copeptin, pmol/L	…	0.354**	0.081	0.261**
MR-proANP, pmol/L	0.354**	…	0.173**	0.419**

GA, gestational age; MAP, mean arterial pressure; MR-proANP; midregional proatrial natriuretic peptide; PlGF, placental growth factor; sFlt-1, soluble Fms-like tyrosine kinase-1; uPCR, urinary protein-to-creatinine ratio. *P < 0.05, **P < 0.01.

Table S4. Multiple linear regression to assess variables affecting mean arterial blood pressure (MAP) and urinary protein-to-creatinine ratio (uPCR).

Variable	**MAP, mmHg**	
	Coefficient	***P* value**
sFlt-1, pg/mL	2.574	0.169
PlGF, pg/mL	-5.544	0.000
Creatinine, umol/L	9.400	0.184
MR-proANP, pmol/L	-0.006	0.739
uPCR, mg/mmol	1.830	0.152
MAP, mm Hg	…	…
R Square	**0.12**	
	uPCR, mg/mmol	
	Coefficient	***P* value**
sFlt-1, pg/mL	-0.062	0.485
PlGF, pg/mL	-0.237	0.001
Creatinine, umol/L	-0.552	0.110
Copeptin, pmol/L	0.437	0.000
MR-proANP, pmol/L	0.005	0.000
uPCR, mg/mmol	…	…
MAP, mm Hg	0.004	0.140
G.A measurements, weeks	-0.012	0.057
R Square	**0.33**	

GA, gestational age; MAP, mean arterial pressure; MR-proANP; midregional pro-atrial natriuretic peptide; PlGF, placental growth factor; sFlt-1, soluble Fms-like tyrosine kinase-1; uPCR, urinary protein-to-creatinine ratio. All variables except MR-proANP, MAP and G.A at time of measurement were log transformed to achieve linearity.

CHAPTER 05

Angiogenic Markers during Preeclampsia: Are They Associated with Hypertension 1 year Postpartum?

Rugina I. Neuman, Aveline M.J Figaroa, Daan Nieboer, Langeza Saleh, Koen Verdonk, A.H. Jan Danser, Hans J.J Duvekot, Anton H. van den Meiracker, Jeanine Roeters van Lennep, Willy Visser

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ABSTRACT

Objectives: Preeclampsia is associated with hypertension in later life, but the underlying pathophysiological mechanisms remain uncertain. We aimed to explore whether the angiogenic markers soluble Fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PlGF) measured in women with preeclampsia could be associated with hypertension 1 year after delivery.

Methods: This is a secondary analysis of a prospective cohort study, originally aimed to evaluate the use of sFlt-1/PlGF ratio to predict adverse outcome in women with (suspected) preeclampsia. Office blood pressure (BP) was evaluated at 1 year postpartum in women who had a confirmed diagnosis of preeclampsia within one week of biomarker measurement.

Results: Eighty women were included with a median (interquartile range) gestational age (GA) at biomarker measurement of 30 (27 – 33) weeks. Twenty-three (29%) women had hypertension 1 year postpartum. These women showed higher median SBP during their pregnancy and lower GA at PE diagnosis compared to women without hypertension. Median PlGF levels were lower in women with hypertension 1 year postpartum compared to women without hypertension (23 vs. 48 pg/mL, p = 0.017), while no differences in sFlt-1 or sFlt-1/PlGF ratio were observed. Multivariable analysis adjusted for GA did not show significant association between PlGF (nor sFlt-1, sFlt-1/PlGF ratio) and hypertension 1 year postpartum (OR [95% CI] 0.9 [0.2-4.4], p = 0.97).

Conclusion: Our data indicate that sFlt-1, PlGF or their ratio measured during pregnancy are not suitable for the prediction of hypertension 1 year postpartum and hence guiding follow-up of women with previous preeclampsia.

INTRODUCTION

Preeclampsia is a severe hypertensive disorder affecting 5 – 7 % of all pregnancies. It is characterized by the new onset of hypertension accompanied by either proteinuria, utero-placental dysfunction such as intrauterine growth restriction and/or other maternal organ dysfunction at or after 20 weeks gestation [1]. Preeclampsia not only has significant impact on maternal and fetal health during pregnancy [2], but has also been established as a risk factor of cardiovascular disease for both mother and offspring [1]. A recent study reported that about 42% of women with severe preeclampsia already show some form of hypertension 1 year after pregnancy [3]. Unfortunately, our knowledge of factors that could predict the development of hypertension (or other cardiovascular disease) or not, is limited. Identification of these factors could enable clinicians to determine which women with previous preeclampsia require earlier follow-up after delivery.

Although the underlying pathophysiology of preeclampsia is not completely elucidated, an imbalance between circulating pro- and antiangiogenic factors, reflected by elevated soluble Fms-like tyrosine kinase-1 (sFlt-1) and low placental growth factor (PlGF) levels has been well established [1]. This high antiangiogenic state inducing a pro-inflammatory state and endothelial dysfunction is thought to play a key role in the disorder. In fact, endothelial dysfunction has been reported to persist up to 15 years after preeclampsia [4].

We have reported that plasma PlGF levels were independently associated with mean arterial pressure during pregnancy [5] whereas the severity of hypertension itself is a predictor for the development of future hypertension [6]. Although sFlt-1, PlGF and their ratio have been investigated widely for the prediction of preeclampsia [7] and preeclampsia-related pregnancy outcomes [8], their role in prediction of postpartum hypertension or cardiovascular disease in later life has not yet been determined.

Therefore, we aimed to evaluate whether the angiogenic imbalance during preeclampsia could predict hypertension 1 year after pregnancies complicated by preeclampsia.

METHODS

Study design and participants

This was a secondary analysis of a prospective observational cohort study conducted from 2012 to 2016 at the Erasmus Medical Center, Rotterdam, the Netherlands, originally aimed to evaluate the usefulness of the sFlt-1/PlGF ratio to predict adverse pregnancy outcome in 408 women with suspected or confirmed preeclampsia. Written informed consent to participate in the study was approved by the local research ethics committee (MEC-2013-202) and was obtained from all participants. For the current analysis, women with pre-existing hypertension and/or proteinuria were excluded (n = 117). In order to assess the biomarker values at the time

of confirmed preeclampsia, we excluded women who did not have confirmed preeclampsia within one week of study entry (time of biomarker measurement) (n = 136). Women who had a follow-up appointment at the Internal Medicine clinician and/or at the Follow-Up Pre-Eclampsia (FUPEC) Outpatient Clinic in Erasmus Medical Center, Rotterdam, within 9 to 15 months postpartum were included in the analysis.

Preeclampsia diagnosis

Preeclampsia was defined according to the definition of the International Society for the Study of Hypertension in Pregnancy (ISSHP) of 2018; de novo hypertension (diastolic blood pressure [DBP] of ≥90 mmHg or systolic blood pressure [SBP] of ≥140mmHg) accompanied by ≥1 of the following new-onset conditions at or after 20 weeks' gestation: proteinuria (urinary protein-to-creatinine ratio [uPCR] ≥30 mg/mmol or ≥300 mg/24 hours or 2+ dipstick), acute kidney injury (AKI) (creatinine ≥90 μmol/L; 1 mg/dL), neurological complications (e.g eclampsia), hematological complications (thrombocytopenia -platelet count <150.000/μL, disseminated intravascular coagulation, hemolysis), liver involvement (elevated transaminases, e.g: alanine aminotransferase [ALAT] or aspartate aminotransferase >40 IU/L) with or without right upper quadrant or epigastric abdominal pain, or uteroplacental dysfunction (fetal growth restriction, abnormal umbilical artery (UA) Doppler wave form analysis or stillbirth). [9] HELLP syndrome, defined as hemolysis, elevated liver enzymes and low platelet count, was now also considered as preeclampsia according to the ISSHP 2018 criteria [9].

Data collection

Serum for the analysis of sFlt-1 and PlGF was collected at inclusion of the original study. Serum was stored at -80° C after centrifugation, until analysis. All samples were measured postpartum. Measurement of sFlt-1 and PlGF was performed using an automated analyzer (Cobas 6000, e-module; Roche Diagnostics, Mannheim, Germany). Clinical data during and after pregnancy including demographic information, gestational age (GA) at biomarker measurement, diagnosis and delivery, physical examination, laboratory test results and pregnancy outcome were attained from the electronic medical records of the patients and ascertained by two independent researchers (R.I.N and A.M.J.F). Time to delivery was defined as the amount of days between study entry (at time of biomarker measurement) and delivery.

Outcome measures at 1-year follow-up

A trained nurse or research assistant measured office BP with the participant in the upright sitting position after 5 minutes of rest. The appropriate arm cuff was placed around the right upper arm to measure BP with a validated oscillometric device. Women were not allowed to speak during BP measurements.

Hypertension based on office BP was defined according to the European Society of Hypertension and European Society of Cardiology: office hypertension (average SBP of ≥140mmHg and/or an average DBP of ≥90mmHg) and/or the use of antihypertensive medication.

Statistical analysis

Data are reported as median (interquartile range) for continuous variables and as number (percentage) for categorical variables. Normal distribution for continuous variables was assessed using the Shapiro-Wilk W test. To investigate the difference between non-parametric continuous data, Mann Whitney U-test was performed. The Fisher's exact (in the case of a small sample size, ≤5) and Chi-square were used to assess differences between two categorical variables. A non-response analysis was performed to evaluate baseline characteristics between women that were included and women lost to follow-up. Logistic regression analysis was performed to study the association between potential predictors (i.e., biomarkers) and postpartum hypertension at 1 year follow-up. Biomarkers evaluated in univariable analysis included sFlt-1, PlGF and sFlt-1/PlGF ratio. Due to the fact that GA at time of biomarker measurement can affect the levels of these biomarkers, multivariable analysis was performed to correct for GA at biomarker measurement. Because of the limited number of events (n = 23), we were unable to adjust for additional confounders. Clinical parameters such as nulliparity, highest SBP during pregnancy, preconceptional BMI, time to delivery, GA at delivery and at preeclampsia diagnosis were evaluated as predictors in univariable analysis. The discriminative ability of the models was assessed using concordance-statistic (C-statistic) which is equivalent to the area under the ROC curve for dichotomous outcomes. To evaluate the added value of sFlt-1, PlGF or their ratio when corrected for GA at measurement, we fitted a logistic regression model containing sFlt-1, PlGF or sFlt-1/PlGF ratio and a logistic regression model containing both GA at measurement and one of the angiogenic markers. sFlt-1, PlGF or sFlt-1/PlGF ratio were considered to have additional value if the likelihood ratio test comparing both models was statistically significant. SPSS Statistics 21 (IBM Corporations) and R Software were used for the statistical analysis.

RESULTS

Patient demographics

The final population for analysis consisted of 80 women (Figure 1). Patient characteristics during pregnancy and at 1 year follow-up of all participants (aged 20 – 43) are shown in Table 1. Median (interquartile range) GA at study entry (biomarker measurement) was 30 weeks (27 - 33). Fifty-nine women (74%) were nulliparous, and eight women (10%) had a previous history of preeclampsia. Preconceptional BMI (kg/m^2) was 23 (22 - 27). The median highest SBP during pregnancy was 157 (140 – 165) mmHg, the median highest uPCR was 98 (38-

262) g/mol and the median sFlt-1/PlGF ratio was 296 (68 – 602). GA at delivery was 30 (28 – 34) weeks. At 1 year follow-up, the overall SBP normalized to 124 (114 – 135) mmHg whereas 3 women (4%) still had proteinuria (uPCR≥30 g/mol).

Patient characteristics and angiogenic markers according to hypertension at 1 year follow-up

Of the 80 women, 23 (29%) had hypertension 1 year after pregnancy (Table 1). Compared to women without hypertension, participants with hypertension had a lower GA at PE diagnosis (27 vs. 30 weeks, p <0.01) and were more often nulliparous (91% vs 67%, p = 0.03). The highest median SBP during pregnancy was higher in women with hypertension 1 year postpartum (168 vs. 155 mmHg, p = 0.02), while their GA at delivery (28 vs. 31, p<0.01) and birth weight (955 vs. 1350, p < 0.01) were lower in comparison to women without hypertension (Table 1). Women with hypertension at 1 year more often had HELLP syndrome during their pregnancy (57% vs. 28%, p = 0.02), but this difference was most likely due to higher SBP in this group. Nine women were still using antihypertensive medication after 1 year, suggesting they had persistent hypertension. Median gestational PlGF levels were lower in women with hypertension 1 year postpartum in comparison to women without hypertension (23 [14-50] vs. 48 [23 – 80] pg/mL, p = 0.02), while no differences in sFlt-1 or sFlt-1/PlGF ratio were observed between the two groups (Table 1, Figure 2). Median GA at study entry (biomarker measurement) was three weeks earlier in the hypertension group (Table 1). The non-response analysis showed that women without follow-up had milder forms of preeclampsia, as observed by the later GA at study entry (35 vs. 30 weeks, p<0.01), GA at delivery (37 vs. 30 weeks, p<0.01) and lower sFlt-1/PlGF ratio (67 vs. 296, p<0.01). No differences in age, race or parity were observed (Table 2).

Prediction of hypertension based on office BP at 1 year follow-up

The clinical parameters nulliparity and highest SBP were significantly associated with the occurrence of hypertension at 1 year follow-up, although their discriminative ability was limited (C-index of 0.62, p =0.04 and C-index of 0.65, p =0.04) (Table 3A). The GA at delivery showed good value to predict postpartum hypertension with a C-index of 0.72, while GA at diagnosis performed significantly better as a continuous value in comparison to the cut-off value of 34 weeks (C-index = 0.73, p <0.001 vs. C-index of 0.61, p = 0.06). PlGF was not significantly associated with the occurrence of hypertension at 1 year (C-index = 0.67, OR [95% CI] 0.3 [0.1 – 0.9], p = 0.06), neither were sFlt-1 or the sFlt-1/PlGF ratio. When corrected for GA at biomarker measurement in multivariable analysis, the model with angiogenic markers showed no significant association with the occurrence of hypertension at 1 year postpartum (Table 3B).

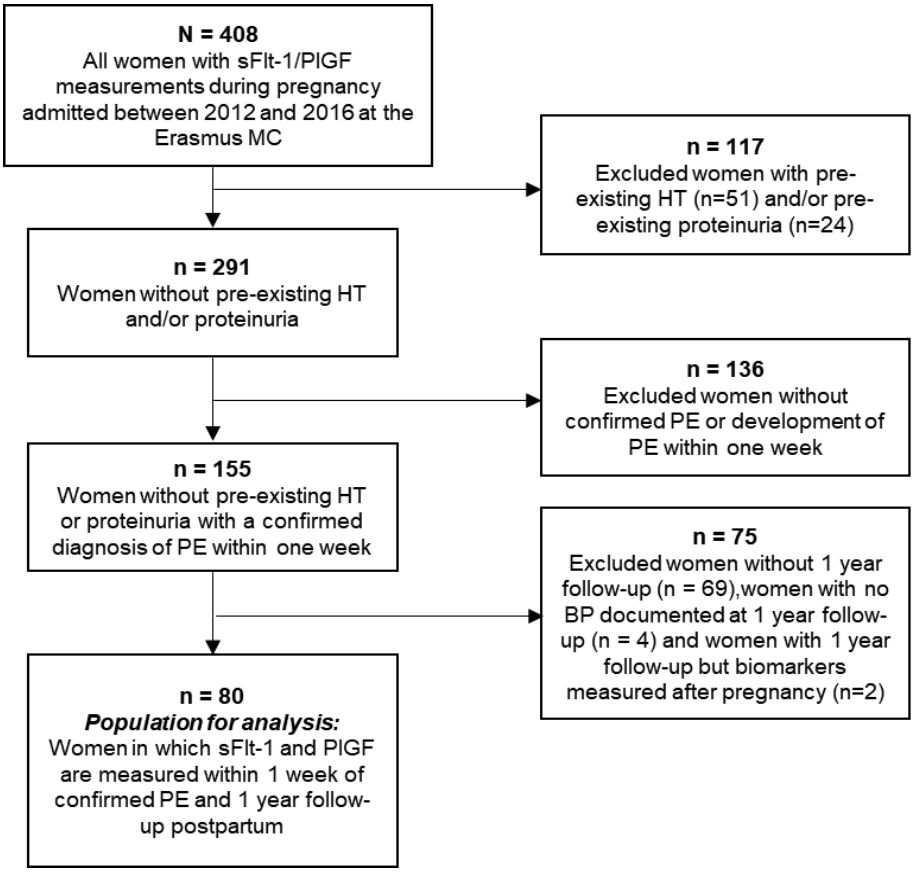

Fig. 1. Flowchart of in- and exclusion criteria in this study. PE indicates preeclampsia. HT indicates hypertension; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

Table 1. Patient Demographics of All Included Women and According to Hypertension based on office BP.

Parameter	All Women	No Hypertension	Hypertension	P-Value
Characteristics during pregnancy	80	57	23	
Age at study entry, yrs	30 (27 - 34)	30 (27 - 35)	29 (27 - 34)	0.59
GA at study entry*, wks	30 (27 - 33)	30 (29 – 33)	27 (26 - 30)	<0.01
Preconceptional BMI, kg/m^2	23 (22 - 27)	23 (21 - 26)	26 (23 - 30)	0.07
Race, n (%)				
White	56 (70)	37 (65)	19 (83)	0.18
Black	9 (11)	6 (11)	3 (13)	0.71
Other	15 (19)	14 (25)	1 (4)	0.06
Nulliparous, n (%)	59 (74)	38 (67)	21 (91)	0.03
Smoking at inclusion, n (%)	4 (5)	3 (5)	1 (4)	1.00
History of preeclampsia, n (%)	8 (10)	8 (14)	0 (0)	0.10
Clinical parameters				
GA at diagnosis PE, wks	30 (27 - 33)	30 (29 - 34)	27 (26 - 30)	<0.01
Highest SBP, mmHg	157 (140 - 165)	155 (140 -162)	168 (146 - 174)	0.02
Highest DBP, mmHg	97 (90 - 105)	95 (90 - 105)	100 (91 - 110)	0.18
Antihypertensive drug use at study entry, n (%)	56 (70)	40 (70)	16 (70)	0.96
Highest uPCR, g/mol	98 (38 - 262)	104 (40 - 240)	59 (24 - 397)	0.37
Highest Uric acid, mmol/L	0.37 (0.32 - 0.44)	0.37 (0.32 - 0.43)	0.38 (0.29 - 0.47)	0.84
Highest Creatinine, µmol/L	67 (58 - 73)	65 (58 - 73)	68 (60 - 73)	0.74
Highest ALAT, U/L	47 (24 - 175)	33 (20 - 169)	81 (38 - 193)	0.06
Highest LD, U/L	289 (223 - 443)	269 (219 - 419)	323 (254 - 602)	0.09
Lowest Platelet Count,10^9/L	149 (100 - 203)	158 (104 - 223)	125 (66 -172)	0.04
HELLP syndrome, n (%)	29 (36)	16 (28)	13 (57)	0.02
Highest SBP	159 (140 – 170)	148 (140 – 160)	168 (149 – 178)	0.03
Highest DBP	96 (88 – 105)	90 (86 – 103)	100 (95 – 107)	0.09
Pregnancy outcome				
GA at delivery, weeks	30 (28 - 34)	31 (29 - 34)	28 (27 - 31)	<0.01
Girls, n (%)	40 (50)	27 (47)	13 (57)	0.35
Birth weight, grams	1210 (863 - 1798)	1350 (1070 - 2083)	955 (760 - 1440)	<0.01
Birth weight percentile <10, n (%)	20 (25)	15 (26)	5 (22)	1.00
Time to delivery‡, days	2 (1 - 8)	2 (1 - 8)	4 (2 - 12)	0.06
Angiogenic markers				
sFlt-1, pg/mL	9405 (5066 - 15839)	10295 (4741 - 16809)	8801 (5283 - 12888)	0.29
PlGF, pg/mL	32 (18 - 69)	48 (23 - 80)	23 (14 - 50)	0.02
sFlt-1/PlGF ratio	296 (68 - 602)	208 (62 - 580)	498 (92 - 704)	0.24
sFlt-1/PlGF ratio ≤38, n (%)	11 (14)	8 (14)	3 (13)	1.00
sFlt-1/PlGF ratio >85, n (%)	56 (70)	38 (67)	18 (78)	0.42
Characteristics at 1-year Follow-up				
Office SBP, mmHg	124 (114 - 135)	119 (111 - 128)	141 (131 - 150)	<0.01
Office DBP, mmHg	76 (70 - 83)	73 (68 - 80)	86 (79 - 95)	<0.01
BMI, kg/m^2	25 (23 – 30)	24 (22 – 29)	29 (24 – 31)	0.17
Antihypertensive drug use, n (%)	9 (11)	0 (0)	9 (39)	<0.01
uPCR ≥30 g/mol	3 (4)	3 (5)	0 (0)	0.25

Hypertension defined as office SBP ≥140mmHg and/or office DBP≥90mmHg and/or antihypertensive drug use. Values are median (interquartile range) or number (percentage). GA, gestational age; BMI, body mass index; PE, preeclampsia; SBP, systolic blood pressure; DBP, diastolic blood pressure; uPCR, urinary protein-to-creatinine ratio; LD, lactate dehydrogenase; ALAT, alanine-aminotransferase; sFlt-1 indicates soluble FMS-like tyrosine kinase-1; PlGF, placental growth factor. P-value depicts difference between hypertension and no hypertension group. *Biomarkers were determined at time of study entry. ‡Time to delivery is defined as the amount of days between study entry and delivery.

Correlations between angiogenic markers and BP during and after pregnancy

Angiogenic markers were evaluated in women during pregnancy (with and without follow-up, n = 155) and at follow-up, as depicted in Table 4. sFlt-1 and sFlt-1/PlGF ratio showed a significant negative correlation with GA at inclusion ($r = -0.193$ and $r = -0.539$, $p<0.001$), while PlGF showed a positive correlation with GA at inclusion ($r = 0.672$, $p <0.001$). The angiogenic markers did not significantly correlate with SBP or DBP at inclusion, but both PlGF and sFlt-1/PlGF ratio showed a significant correlation with highest SBP and DBP during pregnancy. No significant correlation between sFlt-1, PlGF or sFlt-1/PlGF ratio and office BP at 1 year follow-up were observed.

DISCUSSION

In this study of 80 women with previous preeclampsia, we examined whether the angiogenic factors sFlt-1, PlGF and sFlt-1/PlGF ratio could be associated with postpartum hypertension at 1 year follow-up based on office BP measurements. Twenty-nine percent of women in our cohort showed office hypertension 1 year after pregnancy. This percentage was lower than recently reported in 200 women with severe preeclampsia (42%) [3], however in that study hypertension was diagnosed by 24-hour ambulatory BP measurements (ABPM). Hence, the discrepancy between these numbers is most likely explained by women with masked hypertension who are missed by an office BP measurement alone. Indeed, about 18% of women in that study had masked hypertension [3].

When evaluating the angiogenic factors, we found lower levels of the proangiogenic factor PlGF in women with office hypertension at 1 year follow-up. However, this marker did not show significant value to predict hypertension 1 year after delivery, even when corrected for GA at time of biomarker measurement in multivariable analysis. Moreover, both sFlt-1 and sFlt-1/PlGF ratio showed limited predictive performance to determine whether women had hypertension both in uni- and multivariable analysis, suggesting that their levels during preeclampsia are not associated with persistence or the development of hypertension 1 year after delivery. These observations remained similar when evaluating hypertension based on 24-hour ABPM at 1 year postpartum in a small subset of women (n = 49) (data not shown).

That women with previous preeclampsia are at increased risk of CVD including chronic hypertension in later life has been well established [10]. Whether this is an effect of pre-pregnancy cardiovascular risk factors or a direct consequence of preeclampsia itself, remains a matter of debate. Despite our understanding that a high antiangiogenic state (reflected by elevated sFlt-1 and low PlGF levels) is a key mechanism underlying endothelial dysfunction in preeclampsia [5], only a few studies evaluated the relationship between these factors and the occurrence of hypertension postpartum.

In a cohort of 988 women, Goel et al. [11] demonstrated that antepartum levels of the angiogenic markers were independently associated with persistent or de novo hypertension

postpartum. While these findings are in contrast with our observations, they only evaluated the development of hypertension up to six weeks postpartum. Interestingly, a recent large study (n = 5475) showed that lower mid-pregnancy (mean 20.6 weeks of gestation) serum PlGF levels were associated with higher systolic blood pressure six and nine years after pregnancy [12]. Of note, the latter study mostly evaluated PlGF levels in uncomplicated pregnancies, which differs from our population consisting only of women with preeclampsia. In addition, the effect of lower mid-pregnancy PlGF levels on systolic blood pressure was very limited (i.e regression coefficient β [95% CI] of 1.8 [0.35 – 3.2] mmHg in comparison to women with high PlGF) [12]. It is also important to note that the GA at time of biomarker measurement in our cohort was much later (median 27 weeks). Possibly, the contribution of PlGF to blood pressure is evident only when considering all pregnancies (including cases of mild preeclampsia or gestational hypertension) and can no longer be detected when focusing on women with only severe features of preeclampsia. Indeed, when we compared pregnancy characteristics between the women included in this study and the women that were lost to follow-up, it seems that our cohort consisted mostly of women with severe forms of preeclampsia, as reflected by the higher sFlt-1 and lower PlGF levels, and earlier GA at study entry and delivery (Table 2). We also evaluated other factors during pregnancy that could be associated with postpartum hypertension and found that the GA when preeclampsia occurred showed the highest value to predict the occurrence of hypertension 1 year postpartum. Interestingly, the continuous value of GA showed significantly better prediction than a cut-off value of 34 weeks (early-onset vs. late-onset). Indeed, some studies have shown that both women with early and late-onset preeclampsia are at risk of developing hypertension postpartum [13, 14] while others have reported increased risk of chronic hypertension when preeclampsia occurred <37 weeks in comparison to >37 weeks [10]. However, none of these studies reported the continuous value of GA, which should be considered in future studies. Our observation that GA at delivery, highest SBP during pregnancy and nulliparity were significantly associated with postpartum hypertension, supports the concept that the severity of the features of preeclampsia is an important determining factor [3, 6, 15]. Factors at 1 year postpartum that could influence blood pressure such as oral contraceptive use or breastfeeding were not taken into account due to the retrospective nature of this study. However, an effect of breastfeeding is not expected since ~85% of women already stop breastfeeding after 6 months [16].

Our study has some limitations. First of all, the number of women evaluated in this study is limited. A significant proportion of the initial study population (~50%) were lost to follow-up, which were mostly women with milder forms of preeclampsia. Nevertheless, our findings indicate that in women with (mostly severe forms of) preeclampsia, angiogenic factors are not a determining factor for the occurrence of hypertension at 1 year postpartum. Future studies

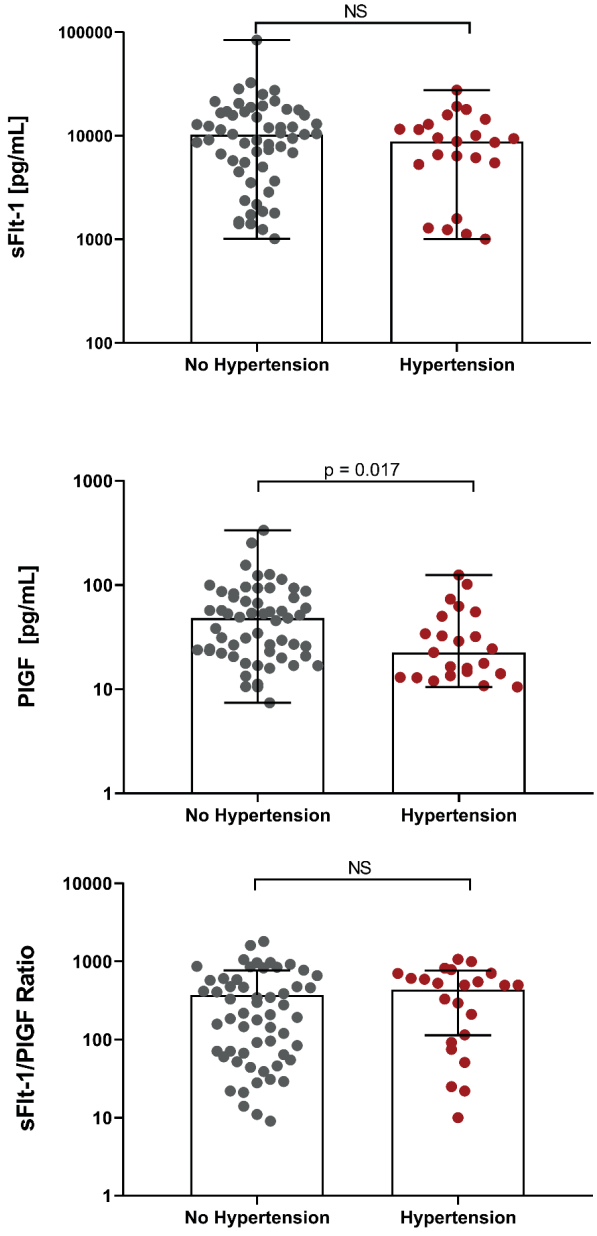

Figure 2. sFlt-1, PlGF and sFlt-1/PlGF ratio levels in the 80 women according to the occurrence of hypertension at 1 year follow-up. sFlt-1 indicates soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

Table 2. Difference in Pregnancy Characteristics between Included Women (n = 80) and Women that were Lost to Follow-Up (n = 75)

Parameter	Follow-Up	No Follow-Up	*P-Value*
Characteristics during pregnancy	80	75	
Age at study entry, yrs	30 (27 - 34)	32 (27 - 35)	0.39
GA at study entry, wks	30 (27 - 33)	35 (31 - 37)	<0.01
Pre-conceptional BMI, kg/m^2	23 (22 - 27)	25 (22 - 30)	0.11
Race, n (%)			
White	56 (70)	46 (61)	0.30
Black	9 (11)	16 (21)	0.08
Other	15 (19)	12 (16)	0.68
Nulliparous, n (%)	59 (74)	46 (61)	0.10
Smoking at inclusion, n (%)	4 (5)	4 (5)	1.00
History of preeclampsia, n (%)	8 (10)	9 (12)	0.69
Clinical parameters			
GA at diagnosis PE, wks	30 (27 -33)	36 (31 - 40)	<0.01
Highest SBP, mmHg	157 (140 - 165)	150 (140 - 160)	0.41
Highest DBP, mmHg	97 (90 - 105)	99 (90 -100)	0.94
Antihypertensive drug use, n (%)	56 (70)	44 (59)	0.18
Highest uPCR, g/mol	98 (38 - 262)	57 (32 - 153)	0.18
Highest Uric acid, mmol/L	0.37 (0.32 - 0.44)	0.36 (0.31 - 0.41)	0.44
Highest Creatinine, µmol/L	67 (58 - 73)	69 (56 - 78)	0.56
Highest ALAT, U/L	47 (24 - 175)	18 (13 - 35)	<0.01
Highest LD, U/L	289 (223 - 443)	235 (198 - 292)	<0.01
Lowest Platelet Count,10^9/L	149 (100 - 203)	163 (137 - 216)	0.04
Pregnancy outcome			
GA at delivery, weeks	30 (28 - 34)	37 (32 - 38)	<0.01
Girls, n (%)	40 (50)	30 (40)	0.43
Birth weight, grams	1210 (863 - 1798)	2505 (1435 - 3190)	<0.01
Birth weight percentile <10, n (%)	20 (25)	14 (19)	0.44
Time to delivery‡, days	2 (1 - 8)	5 (2 - 12)	0.08
Angiogenic markers			
sFlt-1, pg/mL	9405 (5066 - 15839)	6435 (1977 - 10495)	0.01
PlGF, pg/mL	32 (18 - 69)	74 (44 - 127)	<0.01
sFlt-1/PlGF ratio	296 (68 - 602)	67 (25 - 197)	<0.01

Values are median (interquartile range) or number (percentage). PE indicates preeclampsia; GA, gestational age: SBP, systolic blood pressure; DBP, diastolic blood pressure; uPCR, urinary protein-to-creatinine ratio; LD, lactate dehydrogenase; ALAT, alanine-aminotransferase; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor. *Biomarkers were determined at time of study entry. ‡Time to delivery is defined as the amount of days between study entry and delivery.

Table 3A. Univariable Analysis for the Prediction of Hypertension at 1 year Follow-up

All Women (n = 80)	Univariable Analysis		
	Odds Ratio	**C-index**	**P-Value**
Nulliparity	5.3 (1.1 – 25)	0.62	0.04
Highest SBP	2.1 (0.8 - 6.0)	0.65	0.04
Preconceptional BMI	2.9 (0.9 - 10)	0.66	0.15
GA at study entry*	0.3 (0.1 - 0.7)	0.74	<0.001
GA at PE diagnosis	0.3 (0.1 - 0.7)	0.73	<0.001
GA at PE diagnosis < 34wks	4.7 (0.9 - 21)	0.61	0.06
Time to delivery‡, (days)	1.4 (0.9 – 2.3)	0.64	0.09
GA at delivery	0.3 (0.1 - 0.7)	0.72	<0.001
sFlt-1	0.8 (0.3 - 1.7)	0.55	0.54
PlGF	0.3 (0.1 - 0.9)	0.67	0.06
sFlt-1/PlGF ratio	2.4 (0.8 - 7.7)	0.63	0.32

Hypertension defined as office SBP ≥140mmHg and/or office DBP≥90mmHg and/or antihypertensive drug use. Interquartile odds ratio and associated 95% confidence interval was calculated to aid interpretation of continuous predictors. It is defined as comparing the risk of hypertension after 1 year at the 75th percentile of the marker value versus the 25th percentile. SBP indicates systolic blood pressure; BMI, body mass index; GA, gestational age; PE, preeclampsia; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor. *Biomarkers were determined at time of study entry. ‡Time to delivery is defined as the amount of days between study entry and delivery.

Table 3B. Multivariable analysis for the Prediction of Hypertension at 1 year Follow-Up

All Women (n = 80)	Multivariable Analysis		
	Odds Ratio	**C-index**	**P-Value**
sFlt-1	0.7 (0.3 - 1.6)		0.68
GA at biomarker measurement	0.3 (0.1 - 0.7)	0.74	<0.01
PlGF	0.9 (0.2 - 4.4)		0.97
GA at biomarker measurement	0.3 (0.1 - 0.7)	0.74	0.03
sFlt-1/PlGF ratio	0.9 (0.2 - 3.6)		0.98
GA at biomarker measurement	0.3 (0.1 - 0.7)	0.74	<0.01

Hypertension defined as office SBP ≥140mmHg and/or office DBP≥90mmHg and/or antihypertensive drug use. Multivariable analysis includes GA at biomarker measurement with one of the angiogenic markers. Interquartile odds ratio and associated 95% confidence interval was calculated to aid interpretation of continuous predictors. It is defined as comparing the risk of hypertension after 1 year at the 75th percentile of the marker value versus the 25th percentile. GA indicates gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

Table 4. Correlations between Angiogenic Markers and Blood Pressure Profiles

Parameter	sFlt-1	PlGF	sFlt-1/PlGF ratio
During Pregnancy (n = 155)			
GA at study entry	-0.193*	0.672**	-0.539**
SBP at study entry	0.023	-0.144	0.118
DBP at study entry	-0.077	-0.077	0.020
Highest SBP	0.105	-0.216*	0.210*
Highest DBP	0.088	0.210*	0.213*
At 1 year Follow-Up (n = 80)			
Office SBP	-0.091	-0.103	0.014
Office DBP	0.029	-0.118	0.154

GA indicates gestational age; SBP, systolic blood pressure; DBP, diastolic blood pressure; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor. *p < 0.05, **p < 0.01.

should be conducted in a larger and a more heterogeneous group of women to establish whether this finding is specific to severe pre-eclampsia or that mild pre-eclamptic pregnancies show similar findings. Secondly, since the angiogenic markers vary with GA, it is important to evaluate them at a fixed time-point, preferably at the end of gestation when the largest alteration in biomarker levels occurs. Lastly, future studies should focus on defining hypertension based on 24-hour ABPM, since this is the most reliable method to diagnose hypertension and to identify participants with masked and white-coat hypertension.

In conclusion, this study is the first to assess the relationship between angiogenic markers and the occurrence of hypertension 1 year after delivery in a cohort of preeclamptic women. Our data illustrates that sFlt-1, PlGF and sFlt-1/PlGF ratio are not associated with hypertension 1 year postpartum, indicating they are not suitable for the prediction of hypertension and guiding of follow-up of women with previous (mostly severe) preeclampsia. We encourage future prospective studies to 1) validate our findings in a larger cohort of preeclamptic women 2) evaluate other cardiovascular biomarkers during pregnancy that could be associated with postpartum hypertension and other cardiovascular disease and lastly 3) to develop prognostic models to adequately stratify women who are at increased risk for developing chronic hypertension after preeclampsia.

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CHAPTER 06

Transfer and vascular effect of endothelin receptor antagonists in the human placenta

Emilie Hitzerd, **Rugina I. Neuman**, Michelle Broekhuizen, Sinno H.P Simons, Sam Schoenmakers, Irwin K.M. Reiss, Birgit C.P. Koch, Anton H. van den Meiracker, Jorie Versmissen, Willy Visser, A.H. Jan Danser

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ABSTRACT

Increasing evidence suggests a role for the endothelin (ET) system in preeclampsia (PE). Hence, blocking this system with endothelin receptor antagonists (ERAs) could be a therapeutic strategy. Yet, clinical studies are lacking due to possible teratogenic effects of ERAs. In this study we investigated the placental transfer of ERAs and their effect on ET-1-mediated vasoconstriction. Term placentas were dually perfused with the selective ET type A receptor (ET_AR) antagonists sitaxentan and ambrisentan or the non-selective ET_AR/ET_BR antagonist macitentan and subsequently exposed to ET-1 in the fetal circulation. ET-1 concentration-response curves after incubation with sitaxentan, ambrisentan, macitentan or the selective ET_BR antagonist BQ-788 were also constructed in isolated chorionic plate arteries using wire-myography, and gene expression of the ET-system was quantified in healthy and early onset PE placentas. At steady state, the mean fetal-to-maternal transfer ratios were 0.32±0.05 for sitaxentan, 0.21±0.02 for ambrisentan, and 0.05±0.01 for macitentan. Except for BQ-788, all ERAs lowered the response to ET-1, both in the perfused cotyledon and isolated chorionic plate arteries. Placental gene expression of ECE-1, ET_AR and ET_BR were comparable in healthy and PE placentas, while ET-1 expression was higher in PE. Our study is the first to show direct transfer of ERAs across the term human placenta. Furthermore, ET_AR exclusively mediates ET-1-induced constriction in the fetoplacental vasculature. Given its limited transfer, macitentan could be considered as potential PE therapy. Extending knowledge on placental transfer to placentas of PE pregnancies is required to determine whether ERAs might be applied safely in PE.

INTRODUCTION

Preeclampsia (PE) is a severe placenta-related pregnancy complication, characterized by de novo hypertension after 20 weeks of gestation, accompanied by evidence of maternal organ damage (e.g. proteinuria, elevated liver enzymes, pulmonary - or cerebral edema) and/or fetal growth restriction.[1] Besides increasing the risk of maternal and fetal morbidity and mortality during pregnancy, PE is associated with maternal and offspring health problems in later life.[2, 3] Over the last years, increased activity of the endothelin (ET) system has been recognized as a key factor in the pathogenesis of PE.[4] ET is a family of three potent vasoconstrictors (i.e. ET-1, -2 and -3), with ET-1 being the most abundantly synthesized by endothelial cells and syncytiotrophoblasts of the placenta.[5] Binding of ET-1 to the ET type A receptor (ET_AR) or ET type B receptor (ET_BR) on vascular smooth muscle cells leads to vasoconstriction and cell proliferation. In contrast, activation of ET_BR on endothelial cells stimulates vasodilation through the release of nitric oxide and prostacyclin.[6] It has been previously shown that ET-1 plasma levels are increased in women with PE compared to healthy pregnancy, and that ET-1 is an independent predictor of proteinuria in PE.[7, 8] Also, a decrease in ET_BR expression in vascular endothelial cells has been found in women with PE.[9] Similarly, PE animal models have shown a significant increase in the expression of the precursor peptide prepro-ET-1, likely leading to higher levels of ET-1, causing hypertension and renal dysfunction.[10-12] Blocking the effect of ET-1 with endothelin receptor antagonists (ERAs) alleviated maternal PE symptoms and improved fetal growth in animal studies.[13-16] However, developmental toxicity studies have also shown serious teratogenic effects, mainly craniofacial and cardiovascular malformations, in offspring of animals treated with ERAs during pregnancy, arguing against clinical trials in pregnant women.[17-19] It should be noted that teratogenic effects might be species-specific – indeed, certain drugs (e.g. corticosteroids) are known to be teratogenic in mice and rats but safe in humans.[20] Moreover, 39 cases of ERA use during pregnancy in women with pulmonary hypertension have been presented in the literature, and none of these reported teratogenic effects.[21] This raises the possibility that ERA treatment might still be an option for severe PE if applied later in pregnancy, thereby avoiding potential teratogenic effects. Since knowledge is lacking regarding the use of ERAs in human pregnancy, the aim of this study was to investigate the placental transfer of different ERAs making use of an *ex vivo* placental perfusion model, and to evaluate the effect of ERAs on ET-1 mediated vasoconstriction in the fetoplacental vasculature, comparing both healthy and PE placentas.

METHODS

Patients and setting

Placentas of women with uncomplicated singleton pregnancies who underwent an elective cesarean section, or women with severe early onset PE (diagnosis ≤ 34 weeks of gestation[22])

were collected immediately after delivery at the Erasmus Medical Center, Rotterdam, the Netherlands. Baseline characteristics were obtained from the digital medical files. The study was exempted from approval by the local institutional Medical Ethics Committee according to the Dutch Medical Research with Human Subjects Law (MEC-2016-418 and MEC-2017-418). All women who donated their placenta provided written informed consent for the use of their placenta and personal data regarding their pregnancy.

Perfusion experiments

The perfusion model used in the current study was previously described extensively by Hitzerd *et al*.[23] Perfusion experiments were conducted in healthy placentas only, given the extreme difficulty to successfully perfuse a preterm (PE) placenta.[23] In brief, maternal and fetal perfusion media consisted of Krebs-Henseleit buffer at 37ºC, supplemented with heparin (final concentration; 2500 IU/L) and aerated with 95% O2 - 5% CO2. The fetal circulation (closed-circuit; flow rate 6 mL/min) was established by cannulating the chorionic artery and corresponding vein of an intact cotyledon. Maternal circulation (closed-circuit; flow rate 12 mL/min) was created by placing four blunt cannulas in the intervillous space. At t=0, at a concentration of ~10x C_{max}, either one of the selective ET_AR antagonists sitaxentan (100 mg/L,[24] a kind gift of dr. M. Iglarz, Actelion, Allschwill, Switzerland) or ambrisentan (10 mg/L,[25] Sigma-Aldrich Chemie, Schnelldorf, Germany), or the non-selective antagonist macitentan (2 mg/L,[26] a kind gift of dr. M. Iglarz) was added to the maternal circulation. Such high concentrations were chosen to prevent underestimation of transfer. To prove good overlap between maternal and fetal circulations antipyrine (100 mg/L) was also added to the maternal buffer. FITC-dextran (40 kDa, 36 mg/L) in the fetal circulation was used as a marker of integrity of the capillary bed. Samples of the maternal and fetal circulations were taken at eight set time points, and immediately stored at -80°C. After 180 min of perfusion, ET-1 (0.1-100 nmol/L) was added to the fetal circulation to construct a concentration-response curve (CRC). These concentrations are higher than the ET-1 concentrations observed in blood,[7] in agreement with the concept that ET-1 normally is synthesized locally, resulting in abluminal concentrations that are far above those in the circulation. An ET-1 CRC was also performed in placentas that were perfused for the same duration without an ERA, to serve as controls. Changes in pressure were measured by pressure transducers and recorded using acquisition software (Biopac, Goleta, CA, USA).

Quality control

An experiment was considered successful when the fetal-to-maternal (F/M) ratio of antipyrine was >0.75 and the maternal-to-fetal (M/F) ratio of FITC-dextran <0.03 at t=180.

Analysis of antipyrine and FITC-dextran

For measuring antipyrine concentration, samples were first deproteinized with perchloric acid 6%, and subsequently a mixture of 0.2 mg/mL $NaNO_2$ and 0.6% H_2SO_4 was added in a 1:1 ratio to form nitroantipyrine. Absorption was measured at 350 nm using ultraviolet–visible spectroscopy (Shimadzu UV-1800). For analysis of FITC-dextran, fluorescence was measured using a Multiwell Plate Reader (Victor X4 Perkin Elmer, excitation/emission 485/519 respectively).

LC-MS analysis of endothelin receptor antagonists

Ambrisentan, macitentan and sitaxentan concentrations were measured in the perfusate by using UPLC-MS/MS. The method was validated in a linear range of 20.28 - 2028 µg/L for ambrisentan, 4.052 - 405.2 µg/L for macitentan and 205.4 – 10 270 µg/L for sitaxentan. The method was successfully validated according to FDA guidelines and is used in our pharmacy laboratory for research and patient analysis.

Wire-myography experiments

Second order branches of chorionic plate arteries of both healthy and PE placentas were cut into segments of 2 mm and mounted in 6-mL organ baths (Danish Myograph Technology, Aarhus, Denmark), filled with Krebs-Henseleit buffer at 37°C and aerated with 95% O_2 – 5% CO_2. Tension was normalized to 90% of the estimated diameter at 100 mmHg effective transmural pressure. Maximum contractile responses were determined using 100 mmol/L potassium chloride (KCl). After washout of the KCl, vessel segments were incubated with sitaxentan (200 µmol/L), macitentan (3 µmol/L), ambrisentan (10 µmol/L) or the selective ET_BR antagonist BQ-788 (10 nmol/L, Sigma-Aldrich Chemie, Schnelldorf, Germany). For sitaxentan, macitentan and ambrisentan the same concentrations were used as in the perfusion experiments (~10x C_{max}) and the concentration of BQ-788 was based on previous experiments with this antagonist.[27] Vessel segments without any inhibitor were used as control. After an incubation period of 30 minutes, CRCs to ET-1 (0.1-100 nmol/L) were constructed.

Quantitative PCR (qPCR) Analysis

Gene expression levels of ET-1 (EDN1), ET_AR, ET_BR and endothelin converting enzyme-1 (ECE-1) were measured with qPCR analysis. ECE-1 is an enzyme involved in converting the precursor ET-1 gene into biologically active ET-1.[28] After delivery of the placenta, pieces of placental tissue were immediately dissected from both the decidual (‘maternal’) and the amniotic (‘fetal’) side of the placenta, and were subsequently snap frozen in liquid nitrogen. As described previously by Hitzerd *et al.,*[23] small tissue pieces were homogenized in RLT

lysis buffer (Qiagen, Venlo, the Netherlands) with β-mercaptoethanol for RNA extraction. Total RNA was extracted (RNeasy Fibrous Tissue Mini Kit, Qiagen) after proteinase K treatment (Invitrogen, Breda, the Netherlands) for ten minutes at 55°C. RNA was eluted in RNase free water and concentration and purity were assessed with a NanoDrop1000 Spectrophotometer (Thermo Fisher Scientific, Bleiswijk, the Netherlands). Complimentary DNA (cDNA) was synthesized from 0·5 µg RNA template with the SensiFast cDNA Synthesis Kit (Bioline, London, UK) according to the manufacturer's instructions. This cDNA was used for qPCR using the SYBR Green qPCR Kit (Bioline, London, UK) and specific primer pairs on a CFX-96 light cycler (Bio-Rad, Hercules, CA, USA). The primer pairs used in this article are listed in Table S1. Target genes were normalized against the reference genes β-actin and Peptidylprolyl Isomerase A (PPIA) and relative gene expression was calculated by the ΔΔCt method. qPCR was performed according to the following conditions: initial denaturation at 95°C for eight min and 30 s, followed by 40 cycles comprising 15 s at 95°C, and one min at 60°C.

Statistical analysis

Data are presented as mean ± SEM for normally distributed data or median (interquartile range) in case of skewed distributions. Statistical analysis was performed with GraphPad Prism (version 5, La Jolla, CA, USA) and SPSS (version 21, SPSS Chicago, IL, USA) on Windows. To compare groups the Student's *t* test or Mann-Whitney U test (in case of non-normally distributed data) were used. For the comparison of continuous variables between more than two groups, one-way ANOVA or Kruskall-Wallis test (in case of skewed distributions) was applied, with a Dunnet or Bonferroni correction for multiple testing. A P-value of <0.05 was considered to be statistically significant.

RESULTS

Placental transfer of endothelin receptor antagonists

Forty-three women were initially included in the study. Twenty-three out of 43 cotyledons met the quality control criteria and were included in the analysis, leading to a success percentage of 53%, which is comparable to previous research from our lab.[23] Maternal characteristics as well as clinical characteristics of the placentas and offspring are shown in Table 1. There were no significant differences between groups. At t=180 the mean F/M ratio for antipyrine was 0.92±0.01, indicating adequate overlap between fetal and maternal circulations (Figure S1). Figure 1 shows the placental transfer of sitaxentan, ambrisentan and macitentan. Only in the case of macitentan, the t=0 level was lower than its level thereafter, suggesting that the distribution across the maternal reservoir occurred somewhat slower than that of sitaxentan and ambrisentan. After 180 min of perfusion the F/M ratio for sitaxentan (n=5) was

0.32±0.05 (Figure 1A). At this steady state condition, only 33±4% of the total added sitaxentan concentration was recovered in the fetal and maternal circulations together. Adherence experiments (running the experiment without a placenta) showed only ~9% tube adherence, indicating that ~60% of the added sitaxentan had accumulated in the placental tissue. For ambrisentan (n=5), the F/M ratio was 0.21±0.02 and 80±6% of the starting concentration was retrieved after 180 min of perfusion (Figure 1B). No tubal adherence was observed. Only minimal amounts of macitentan (n=5) passed the placental barrier (F/M ratio 0.05±0.01), resulting in a fetal concentration of around 150 nmol/L (5%) after three hours of perfusion. Most of the added concentration (86±6%) was still detectable in the maternal and fetal circulations at t=180, therefore no tissue accumulation occurred (Figure 1C).

Since no albumin was used in the current setup, corrections for protein binding were applied by adjusting the F/M ratios using the method of Hill and Abramson (for the exact calculation see Supplemental Methods).[29] No major changes were seen in the F/M ratios of sitaxentan, ambrisentan and macitentan (adjusted ratios 0.37, 0.25 and 0.06, respectively). The F/M ratios for all drugs are summarized in Table S2.

Placental vascular reactivity

After 180 min of perfusion, placentas were exposed to increasing concentrations of ET-1 in the fetal circulation to evaluate the effect of maternally applied ERAs on the fetoplacental vasculature. There was no significant difference in baseline pressure at start of the ET-1 curve between controls and ERA-exposed placentas (Figure 2A). After adding the highest concentration of ET-1 (100 nmol/L) to the fetal side of control placentas, the pressure was increased to 181±16 mm Hg (Figure 2A and 2B). Placentas that had been exposed maternally to sitaxentan, ambrisentan and macitentan all showed attenuated pressure increases to fetally applied ET-1 (pressures of 76±19, 88±32 and 120±15 mm Hg, respectively), which were significant for sitaxentan (P=0.002) and ambrisentan (P=0.01), but not macitentan (P=0.09, Figure 2A and 2B).

Wire-myography experiments

Chorionic plate arteries of 11 healthy and five PE placentas were included in these experiments. The clinical characteristics of these placentas are shown in Table 2.

Table 1. Clinical characteristics of perfused placentas.

	Sitaxentan (n=5)	**Ambrisentan (n=5)**	**Macitentan (n=5)**	**Control (n=8)**
Maternal age (y)	34 (33-37)	30 (25-37)	34 (28-35)	35 (32-36)
Parity	1 (1-2)	1 (0.5-1)	1 (1-3)	1 (1-2)
Western ethnicity (n)	2	2	3	4
Body mass index (kg/m^2)	26.0 (21.2-34.0)	28.0 (20.8-32.5)	21.8 (20.8-39.9)	29.4 (22.5-34.6)
Smoking (n)	0	0	0	1
Highest DBP (mm Hg)	80 (73-83)	75 (65-81)	80 (65-80)	78 (71-83)
Gestational age (weeks)	39 (39-40)	39 (39-39)	39 (39-39)	39 (38-39)
Fetal sex (M/F)	2/3	1/4	2/3	2/6
Birth weight (g)	3620 (3363-4078)	3500 (3258-4085)	3360 (3320-3828)	3428 (3153-3610)
Birth weight (centile)	63 (55-95)	68 (44-94)	52 (47-87)	59 (43-63)
Placental weight (g)	645 (597-790)	618 (543-747)	773 (616-827)	631 (610-689)

Data are presented as median (interquartile range). There were no significant differences between groups (Kruskal-Wallis test). DBP = diastolic blood pressure; F = female; M = male.

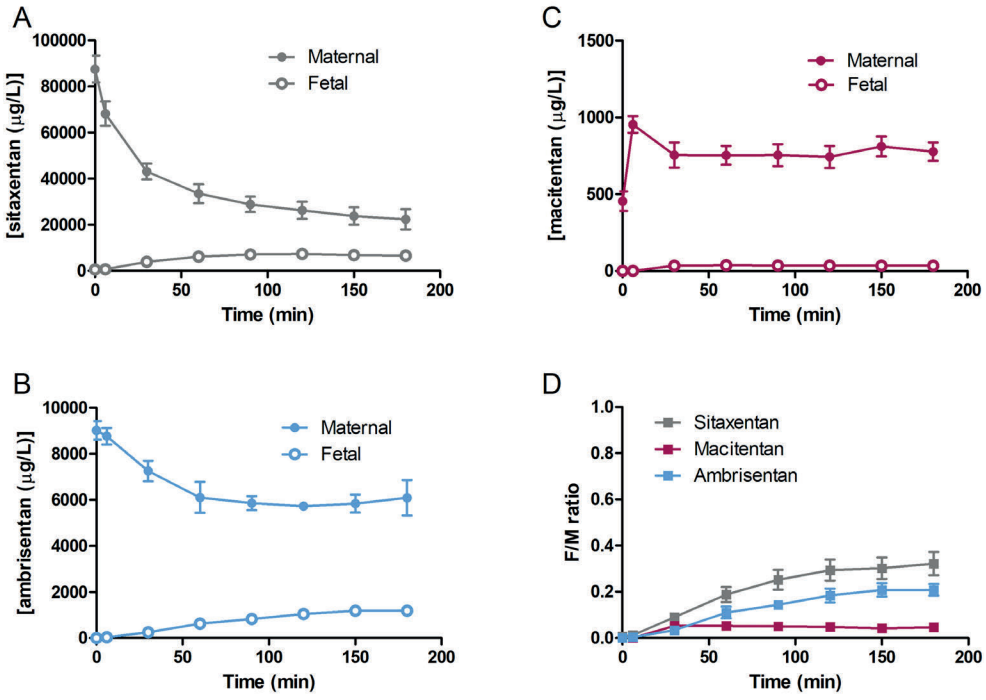

Figure 1. Placental transfer of sitaxentan (A), ambrisentan (B), and macitentan (C). Measured concentrations in the maternal (closed circles) and fetal (open circles) circulations are expressed as micrograms per liter, n=5 per group. D, Shows the fetal-to-maternal (F/M) transfer ratios over time.

The results of these experiments are shown in Figure 3. At its highest concentration (100 nmol/L), ET-1 elicited a constriction corresponding with 132±9% of KCl constriction in control segments of healthy placentas (Figure 3A). Vessel segments that had been pre-incubated with sitaxentan, ambrisentan and macitentan all displayed a significant decreased response to 100 nmol/L (response 1±1, 2±1 and 10±3% of KCl constriction, respectively, $P<0.0001$ for all), whereas incubation with BQ-788 did not alter the response to 100 nmol/L ET-1 (130±14% of KCl constriction). Data on vessel segments that had been pre-incubated with different concentrations of macitentan are shown in Figure S2, and confirm the concentration-dependency of its blocking effect. Vessel segments of PE placentas displayed similar ET-1 responses as those of healthy placentas (Figure 3B). The effect of 100 nmol/L ET-1 in control segments was 112±38% of KCl constriction, compared to 1±1, 1±1 and 6±4% of KCl constriction for segments pre-incubated with sitaxentan, ambrisentan and macitentan, respectively ($P<0.01$ for all). There were no differences in the response to 100 nmol/L ET-1 between healthy and PE placentas (Figure 3C).

Gene expression

Gene expression of ET-1 (EDN1), ET_AR and ET_BR, but not ECE-1, was lower on the amniotic side of the placenta compared to the decidual side, both in healthy and PE placentas (Figure 4). In PE placentas, there was increased gene expression of ET-1 on both the decidual and amniotic side (P=0.02 and 0.06, respectively). No changes in the expression of ET_AR, ET_BR and ECE-1 were observed in PE.

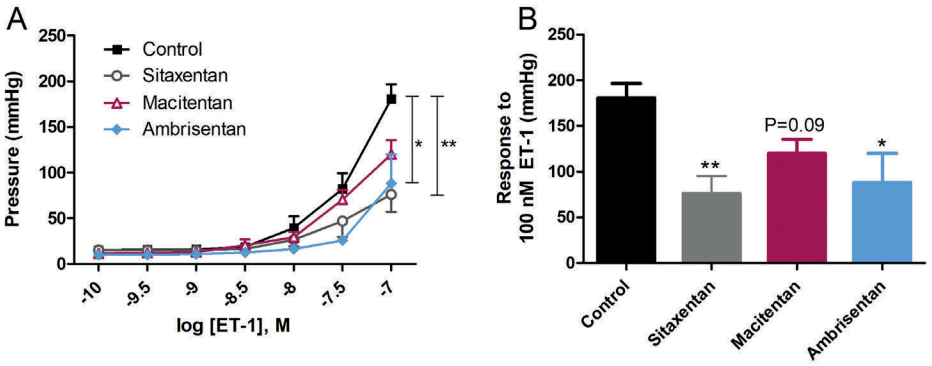

Figure 2. A, Shows the concentration response curves for fetally applied ET-1 (endothelin-1) in the perfused cotyledon without (control, squares) or with prior exposure of the maternal circulation to the endothelin receptor antagonists sitaxentan (circles), macitentan (triangles), or ambrisentan (diamonds). The effect achieved at 100 nmol/L ET-1 is shown in (B). Responses are expressed as mean±SEM of n=5–8. *P<0.05, **P<0.01 vs control (1-way ANOVA with Dunnett post hoc evaluation).

Table 2. Clinical characteristics of placentas used for wire-myography experiments.

	Healthy (n=11)	**PE (n=5)**
Maternal age (y)	32 (28-34)	29 (27-32)
Parity	1 (1-3)	0 (0-0.5)*
Western ethnicity (n)	6	4
Body mass index (kg/m^2)	25.7 (22.3-30.5)	24.9 (19.5-25.7)
Smoking (n)	0	0
Highest DBP (mm Hg)	80 (75-80)	103 (98-111)*
Gestational age (weeks)	39 (39-40)	30 (28-31)*
Fetal sex (M/F)	5/6	3/2
Birth weight (g)	3410 (3200-3645)	920 (708-1281)*
Birth weight (centile)	52 (43-68)	1 (0.3-2.3)*
Placental weight (g)	654 (610-769)	285 (243-395)*

Data are presented as median (interquartile range). *P<0.05, Mann-Whitney U test. DBP = diastolic blood pressure; F = female; M = male; PE = preeclampsia.

DISCUSSION

This study is the first to show direct transfer of ERAs across the human placental barrier. Importantly, the transfer of macitentan was limited (F/M ratio <0.1), while that of sitaxentan and ambrisentan was substantial although no accumulation occurred (F/M ratio <1.0). In line with this, only sitaxentan and ambrisentan (when applied maternally) significantly reduced the contractile effect of fetally applied ET-1 in the cotyledon set-up. Yet, all antagonists, when applied at a concentration corresponding with 10 times C_{max} to isolated chorionic plate arteries, were capable of fully blocking the contractile effect of ET-1. In contrast, the selective ET_BR antagonist BQ-788 did not affect the ET-1 response, arguing against a role for ET_BR. No differences were observed in the vascular response to ET-1 between healthy and PE placentas. Gene expression of ET-1 and its receptors was lower on the amniotic side of the placenta compared to the decidual side. Furthermore, PE placentas showed an increased expression of ET-1, while no changes in the expression of ET_AR, ET_BR and ECE-1 were found.

A significant role for the ET-system has been implicated in the pathogenesis of PE, contributing to hypertension, endothelial- and renal dysfunction with proteinuria.[7, 30] Aside from increased circulating ET-1 levels in preeclamptic women, placental ET-1 levels are similarly elevated, which may account for the increased placental vascular resistance *in vivo*.[8] Thus, administration of ERAs may prove beneficial through alleviation of maternal symptoms and by improving vascular resistance in the placenta. Interestingly, we and others found no difference in ET-1 induced vasoconstriction between chorionic plate arteries of healthy and PE placentas, indicating that there is no altered response to ET-1 during PE. Moreover, while administration of an ERA significantly attenuated ET-1 induced vasoconstriction, this effect was not different between healthy or PE placentas. Taken together, these data, obtained in two different models (the perfusion setup, representing predominantly microcirculatory vasculature, and the myography setup, involving larger second-order arteries) imply that elevated ET-1 levels, but not an enhanced response, accounts for the increased placental vascular resistance during PE. Indeed, we observed elevated expression of the ET-1 gene in PE placentas, while ET_AR and ET_BR expression did not differ between healthy and PE placentas. In a previous study by Benoit *et al*. blockade of ET_AR, but not ET_BR reduced vasoconstriction in healthy placentas when they were exposed to extracts from PE placentas.[31] In the current study, we observed similar results regarding exposure to ET-1, indicating that ET-1-induced vasoconstriction in the fetoplacental vasculature is exclusively mediated by ET_AR. It should be mentioned however, that we were not able to extend these studies towards uterine spiral arteries, since we did not obtain myometrial biopsies.

Given the observed fetotoxicity of ERAs in developmental toxicity studies in animals, no clinical trials in pregnant women have been performed with these drugs, and therefore knowledge regarding the placental transfer of ERAs in humans is virtually non-existent.

However, the placenta is the most species-specific organ, making direct translation of the results of animal studies to humans challenging.[32] As treatment for PE is generally started in the second or third trimester, i.e. when fetal organogenesis has already been completed, one should not disregard ERAs as potential treatment for PE. Moreover, sporadic cases of pregnant women with pulmonary arterial hypertension (PAH) using ERAs in the second or third trimester did not report an increased incidence of fetal birth defects.[21] Importantly, it should be noted that sitaxentan has been withdrawn from therapeutic use in 2010 due to drug-induced hepatotoxicity, while ambrisentan and macitentan are both registered for the treatment of PAH.

A striking finding of our study is the limited transfer observed of the non-selective antagonist macitentan. Whereas all three ERAs used in this study have low molecular weights (<600 g/mol) and lipophilic properties, which in general would favor placental transfer,[33] macitentan is characterized by sustained receptor binding and enhanced tissue penetration.[34] However, this does not seem to explain the limited transfer, since 86% of the starting concentration was recovered at the end of an experiment in the fetal and maternal circulations together. Although the absence or presence of albumin in the perfusion system should not affect the F/M ratio at steady state,[35] to better predict *in vivo* fetal exposure to ERAs we also corrected the results of the current study for protein binding. Yet, this did not alter the outcome of the study, nor did lowering the fetal pH to 6.9.

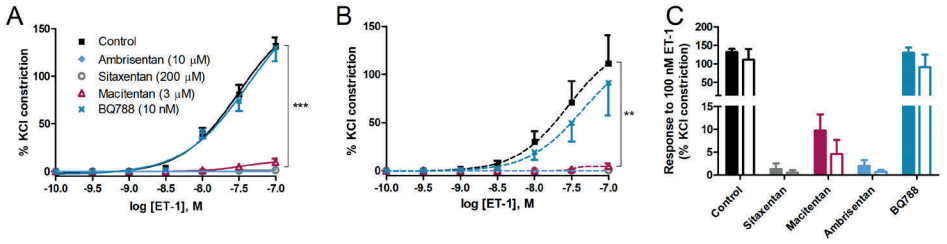

Figure 3. Vascular responses of isolated chorionic plate arteries of healthy (A) and preeclamptic (B) placentas to ET-1 (endothelin-1) in the absence (control, squares) or presence of sitaxentan (circles), macitentan (triangles), ambrisentan (diamonds), or BQ-788 (cross marks). C, Shows the response to 100 nmol/L ET-1 of healthy (closed bars) compared with preeclamptic (open bars) placentas. Responses are expressed as mean±SEM of n=5 to 11. *P<0.05, **P<0.0001 vs control (1-way ANOVA with Dunnett post hoc evaluation).

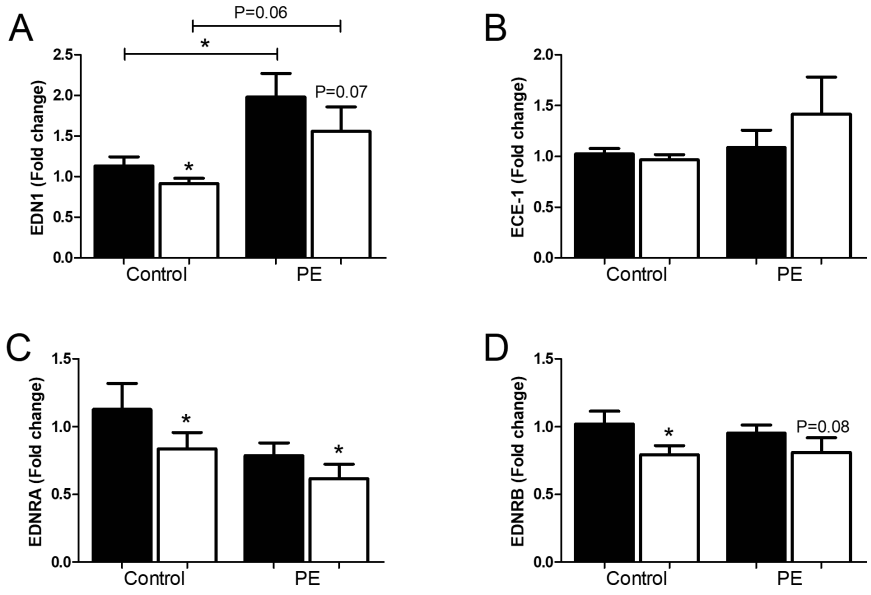

Figure 4. Gene expression of endothelin-1 (A), endothelin converting enzyme-1 (B), endothelin-receptor type A (C), and endothelin- receptor type B (D) in healthy and preeclamptic placentas (n=12 per group). Expression was measured on both the decidual side (black bars) and amniotic side (white bars) of the placenta. Data are expressed as fold change of control samples from the decidual side. *P<0.05 (unpaired or paired Student t-test where appropriate). PE indicates preeclampsia.

Despite its almost absent placental transfer, the fetal vascular bed of the macitentan-perfused placentas did display a (non-significant) decrease in contractile response to ET-1 of approximately 34%. Here it should be noted that we applied a maternal macitentan concentration of 3 µmol/L, resulting in a fetal concentration of around 150 nmol/L (5%) after three hours of perfusion. Wire-myography experiments with chorionic plate arteries in which different concentrations of macitentan were used, revealed that such a reduction would indeed be expected at 150 nmol/L (Figure S2). The approved macitentan dosage for PAH treatment is 10 mg per day,[36] and with this dosage the C_{max} in plasma of (non-pregnant) Caucasian women is 234 ng/mL, or, at a MW of 588, 0.4 µmol/L.[26] If indeed 5% of this concentration would reach the fetus (~20 nmol/L), it cannot be excluded that a modest degree of blockade occurs in the fetus. Blocking the ET-system in the fetus could lead to undesirable effects, since ET-1 plays an important role in the maintenance of high vascular resistance crucial for fetal lung development.[37] The ET_AR is abundantly expressed in fetal lungs during pre- and postnatal periods, and lung maturation is not completed at the end of third trimester.[38] On the other hand, ET_AR blockade could prove beneficial, since elevated serum ET-1 levels are observed in babies that are born from PE pregnancies.[39] Moreover, one of the standard treatments for neonates with pulmonary hypertension is the non-selective ERA bosentan.[40, 41] Although no

long-term follow-up studies have been performed, short-term follow-up showed no adverse effects on lung- and brain development in these children.[40] Another point of concern could be patency of the ductus arteriosus after birth. However, the evidence regarding the effect of ERAs on closure of the ductus is conflicting. Although it has been shown that oxygen-triggered ET-1 release regulates closure of the ductus through binding to ET_AR on vascular smooth muscle cells,[42] ET_AR blockade does not seem to prevent the ductus from closing.[43, 44]

PERSPECTIVES

Given its key role in the pathogenesis of PE, targeting the ET-system would be an interesting approach in the treatment of this severe placenta-related disease. This study was the first to evaluate transfer of ERAs across the human placenta. Since macitentan only displayed very limited placental transfer, and is already registered for treatment of PAH, it could be a promising drug to further investigate for PE treatment. Expanding this knowledge to early onset PE placentas is needed to further evaluate whether it can be safely applied in pregnancy. Furthermore, third-trimester toxicology studies in animals with a longer gestation than rodents are warranted. Subsequently, as described previously,[21] we would suggest a proof of principle study in women with severe early onset PE (< 24 weeks of gestation), when medically indicated termination of pregnancy is considered because of disease severity, to evaluate the effect on maternal PE symptoms and neonatal outcome.

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SUPPLEMENTAL DATA

METHODS

Protein binding adjustment

Albumin was not added to the experimental system, since it is very difficult to mimic exact physiological concentrations. Although the F/M ratio of the free drug concentration at steady state should not be affected by the presence or absence of albumin,[1, 2] the obtained F/M ratios were adjusted for protein binding to estimate the fetal exposure as adequate as possible. The ratios were adjusted using the following formula:[3]

$$\text{F/M ratio} = \frac{\%\ \text{unbound}_M}{\%\ \text{unbound}_F} \times \frac{1+10^{pKa - pH(F)}}{1+10^{pKa - pH(M)}} \times \frac{CL_{MF}}{CL_{FM} + CL_f}$$

In this formula the differences in protein binding between both circulations are taken into account, as the % unbound_F and the % unbound_M represent the free drug concentrations in the maternal and fetal circulations, respectively. pKa is the acid-base dissociation constant of the drug, and pH(F) and pH(M) are the pH values of fetal (7.35) and maternal (7.40) blood, respectively. The last part of the equation stands for drug clearance (MF = maternal-to-fetal, FM = fetal-to-maternal and f = fetus). In the closed perfusion setup, CL_{MF}/CL_{FM} can be taken as the F/M ratio at steady state. The assumption was made that clearance by the fetus was negligible. Because ERAs are strictly contraindicated, there is no available data regarding the % protein binding in plasma of pregnant women and their fetus. However, it is known that in non-pregnant adults all three ERAs used in the current study bind for ~99% to albumin. With this information, protein binding in maternal and fetal plasma could be estimated using the method of Hill and Abramson:[1]

$$\%\ \text{unbound} = 100 - \frac{100 \times (B/F)}{(B/F) + 1}$$

B/F is the ratio between bound (B) and free (F) drug concentrations. This can be calculated for the maternal and fetal circulations, using the known B/F of non-pregnant adults and the plasma protein ratios for albumin (fetal:non-pregnant adult ratio is 0.866 and the maternal:non-pregnant adult ratio is 0.733.[1]

$$(B/F)_{F\ \text{or}\ M} = (B/F)_{\text{non-pregnant}} \times (\text{Plasma albumin ratio})_{F\ \text{or}\ M}$$

All three ERAs have a B/F of 99 in non-pregnant adults. From this we calculated the B/F in the fetal (85.7) and maternal (72.6) circulations, leading to unbound fractions of 1.2% and 1.4%,

respectively. The pKa of macitentan is 6.2, of ambrisentan 3.5 and of sitaxentan 5.0,[4-6] leading to corrected F/M ratios of 0.06, 0.25 and 0.37, respectively.

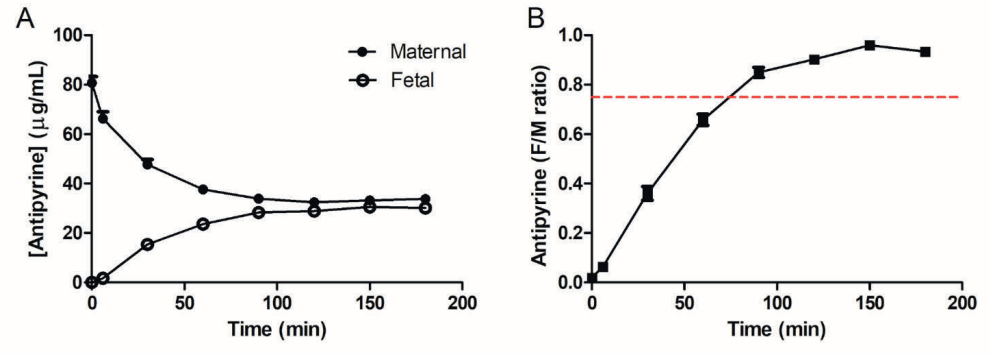

Figure S1. Antipyrine transfer.
Panel A shows the mean maternal (closed circles) and fetal (open circles) concentrations of antipyrine, proving good overlap between both circulations. Panel B shows the mean fetal-to-maternal transfer ratio, with the cutoff point of 0.75 (red dashed line).

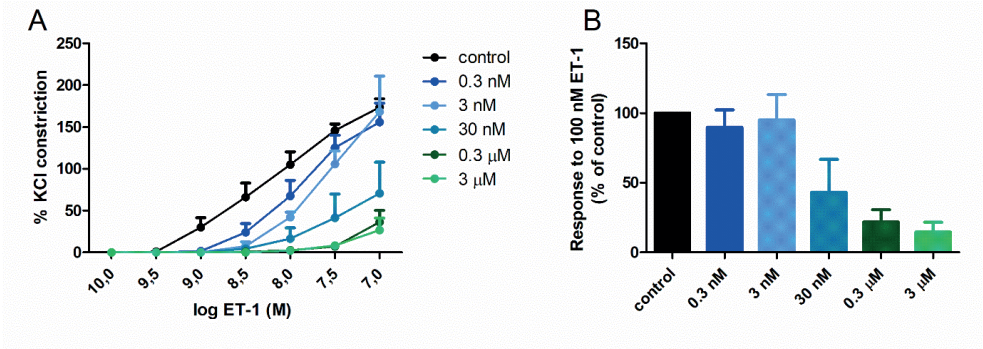

Figure S2. Blocking effect of different macitentan concentrations in chorionic plate arteries.
Concentration-response curves to endothelin (ET)-1 are shown in the absence (control) or presence of different macitentan concentrations (A). Panel B shows the blocking effect of those different concentrations expressed as % of maximum contraction to 100 nmol/L ET-1 in control segments.

Table S1. qPCR Primer Sequences.

Genes	Forward (5' - 3')	Reverse (5' - 3')
EDN1	AAGACAAACCAGGTCGGAGAC	GTCACCAATGTGCTCGGTTG
EDNRA	GTATTTAAGCTGCTGGCTGGG	GAGGTTGAGGACGGTGATCC
EDNRB	ATCACCTAAAGCAGAGACGGG	AGAATCCTGCTGAGGTGAAGG
ECE-1	AAGCTCCTTCCTTGACCAGC	GACAGGTCTTCTTGGTCCCG

Table S2. F/M ratios according to different drugs
Fetal-to-maternal (F/M) ratios are shown for sitaxentan, ambrisentan and macitentan. Adjusted F/M ratio indicates correction for protein binding using the method of Hill and Abramson.

ERA	F/M ratio	Adjusted F/M ratio
Sitaxentan	0.32	0.37
Ambrisentan	0.21	0.25
Macitentan	0.05	0.06

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CHAPTER 07

The sFlt-1 to PlGF Ratio in Pregnant Women with Rheumatoid Arthritis: Impact of Disease Activity and Sulfasalazine Use.

Rugina I. Neuman, Hieronymus T.W Smeele, A.H Jan Danser, Radboud J.E.M Dolhain, Willy Visser

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ABSTRACT

Objectives. An elevated sFlt-1/PlGF-ratio has been validated as a significant predictor of preeclampsia, but has not been established in women with rheumatoid arthritis (RA). We explored whether the sFlt-1/PlGF-ratio could be altered due to disease activity in RA, and could be applied in this population to predict preeclampsia. Since sulfasalazine has been suggested to improve the angiogenic imbalance in preeclampsia, we also aimed to examine whether sulfasalazine could affect sFlt-1 or PlGF levels.

Methods. Making use of a nationwide, observational, prospective cohort study on pregnant women with RA, sFlt-1 and PlGF were measured in the third trimester. A total of 221 women, aged 21 – 42 years, were included, with a median gestational age of 30+3 weeks.

Results. No differences in sFlt-1 or PlGF were observed between women with high, intermediate or low disease activity ($p = 0.07$ and $p = 0.41$) , whereas sFlt-1 and PlGF did not correlate with DAS28-CRP score ($r=-0.01$ and $r=-0.05$, respectively). Four (2%) women with a sFlt-1/PlGF-ratio ≤ 38 developed preeclampsia in comparison to three (43%) women with a ratio>38, corresponding to a negative predictive value of 98.1%. Sulfasalazine users (n=57) did not show altered levels of sFlt-1 or PlGF in comparison to non-sulfasalazine users (n =164, $p=0.91$ and $p=0.11$).

Conclusion. Our study shows that in pregnant women with RA, the sFlt-1/PlGF-ratio is not altered due to disease activity and a cut-off ≤ 38 can be used to exclude preeclampsia. Additionally, sulfasalazine use did not affect sFlt-1 or PlGF levels in this population.

INTRODUCTION

Preeclampsia, a multisystem disorder affecting 2 – 8% of all pregnancies, remains a significant cause of maternal and perinatal morbidity and mortality.[1, 2] While the pathophysiological mechanisms underlying preeclampsia are not entirely elucidated, it is thought that poor placentation triggers the release of the soluble vascular endothelial growth factor receptor-1 (sVEGFR-1, also known as sFlt-1) which antagonizes the proangiogenic VEGF and placental growth factor (PlGF).[1, 2] This anti-angiogenic environment along with increased inflammatory cytokines leads to the widespread endothelial dysfunction typically associated with the disorder.[1] Importantly, the sFlt-1/PlGF ratio, when applied with a cut-off value of ≤38, has become a clinically valuable tool to exclude preeclampsia in women suspected of the disorder.[3] However, the use of this ratio has not been evaluated in pregnant women with diseases characterized by high levels of inflammation, such as rheumatoid arthritis (RA). RA is a common, systemic auto-immune disorder, characterized by synovial inflammation causing joint destruction.[4] In RA, raised sFlt-1 values in both the sera and synovial fluid are observed,[5, 6] whereas serum sFlt-1 levels are positively correlated with disease activity.[6] We hypothesized that the sFlt-1/PlGF ratio is significantly elevated in pregnant women with RA. The first aim of this study was to investigate whether sFlt-1 and/or PlGF are altered in pregnant women with RA in relation to disease activity, and to evaluate whether an sFlt-1/PlGF ratio of ≤38 could be used to exclude preeclampsia in this population.

Very recently, it was proposed on the basis of in-vitro data that sulfasalazine, a commonly used drug to treat RA, has potential to restore the anti-angiogenic imbalance observed in preeclampsia, through lowering placental sFlt-1 secretion and causing an upregulation of PlGF.[7] Sulfasalazine is an anti-inflammatory and anti-oxidant medication that is safe for use during pregnancy.[8] Remarkably, to what degree sulfasalazine itself or its metabolites sulfapyridine and 5-aminosalicylic acid (5-ASA) are responsible for its beneficial effects, is still unknown.[9, 10] Therefore, only an in-vivo study can truly address to what degree sulfasalazine might restore the angiogenic imbalance in preeclampsia. Hence, the secondary objective of this study was to evaluate whether in pregnant women with RA, the use of sulfasalazine is associated with lower sFlt-1 and higher PlGF levels.

METHODS

Study design and population

The present study is embedded within the Pregnancy-induced Amelioration of Rheumatoid Arthritis (PARA) study, a nationwide prospective cohort study conducted between 2002 and 2010 on pregnancy in women with RA in the Netherlands.[11] Patients who had a diagnosis of RA according to the American College of Rheumatology 1987 revised criteria were eligible for participation if they were planning to conceive or were already pregnant. For the current analysis, only those patients who were assessed in the third trimester of pregnancy were evaluated. Twin pregnancies were excluded from the analysis.

All women gave written informed consent to participate in the PARA study, which was approved by the Erasmus MC ethics committee.

Data collection

In the PARA study, clinical data was collected during home visits at preconception, three times during pregnancy (i.e., every trimester) and three times postpartum. During each visit, a blood sample was drawn and disease activity was measured. Patients filled out questionnaires on obstetric history and medication use.

Clinical parameters on pregnancy outcome were documented, including the development of gestational hypertension and preeclampsia, gestational age (GA) at birth, birth weight, birthweight percentile and fetal/neonatal death.

Disease activity was measured using 3 variables: a tender and swollen joint count for 28 joints combined with C-reactive protein (CRP) levels (DAS28-CRP), since this variant of the DAS28 has been validated for use during pregnancy.[11] The disease activity score was categorized into low (<3.2), intermediate (≥ 3.2 and ≤ 5.1) and high (>5.1) according to the recommendations of the European League against Rheumatism (EULAR).

Gestational hypertension was defined as the development of systolic blood pressure of ≥ 140 mmHg and/or a diastolic blood pressure ≥ 90 mmHg without proteinuria after 20 weeks' gestation, in a previously normotensive woman. Preeclampsia was defined as having gestational hypertension in combination with proteinuria (24-h urine collection containing ≥ 0.3 grams protein, or a dipstick reading of 2+ or greater), according to the International Society for the Study of Hypertension in Pregnancy (ISSHP) 2001 criteria. These criteria were in effect at the time of study initiation.[12]

Measurements of sFlt-1 and PlGF

For the analysis of sFlt-1 and PlGF, venous blood that was collected during the third trimester of pregnancy (28 – 32 weeks' GA) was utilized. Measurement of sFlt-1 and PlGF was performed in 221 samples making use of an automated analyzer (Cobas 6000, e-module; Roche Diagnostics, Rotterdam, the Netherlands).

Statistical analysis

Data are reported as median (interquartile range; IQR) for continuous variables and number (percentage) for categorical variables. The normality of continuous data was evaluated using the Shapiro-Wilk W test. For the comparison of the continuous values between >2 groups (i.e., the three categories of DAS28-CRP), the Kruskall-Wallis test was applied with a Bonferroni correction for multiple testing. Multiple comparisons of mean ranks for all groups were performed as post-hoc test. To investigate differences in continuous variables between 2 groups, the Mann-Whitney *U* test was performed. Categorical variables between 2 or more groups were assessed using the χ^2 or Fisher's exact test. Spearman rank order correlation was applied to calculate correlation coefficients. $P<0.05$ was considered statistically significant. Statistical analysis was performed using Prism 8.0 (GraphPad software Inc.) and IBM SPSS Statistics 25.0.

RESULTS

A total of 221 women, aged 21 – 42 years, were included in the current analysis. Table 1 provides a detailed description of their demographic characteristics. Median GA at biomarker measurement (third trimester of pregnancy) was 30+3 weeks (IQR; 29+2 – 31+3) and the median sFlt-1/PlGF ratio was 4 (2 – 7). Disease duration of RA was 4.7 years (IQR; 2.1 – 9.0). Of all 221 women, 153 (69%) were rheumatoid factor positive while 132 women (60%) were anti-citrullinated protein antibody positive. Medication use consisted mostly of prednisone (33%), sulfasalazine (26%), or hydroxychloroquine (2%). Fifty-one percent of women did not use any medication in the third trimester.

Table 1. Demographic Characteristics of All Included Women

Parameter	PARA Cohort
N	221
Age, years	33 (29 - 35)
GA at measurement, weeks^{+days}	30^{+3} (29^{+2} - 31^{+3})
Nulliparity, n (%)	109 (49)
Smoking during pregnancy, n (%)	17 (8)
Ethnicity	
Caucasian, n (%)	204 (92)
Duration of RA, years	4.7 (2.1 – 9.0)
RF-positive, n (%)	153 (69)
ACPA-positive, n (%)	132 (60)
Erosions, n (%)	129 (58)
Drug use in third trimester, n (%)	
No medication	112 (51)
Sulfasalazine	57 (26)
Prednisone	72 (33)
Sulfasalazine and Prednisone	18 (8)
Hydroxychloroquine	4 (2)
Angiogenic Marker Values	
sFlt-1	1645 (1210 - 2297)
PlGF	450 (277 - 718)
sFlt-1/PlGF ratio	4 (2 - 7)

Values are median (interquartile range) or number (percentage). GA indicates gestational age; RA, rheumatoid arthritis; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibody; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

Pregnancy characteristics and angiogenic markers according to disease activity

Disease activity (DAS28-CRP) in the third trimester was documented in 218 women of the 221 women. Fifteen women (7%) had high disease activity, 103 (47%) intermediate activity and 100 (46%) low disease activity during the third trimester. Table 2 shows pregnancy characteristics and angiogenic markers according to disease activity states of DAS28-CRP. A higher proportion of prednisone users was observed in women with intermediate or high disease activity (60% and 45%, respectively) in comparison to women with low disease activity (17%).

Seven women (3%) developed preeclampsia, of whom three (3%) had low disease activity and four (4%) had intermediate disease activity. A total of 17 women had gestational hypertension, of which one patient had missing data for disease activity.

No difference in preeclampsia rate was observed between low, intermediate and high disease activity groups (P=0.72). Women who had high disease activity did not show higher sFlt-1 in comparison to women with intermediate activity or low activity (P=0.07). In addition, no differences in PlGF or sFlt-1/PlGF ratio levels were observed between low, intermediate and high disease activity groups (Table 2 & Figure 1A-1C).

Correlations between sFlt-1, PlGF, sFlt-1/PlGF ratio and DAS28-CRP score and CRP

The values of sFlt-1, PlGF and sFlt-1/PlGF ratio were not significantly correlated with the DAS28-CRP score (Figure 2A, 2C, 2E). CRP correlated weakly with PlGF (r = -0.14, P=0.03, figure 2D), while no correlation was found with sFlt-1 or the sFlt-1/PlGF ratio (Figure 2B, 2F).

Pregnancy outcome according to sFlt-1/PlGF ratio ≤38 versus >38

Women with a ratio≤38 had a higher GA at birth and higher birth weight in comparison to women with a ratio above 38, as shown in Table 3. The proportion of infants with a birth weight percentile <10 was significantly higher in women with a ratio>38 (71%) than in the ratio≤38 group (15%) (P<0.01). When stratified to a sFlt-1/PlGF ratio of 38 or lower only 2% of women developed preeclampsia, compared to 43% of the women with a ratio higher than 38. The observed sensitivity for this cut-off was 42.1%, the specificity 98.1%, and the negative predictive value (NPV) was 98.1%. Time to delivery in women with a ratio>38 was shorter than in women with a ratio≤38 (median 52 vs. 66 days), although this was not significant (P=0.08). Of the 4 women with an sFlt-1/PlGF ratio ≤38 that developed preeclampsia, none of them had pre-existing disorders such as chronic hypertension, chronic kidney disease or systemic lupus erythematosus (SLE). Median time to delivery in these 4 women was 71 days compared to 52 in the 3 women with preeclampsia in the ratio >38 group (data not shown).

sFlt-1 and PlGF concentrations according to sulfasalazine use

There were 57 women using sulfasalazine during the third trimester. Median levels of sFlt-1 or PlGF did not differ between women using sulfasalazine (median [IQR] 1578 [1193 – 2357]) and 500 [295 – 844]) in comparison to women not treated with this drug during pregnancy (1697 [1218 – 2270] for sFlt-1 and 442 [265 – 633] for PlGF) (n=164) (Figure 3). The dosages of sulfasalazine ranged from 3000mg/day (n=3), 2000 mg/day (n=35) and <2000 mg/day (n=19). When sFlt-1 levels were divided according to sulfasalazine dosage, sFlt-1 values were slightly higher in women with dosages <2000mg/day (median [IQR] 2297 [1406 – 2574]), compared to 2000mg/day (1572 [1088 – 2054] pg/mL) and 3000mg/day (median 1546 pg/mL), but this was not significant (P=0.26). Similarly, there were no alterations in PlGF or sFlt-1/PlGF ratio between different sulfasalazine dosages (p=0.85 and p=0.68, respectively). We observed no differences in pregnancy characteristics or disease activity (DAS28-CRP) between sulfasalazine users and non-users (Table S1). The proportion of women using other drugs (i.e., prednisone and hydroxychloroquine) was similar between sulfasalazine users and those not using this drug. Pregnancy outcomes such as GA at delivery, birth weight, birthweight percentile<10 and preeclampsia rate did not differ between sulfasalazine users and non-users (Table S1).

Table 2. Pregnancy Characteristics and Angiogenic Markers according to DAS28-CRP in 218 women[‡]

Parameter	Low[a]	Intermediate[b]	High[c]	*P-Value*
	<3.2	≥3.2 - ≤5.1	>5.1	
N	100	103	15	
Maternal age, years	33 (30 - 35)	33 (29 - 35)	32 (29 - 35)	0.80
GA at measurement, weeks^{+days}	30^{+2} (29^{+2} - 31^{+1})	30^{+4} (29^{+2} - 31^{+5})	30^{+0} (29^{+1} - 30^{+3})	0.13
Nulliparity, n (%)	51 (51)	50 (49)	6 (40)	0.72
Smoking during pregnancy, n (%)	9 (9)	8 (8)	0 (0)	0.30
Ethnicity, n (%)				
Caucasian	96 (96)	93 (90)	14 (93)	0.28
Drug use in third trimester, n (%)				
Sulfasalazine	29 (29)	27 (26)	1 (7)	0.19
Prednisone	17 (17)	46 (45)[a]	9 (60)[a]	<0.001
Hydroxychloroquine	3 (3)	1 (1)	0 (0)	0.48
Pregnancy outcome				
GA at birth, weeks^{+days}	40^{+3} (38^{+1} - 40^{+4})	39^{+2} (38^{+0} - 40^{+4})[a]	39^{+3} (38^{+1} - 40^{+4})	0.04
<34	2 (2)	0 (0)	0 (0)	0.30
34 - 37	4 (4)	13 (13)	1 (7)	0.08
Male	55 (55)	52 (50)	10 (67)	0.47
Birth weight, grams	3550 (3093 - 3870)	3230 (2840 - 3700)[a]	3085 (2800 - 3800)	<0.01
Maternal outcome				
Gestational Hypertension	8 (8)	8 (8)	0 (0)	0.42
Preeclampsia	3 (3)	4 (4)	0 (0)	0.72
Fetal outcome				
Birth weight percentile <10	14 (14)	20 (19)	4 (27)	0.37
Fetal/Neonatal death	0 (0)	1 (1)	0 (0)	0.57
Angiogenic markers				
sFlt-1, pg/mL	1716 (1327 - 2328)	1589 (1107 - 2174)	2137 (1921 - 2465)	0.07
PlGF, pg/mL	443 (295 - 696)	459 (263 - 754)	295 (205 - 584)	0.41
sFlt-1/PlGF ratio	4 (2 - 7)	4 (2 - 8)	6 (3 - 14)	0.18
≤38	98 (98)	98 (95)	15 (100)	0.39
38>-<85	2 (2)	4 (4)	0 (0)	0.57
≥85	0 (0)	1 (1)	0 (0)	0.57

Data are reported as median (interquartile range) or number (percentage). [‡]Data from 3 women regarding DAS28-CRP were missing. DAS28 indicates disease activity score in 28 joints; CRP, C-reactive protein; GA, gestational age; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor. *a* indicates comparison with low disease activity at a significance level of p<0.05; *b* indicates comparison with intermediate disease activity at a significance level of p<0.05 and *c* indicates comparison with high disease activity at a significance level of p<0.05.

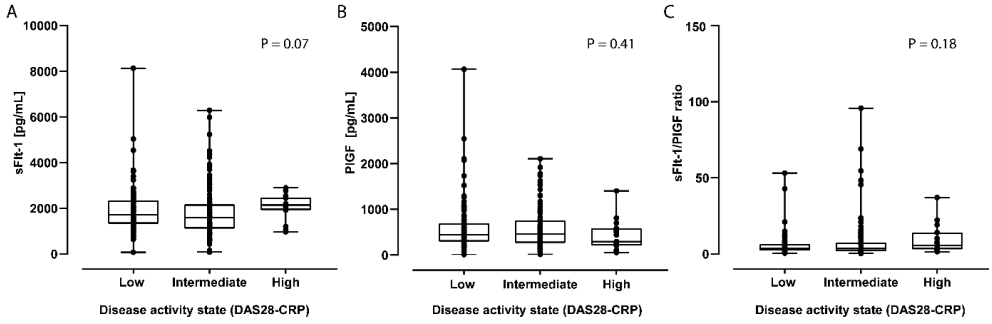

Figure 1. Angiogenic markers according to disease activity (DAS28-CRP).
A-C: Box and whisker plots showing levels of sFlt-1, PlGF and sFlt-1/PlGF ratio according to low (n = 100), intermediate (n =103) and high (n=15) disease activity. Boxes are median (interquartile range) and whiskers are range. sFlt-1 indicates soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; DAS-28, disease activity score in 28 joints; CRP, C-Reactive Protein. P<0.05 is considered statistically significant.

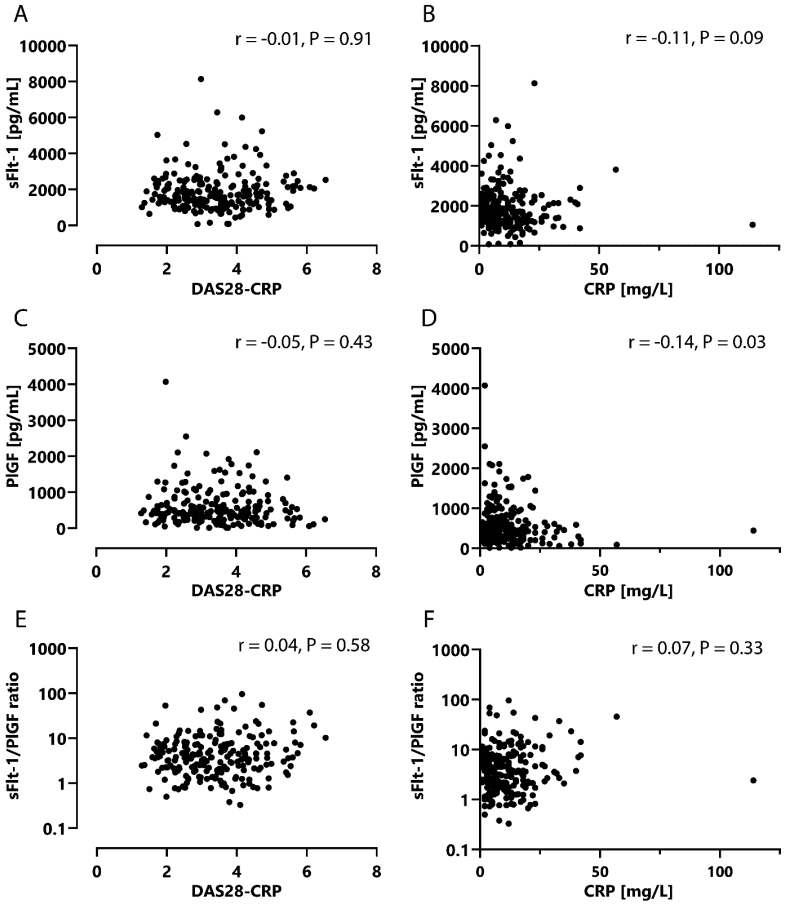

Figure 2. Correlations between sFlt-1, PlGF and sFlt-1/PlGF ratio with DAS28-CRP and CRP. Correlation coefficients for sFlt-1 (A-B), PlGF (C-D) and sFlt-1/PlGF ratio (E-F) with DAS28-CRP and CRP. sFlt-1 indicates soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; DAS-28, disease activity score in 28 joints; CRP, C-Reactive Protein; P<0.05 is considered statistically significant.

DISCUSSION

The sFlt-1/PlGF ratio has become a reliable predictor of preeclampsia in women clinically suspected of the disorder.[3, 13] We examined whether disease activity in pregnant women with RA, a disease characterized by high levels of inflammation, could impact the levels of the sFlt-1/PlGF ratio, and its applicability as a clinical tool to predict preeclampsia in this patient population.

We found that the values of sFlt-1, PlGF and sFlt-1/PlGF ratio were not altered due to disease activity in pregnant women with RA, and that the sFlt-1/PlGF ratio could be used to exclude preeclampsia when a cut-off of ≤38 was applied.

Our findings imply that the levels of sFlt-1 and PlGF in pregnant women with RA are similar to that of a healthy pregnant population, irrespective of disease activity. While we were unable to directly compare sFlt-1 values to healthy non-RA pregnancies, GA-specific reference ranges from previous studies (median [IQR] 1934 [1222 – 2818] pg/mL)[14] fall well within the values we observed in our cohort (median [IQR] 1645 [1210 – 2297] pg/mL, even in the case of high disease activity (median [IQR] 2137 [1921 – 2465]), implying these values are not elevated due to RA in pregnant women. Because the placenta accounts for >99% of the sFlt-1 elevation during pregnancy,[15, 16] it is mostly probable that the (non-placental) sFlt-1 increase observed in patients with RA plays an insignificant role during pregnancy.

Table 3. Pregnancy Outcome according to sFlt-1/PlGF ratio with a cut-off value of 38.

Parameter	Ratio ≤38	Ratio >38	P-value
N	214	7	
GA at birth, weeks	39 (38 - 40)	37 (36 - 40)	0.05
<34	2 (1)	0(0)	1.00
34 - 37	18 (8)	2 (29)	0.12
Male, n (%)	113 (53)	5 (71)	0.45
Birth weight, grams	3420 (2998 - 3800)	2620 (2360 - 2850)	<0.01
Time to delivery, days	66 (52 - 75)	52 (44 - 66)	0.08
Maternal outcome			
Gestational Hypertension	15 (7)	2 (29)	0.09
Preeclampsia	4 (2)	3 (43)	<0.001
Fetal outcome			
Birth weight percentile <10	33 (15)	5 (71)	<0.01
Fetal/Neonatal Death	1 (1)	0 (0)	1.00

Data are reported as median (interquartile range) or number (percentage). sFlt-1 indicates soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; GA, gestational age. Time to delivery is defined as the amount of days between blood sampling and delivery.

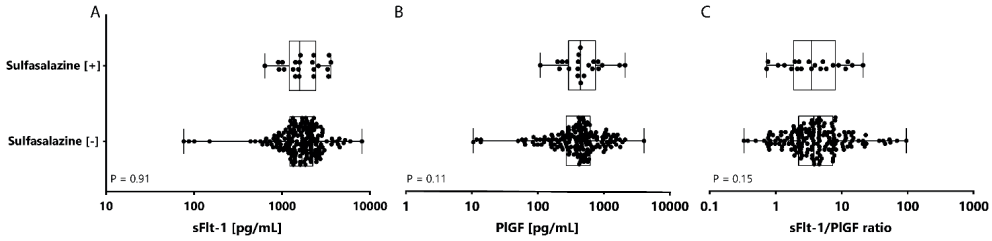

Figure 3. Angiogenic markers according to sulfasalazine use (n=57) versus non-users (n=164). Boxes are median (interquartile range) and whiskers are range. sFlt-1 indicates soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor. p<0.05 is considered statistically significant.

Despite existing studies investigating the levels of sFlt-1 and PlGF in RA, none of them focused on pregnancies with RA, which makes a direct translation to our pregnant population with RA challenging. In a study by Ballara et al., sFlt-1 values measured in 122 subjects with RA were significantly elevated in comparison to 31 healthy controls.[5] Similarly, in a recent cohort involving 157 patients with RA, Kim et al. found raised sFlt-1 values in comparison to 50 controls with osteoarthritis.[6] Our observation that serum sFlt-1 was not correlated with DAS28-CRP or CRP partially agrees with the findings by Kim et al., who reported a modest correlation between sFlt-1 and DAS28, but not with CRP. The finding in their study that synovial fluid sFlt-1 levels strongly correlated with CRP could imply that synovial fluid sFlt-1 rather than circulating sFlt-1 reflects systemic inflammation in RA. On the other hand, we observed a weak negative correlation between PlGF and the inflammatory parameter CRP. In a former study conducted by de Man et al., it was shown that high disease activity was independently associated with lower birth weight, even when corrected for gestational age.[17] Hence, one could hypothesize that a certain degree of inflammation might affect placental development, subsequently impairing PlGF production. Here, it would be interesting to examine whether other inflammatory parameters, such as TNF-α, IL-6, and IL-10 are also correlated with sFlt-1 and PlGF in this population.

The prevalence of preeclampsia in our cohort was 3% which is similar to that observed in a healthy population.[2, 18] When we stratified women according to a sFlt-1/PlGF ratio of 38 or lower, a cut-off point recently identified as useful for predicting the absence of preeclampsia,[3] only 2% of women developed preeclampsia, in comparison to 43% of the women with a ratio >38, corresponding to a negative predictive value (NPV) of 98.1%. These results are comparable to the 99.3% and 94% as previously described by Zeisler et al.[3, 19] to rule out preeclampsia at 1 and 4 weeks, respectively. Since the median time to delivery in the ratio≤38 group was 66 days (Table 3), it seems that these women developed preeclampsia much later, suggesting an even higher NPV to exclude preeclampsia within 4 weeks, in our population.

To our knowledge, we are the first to validate the use of the sFlt-1/PlGF ratio to exclude preeclampsia in a large cohort of singleton pregnancies with RA.

Another interesting finding of our study was that sFlt-1 or PlGF were not altered in women using sulfasalazine in comparison to women not using this drug. In recent years, sulfasalazine has gained increasing interest as a potential drug for the treatment of preeclampsia. This concept originated from Brownfoot *et al*., who observed a significant reduction in sFlt-1 secretion along with upregulated PlGF when sulfasalazine was administered to placental explants from preeclamptic mothers.[7, 20] We are the first to investigate the association between sulfasalazine use and the angiogenic factors in vivo. This is relevant, since sulfasalazine itself is poorly absorbed (~30%), with the majority of the drug being metabolized to sulfapyridine and 5-ASA in the colon.[9, 10] One explanation of our discrepant findings is therefore that they represent an in-vivo situation which is entirely different from the in-vitro placental explant setup.[7] Secondly, the in-vitro doses applied in the study by Brownfoot et al., (equivalent to >4 grams per day) were substantially higher than the dosages applied in our study (i.e., an average of 2 grams per day)[7]. Here, it is important to mention that the recommended dose during pregnancy should not exceed 2 grams per day, due to the potential risk of leucopenia of the fetus.[21]

Whereas the use of alternative drugs affecting sFlt-1 might have influenced our results, the proportion of women using other medication (e.g prednisone, hydroxychloroquine or proton pump inhibitors) was similar in those women with and without sulfasalazine. It could also be the case that a potential effect of sulfasalazine on reducing sFlt-1 is more evident when sFlt-1 values are markedly higher, as observed in women with preeclampsia. Therefore, future clinical trials to investigate the effects of sulfasalazine on sFlt-1 and PlGF in women with early-onset preeclampsia are still warranted.

A few limitations of our study must be considered. The number of women that developed preeclampsia in our cohort was relatively small. Yet, this probably confirms that women with RA do not have an elevated risk of preeclampsia. Also, we were unable to allow conclusions on the sFlt-1/PlGF ratio levels at the time of preeclampsia onset, since biomarker measurements occurred several weeks earlier. Lastly, due to the nature of this cohort, pregnant women without RA were not included as a control group.

In conclusion, our findings illustrate that in pregnant women with RA, the sFlt-1/PlGF ratio is not altered due to disease activity, and a cut-off of ≤38 of this ratio can be used to exclude preeclampsia. With this study, we are the first to validate the use of this ratio in pregnancies with RA. Interestingly, we were not able to show an effect of sulfasalazine use on the values of sFlt-1 or PlGF, implying this drug does not improve the angiogenic imbalance observed in preeclampsia. Future, prospective trials in women with early-onset preeclampsia are still necessary to confirm our findings.

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CHAPTER 08

Omeprazole Administration to Women with Preterm Preeclampsia: Effects on Circulating Soluble Fms-like Tyrosine Kinase-1, Placental Growth Factor and Endothelin-1 Secretion.

Rugina I. Neuman, Milan Baars, Langeza Saleh, Daan Nieboer, Jerome Cornette, Eric A.P Steegers, Henk Russcher, Sjoerd A.A van den Berg, Anton H. van den Meiracker, Willy Visser, A.H Jan Danser

ABSTRACT

Background: Low soluble Fms-like tyrosine kinase (sFlt-1) has been reported in women with suspected or confirmed preeclampsia using proton pump inhibitors (PPI). Here, we examined whether omeprazole administration to women with preeclampsia could acutely reduce their circulating levels of sFlt-1, or endothelin-1 (ET-1) or enhance the levels of PlGF (placental growth factor).

Methods: This was a randomized controlled trial in which women (≥ 18 years) with confirmed preeclampsia and a gestational age between 20^{+0} and 34^{+6} weeks were allocated to receive 40 mg omeprazole once daily or no omeprazole. Blood was collected at baseline and days 1,2,4,8 followed by twice-weekly until delivery. Primary outcome was specified as the difference in sFlt-1, PlGF or CT-proET-1 after 4 days of omeprazole initiation compared to the non-omeprazole group.

Results: Fifty women with preeclampsia were randomly allocated to receive omeprazole (n=26) or no omeprazole (n=24), of which 40 remained pregnant after 4 days. Mean maternal age was 30 years, and median gestational age was 30^{+3} weeks. Baseline sFlt-1 levels did not differ between non-omeprazole and omeprazole group (10743 vs. 7110 pg/mL, p=0.11) and the same was true for PlGF or CT-proET-1 (p=0.14 and p=0.55). After 4 days, sFlt-1 levels remained similar in women receiving omeprazole (8364 pg/mL) compared to women not receiving omeprazole (13017 pg/mL, p=0.14), while levels of PlGF and CT-proET-1 also did not differ between groups. Women receiving omeprazole had a similar length of pregnancy compared with those in the non-omeprazole group (median 15 vs. 14 days, p=0.70). Except for a higher neonatal intubation rate in the non-omeprazole group (31% vs 4%, p=0.02) there were no differences in maternal/perinatal complications.

Conclusions: Administration of 40 mg (once daily) omeprazole to women with confirmed preeclampsia does not alter their circulating levels of sFlt-1, PlGF or ET-1, arguing against a role of this drug as a treatment for this syndrome.

INTRODUCTION

Preeclampsia is a hypertensive syndrome unique to human pregnancy, with significant impact on maternal and fetal wellbeing worldwide.[1, 2] Approximately 5% of all pregnancies are affected by this syndrome, which is defined as the new-onset of hypertension in the presence of maternal organ and/or uteroplacental dysfunction after 20 weeks' gestation.[1, 2] Because there is no curative treatment for preeclampsia other than placental delivery, current management entails weighing the maternal risk of developing severe complications such as eclampsia, renal insufficiency, pulmonary edema and HELLP syndrome (hemolysis, elevated liver enzymes, low platelets), against the danger for the fetus of being born prematurely[1].

The wide variety and complexity of preeclampsia has tempered our ability to precisely understand the pathogenic mechanisms underlying this syndrome. Yet, a well-recognized phenomenon is that the placenta releases excessive amounts of soluble Fms-like tyrosine kinase-1 (sFlt-1) into the maternal circulation, which in turn binds to and reduces free placental growth factor (PlGF). The ensuing highly anti-angiogenic state subsequently triggers the release of the vasoconstrictor endothelin-1 (ET-1), further inducing the widespread maternal endothelial dysfunction typically associated with this syndrome[3, 4]. Ideally, mitigating the release of sFlt-1 and ET-1 or enhancing PlGF production should restore the angiogenic imbalance, or at least, delay disease progression in women with preeclampsia. As such, current studies focus on novel therapies that could target these (anti-) angiogenic factors[5]. Recently, proton pump inhibitors (PPIs) were shown to dose-dependently reduce sFlt-1 release in placental explants or trophoblast cells.[6] PPIs are commonly prescribed for the treatment of gastric acid reflux and are considered safe for use in pregnancy. Following these observations, our group demonstrated that in gestational-age matched women with suspected or confirmed preeclampsia, PPI use was associated with lower sFlt-1 and ET-1 levels compared to women not using these drugs.[7] Since most women (80%) in the former study were using omeprazole[7], we hypothesized that administering this PPI to women with confirmed preeclampsia could actively decrease the levels of circulating sFlt-1. In the present study, we performed a randomized controlled trial to evaluate whether omeprazole treatment could alter the circulating and cord blood levels of sFlt-1, PlGF and CT-proET-1 (C-terminal pro-endothelin-1; a stable surrogate marker of ET-1)[8] in women with confirmed preeclampsia. In addition, making use of the ex vivo placenta perfusion model, we established whether omeprazole and esomeprazole could effectively reduce placental perfusate levels of sFlt-1.

METHODS

Study design

This was a randomized controlled trial with individual randomization to the omeprazole group or non-omeprazole group using blocks according to gestational age (GA). The trial was performed at two maternity units in Rotterdam, the Netherlands (Erasmus University Medical Center and Maasstad Hospital) between 2018 and 2021. All participants gave written informed consent to participate in the trial, which was approved by the Erasmus MC Research Ethics Committee (MEC-2018-071).

Participants

Women were eligible to participate in the study if they had a confirmed diagnosis of preeclampsia with a GA between 20^{+0} and 34^{+6} weeks on the day of randomization, a singleton pregnancy, ≥18 years of age, and were able to give written informed consent. Women were not included in the trial if they were using any PPI at time of randomization, if they had a contraindication or hypersensitivity to PPI use, if they were using medication that could interact with PPI, if there was fetal death or distress at time of inclusion or if the clinicians expected delivery within the next 48 hours.

The diagnostic criteria were based on the ISSHP 2018 criteria (International Society of the Study of Hypertension in Pregnancy) which defines preeclampsia as hypertension accompanied by proteinuria and/or maternal organ dysfunction and/or uteroplacental dysfunction at or after 20 weeks of gestation.[9]

Randomization

Participants were allocated to receive omeprazole 40 mg, once daily or no omeprazole. Randomization was performed using an online, web-based sequence generator. Since the circulating biomarkers could significantly alter with advancing gestation, randomization blocks were stratified according to GA (strata 1 was <25 weeks, strata 2 = ≥25 until <29 weeks, strata 3 = ≥29 until <32 weeks and strata 4 = ≥33 until <35 weeks).

Study procedures

Women allocated in the omeprazole group received 30 capsules that were packaged for oral administration, with no special storage conditions. The omeprazole capsules were packaged and labelled by the pharmacist at the Erasmus Medical Center, which distributed the packs to the other participating center upon request. We advised that capsules should be taken once daily, preferably in the morning on an empty stomach, according to the packs

instructions. Women in the omeprazole group that did not deliver 30 days after randomization would receive an additional omeprazole pack (containing 30 capsules). We recommended that treatment should be continued from enrollment until delivery. Compliance was closely monitored on a daily basis, since most patients were admitted at the maternity units. After birth, all omeprazole packages would be collected by a research team member, to adequately assess compliance, by counting the number of missing capsules.

In the non-omeprazole group, patients would not receive additional medication, and those that developed gastric acid reflux during the trial, were advised to receive other medication than PPIs. If despite these medications participants maintained gastric reflux requiring a PPI, they would be excluded. At study enrollment, baseline data was entered on a web-based program by two independent researchers (R.I.N and M.B).

Blood collection and biochemical measurements

In both the omeprazole and non-omeprazole group, venous blood (serum and EDTA) was collected at baseline (day 0; before starting treatment with omeprazole), and day 1, 2, 4, 8 followed by twice weekly (during routine blood measurements) until delivery. The collected blood samples were stored after centrifugation, at -80°C until analysis. Analysis of sFlt-1, PlGF, CT-proET-1 were performed at the end of the study. Serum sFlt-1 and PlGF were measured using an automated analyzer (Cobas 6000e, Roche Diagnostics, Rotterdam, the Netherlands), while plasma CT-proET-1 levels were analyzed using BRAHMS Kryptor instrument (Thermo Fisher Scientific, BRAHMS GmbH., Henningsdorf, Germany).

Outcomes

The primary outcome of the study was specified as the difference in sFlt-1, PlGF and CT-proET-1 levels 4 days after enrollment between the omeprazole group and the non-omeprazole group. Secondary outcomes comprised the difference in angiogenic markers 8 days after PPI administration, and the difference in longitudinal course or cord blood levels between the omeprazole and non-omeprazole group. Maternal and fetal/neonatal complications in the omeprazole versus the non-omeprazole group were also assessed. Maternal complications included eclampsia, pulmonary edema, placental abruption, subcapsular liver hematoma, cerebral hemorrhage/edema or infarction, visual disturbances, acute renal failure (absolute increase in the serum creatinine concentration of 0.3 mg/dL [26.4 μmol/L from baseline or >50% increase in serum creatinine; or oliguria with <0.5 mL/kg per hour for at least 6 hours) or postpartum hemorrhage (blood loss ≥1 L after delivery). Fetal/neonatal complications comprised of neonatal intensive care unit (NICU) admission; small-for-gestational age infant (birth weight <10th percentile according to Dutch Perinatal Registration); endotracheal

intubation; sepsis; respiratory distress syndrome; bronchopulmonary dysplasia [defined as chronic lung disease developing in preterm neonates treated with oxygen and positive-pressure ventilation, with radiographic signs of inflammation and scarring, in need of artificial ventilation 4 weeks post-partum and at 36 weeks postmenstrual age]; necrotizing enterocolitis and fetal/neonatal death. All outcomes were recorded on the web-based trial database by two independent researchers (R.I.N and M.B).

Placenta studies

Patients and setting

Placentas of women who underwent an elective cesarean section with uncomplicated singleton pregnancies were collected immediately after delivery at the Erasmus Medical Center, Rotterdam, the Netherlands. Placental and clinical characteristics were obtained from the patients' electronic files. All women who donated their placenta gave approval through written informed consent. The study was exempted from approval by the local institutional Medical Ethics Committee according to the Dutch Medical Research with Human Subjects Law (MEC-2016-418 and MEC-2017-418).

Perfusion experiments

The perfusion model used in the current study has been described by Hitzerd et al.[10] Perfusion experiments were conducted in healthy, term placentas. Maternal and fetal perfusion media consisted of Krebs-Henseleit buffer at 37°C, supplemented with heparin (final concentration; 2500 IU/L) and aerated with 95% O2 - 5% CO2. The fetal circulation (closed circuit; flow rate, 6 mL/minute) was established by cannulating the chorionic artery and corresponding vein of an intact cotyledon. Maternal circulation (closed circuit; flow rate 12 mL/minute) was created by placing 4 blunt cannulas in the intervillous space. At t = 0, at a concentration of $10\times C_{max}$, esomeprazole (50 µmol/L) or omeprazole (30 µmol/L), or no drug as a control were added to the maternal circulation. Samples of the maternal and fetal circulations were taken every 30 minutes until the end of the experiment (180 minutes) to determine biomarker concentrations and were immediately stored at −80°C.

Statistical analysis

Data are reported as mean (± SD) or median (interquartile range) for continuous variables and as number (percentage) for categorical variables. The normality of continuous variables was assessed using the Shapiro-Wilk W test. For the comparison of continuous variables between more than two groups, an unpaired t-test or Mann-Whitney U test was performed in the case of non-normal distribution. For the comparison of categorical variables between two groups,

Fisher's exact or Chi-square test was applied. We used linear mixed models to evaluate the effect of treatment (omeprazole group) on the angiogenic markers. To account for within subject correlations, all subjects were added as random effect. Time (days at which blood was taken) and GA at the first measurement were added as fixed effects. Baseline values (at day 0) were added as covariate. For the placenta studies, differences in biomarker levels were studied using general linear model repeated measurements. A p-value less than 0.05 was considered statistically significant. Data were analyzed using IBM SPSS statistics (IBM Corporation, version 25) and R studio Statistical Software.

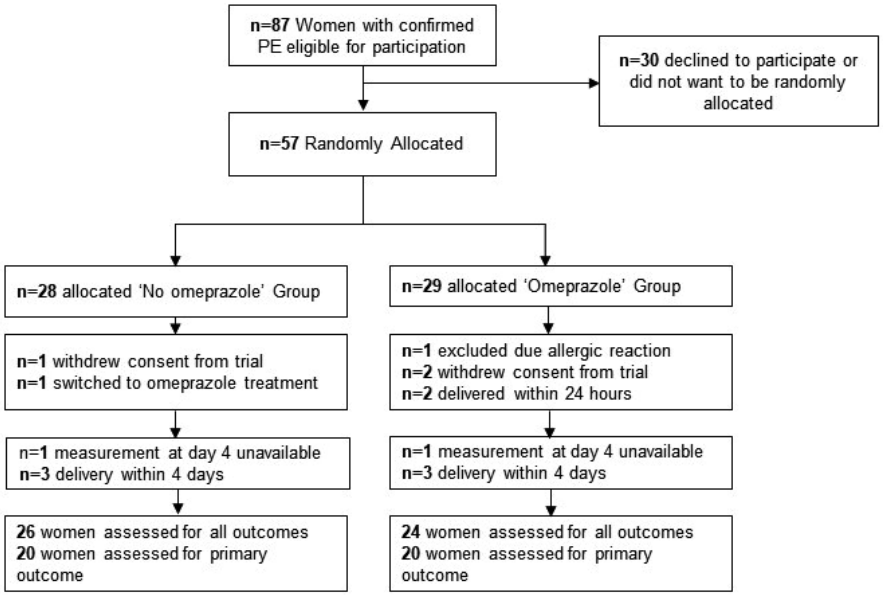

Figure 1. Flowchart of participants in- and excluded from the study. PE indicates preeclampsia.

RESULTS

Between December 2018 and June 2021, a total of 87 women with preeclampsia were found eligible for inclusion, of which 57 women agreed to participate and were randomized. Of these, 50 were included in the outcome analysis of which 26 were allocated to the non-omeprazole group and 24 to the omeprazole group (Figure 1). Baseline characteristics of all participants are displayed in Table 1. Mean maternal age was 30 years, and body mass index 25 kg/m^2. Median GA at enrollment was 30 (28 – 31) weeks, which did not differ between the two groups (p=0.45).

Table 1. Baseline characteristics of all 50 participants at enrollment

	All patients	No Omeprazole group	Omeprazole group	P-Value
N	50	26	24	
Maternal age	30.7 (±5.6)	30.2 (±5.8)	31.2 (±5.5)	0.59
GA at enrollment, weeks+days	30+3 [28+1 - 31+4]	30+0 [27+1 - 31+4]	30+4 [28+2 - 31+5]	0.45
Pregestational BMI, kg/m^2	25.7 (±4.5)	25.9 (±3.8)	25.4 (±5.3)	0.72
Ethnic background				
Caucasian/White	31	18 (69)	13 (54)	0.27
Black/African/Creole	9	2 (8)	7 (29)	0.07
Asian	1	1 (4)	0 (0)	1.00
Hindi/Pakistani	3	1 (4)	2 (8)	0.60
Turkish/Morrocan	2	1 (4)	1 (4)	1.00
Other	4	3 (12)	1 (4)	0.61
Nulliparous	33	18 (69)	15 (63)	0.62
History of PE/HELLP	7	4 (15)	3 (13)	1.00
Pre-existing hypertension	14	9 (35)	5 (21)	0.35
Pre-existing proteinuria	2	1 (4)	1 (4)	1.00
Clinical findings at enrollment				
Systolic blood pressure, mmHg	140 (135 - 150)	140 (135 - 151)	140 (134 - 150)	0.60
Diastolic blood pressure, mmHg	90 (85 - 95)	90 (85 - 95)	90 (80 - 95)	0.66
Anti-hypertensive drug use	42	22 (85)	20 (83)	1.00
uPCR, mg/mmol	52 (34 - 219)	52 (31 - 274)	64 (34 - 178)	0.85
Creatinine, umol/L	58 (51 - 67)	55 (50 - 64)	63 (55 - 73)	0.08
Uric acid, mmol/L	0.33 (0.25 - 0.36)	0.32 (0.28 - 0.36)	0.34 (0.21 - 0.38)	0.73
ALT, U/L	23 (15 - 41)	25 (16 - 58)	19 (13 - 28)	0.24
Lactate dehydrogenase, U/L	216 (198 - 259)	218 (180 - 272)	216 (206 - 247)	0.93
Platelet count, x10^9/L	227 (169 - 265)	223 (189 - 278)	231 (167 - 257)	0.71
(partial) HELLP syndrome	4	2 (8)	2 (8)	1.00
Angiogenic factors at enrollment				
sFlt-1, pg/mL	10679 (4398 - 16474)	10794 (5468 - 18152)	9819 (3365 - 12767)	0.30
PlGF, pg/mL	55 (32 - 106)	50 (33 - 86)	73 (31 - 273)	0.27
sFlt-1/PlGF ratio	206 (37 - 481)	226 (60 - 518)	150 (8 - 383)	0.18
CT-proET-1, pmol/L	65 (38 - 95)	73 (50 - 91)	56 (30 - 103)	0.68

Values are mean (±SD), median (IQR) or number (%). GA indicates gestational age, HELLP; hemolysis, elevated liver enzymes and low platelets; uPCR, urinary protein-to-creatinin ratio; ALT, alanine transaminase; sFlt-1, soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor; CT-proET-1, C-terminal preproendothelin-1.

Table 2. Pregnancy Outcomes according to Treatment Groups.

	No Omeprazole (n = 26)	Omeprazole (n = 24)	P-Value
Prolongation of pregnancy, days	14 (4 - 28)	15 (5 - 33)	0.70
GA at delivery, weeks+days	32+0 [30+1 - 35+5]	33+1 [31+1 - 35+2]	0.36
Male/Female	16/10	11/13	0.27
Birth weight, grams	1569 [± 823]	1758 [± 152]	0.40
Maternal outcomes			
Highest systolic blood pressure, mmHg	160 (153 - 175)	158 (150 - 180)	0.63
Highest diastolic blood pressure, mmHg	105 (99 - 110)	103 (96 - 110)	0.91
Highest uPCR, mg/mmol	85 (35 - 308)	93 (43 - 289)	0.62
Development of HELLP syndrome	2 (8)	1 (4)	1.00
Need for magnesium sulphate	14 (54)	14 (58)	0.75
Need for intravenous antihypertensives	5 (19)	5 (21)	1,00
Eclampsia	0 (0)	0 (0)	1.00
Pulmonary edema	0 (0)	2 (8)	0.23
Placental abruption	2 (8)	1 (4)	1.00
Postpartum hemorrhage	1 (4)	1 (4)	1.00
Acute kidney injury	2 (8)	0 (0)	0.49
Neonatal outcomes			
Neonatal Intensive Care Unit admission	19 (73)	15 (63)	0.42
Small-for-gestational age (percentile <10)	19 (73)	14 (58)	0.27
Endotracheal intubation	8 (31)	1 (4)	0.02
Respiratory distress syndrome	12 (46)	8 (33)	0.36
Sepsis	4 (15)	3 (13)	1.00
Necrotising enterocolitis	1 (4)	0 (0)	1.00
Fetal or neonatal death	2 (8)	0 (0)	0.49

Values are mean (±SD), median (IQR) or number (%). GA indicates gestational age, HELLP; hemolysis, elevated liver enzymes and low platelets; uPCR, urinary protein-to-creatinin ratio; NICU, neonatal intensive care unit.

Biomarker levels 4 and 8 days after omeprazole treatment

In total, 41 women remained pregnant 4 days after enrollment. One of these women was excluded from the primary outcome analysis because biomarker measurements on day 4 were not performed according to protocol (Figure 1). At baseline, levels of sFlt-1, PlGF and sFlt-1/PlGF ratio were not significantly different between the omeprazole group and the non-omeprazole group (7110 *vs.*10743 pg/mL, p=0.11 for sFlt-1; 92 *vs*. 55 pg/mL, p=0.14 for PlGF and 118 *vs*. 208, p=0.08 for sFlt-1/PlGF ratio) (Figure 2A-C). Of these women, those receiving omeprazole did not show lower sFlt-1 levels when measured 4 days after treatment compared to women not using omeprazole (8364 vs. 13017 pg/mL, p=0.14), and the same was true for PlGF (90 vs. 55 pg/mL, p=0.14) and sFlt-1/PlGF ratio (132 vs. 294, p=0.06). Thirty-one women remained pregnant after 8 days, of which those in the omeprazole group displayed lower sFlt-1 (6800 vs. 13349 pg/mL, p=0.01) and sFlt-1/PlGF ratio (59 vs. 373, p=0.03). However, baseline sFlt-1 levels in these 31 women were also significantly lower (p=0.01) in the omeprazole compared to the non-omeprazole group, and the same was true for the sFlt-

1/PlGF ratio (p=0.02) (data not shown). CT-proET-1 measurements were only performed in 31 women, of which the values did not differ at baseline, or at 4 or 8 days after enrollment (Figure 2D).

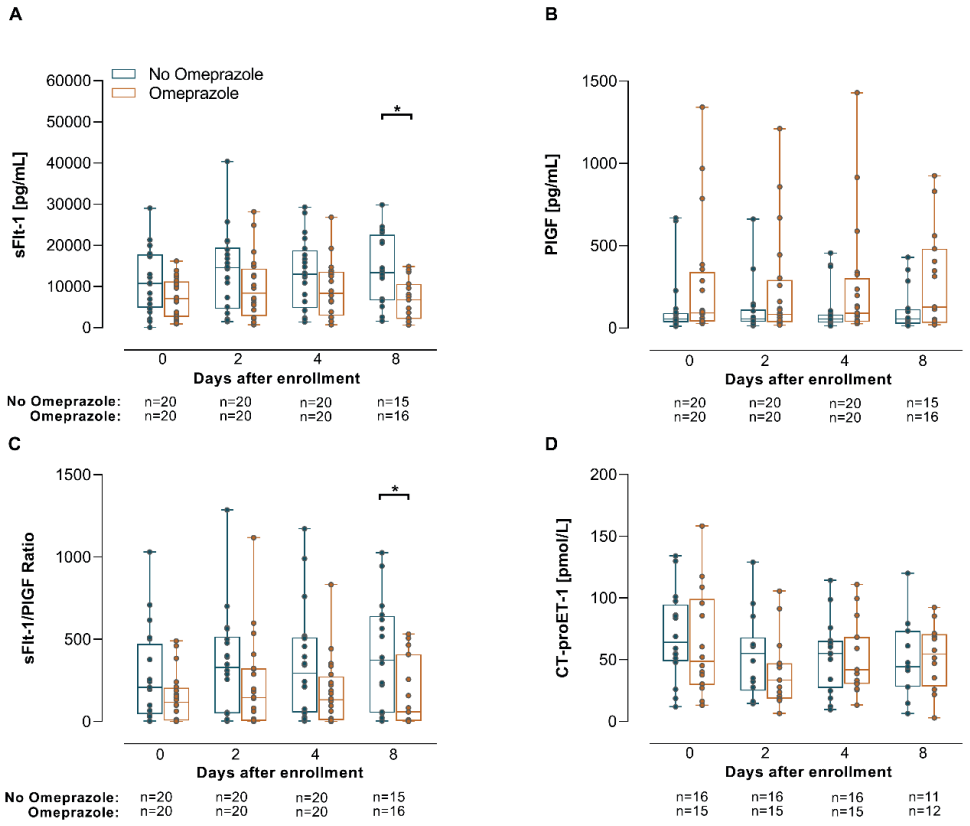

Figure 2A-D. Box-and-whiskers plots showing circulating levels of in 40 singleton pregnancies in the omeprazole and non-omeprazole group according to days 0, 2, 4 and 8 after enrollment; * indicates P < 0.05. Boxes show median and interquartile range, and whiskers are range.

Longitudinal course of biomarkers and cord blood levels

The longitudinal course differences in sFlt-1, PlGF, sFlt-1/PlGF ratio or CT-proET-1 levels could not be attributed to an effect of omeprazole treatment (fixed effect estimate of treatment group for sFlt-1, 281.76 [p=0.74]; PlGF, 36.04 [p=0.12]; sFlt-1/PlGF ratio, -37.6 [p=0.17] and for CT-proET-1, -3.8 [p=0.37]) (Figure 3A-D). Additionally, there were no differences in cord blood levels of the circulating factors between omeprazole group and non-omeprazole group (Figure 4A-D).

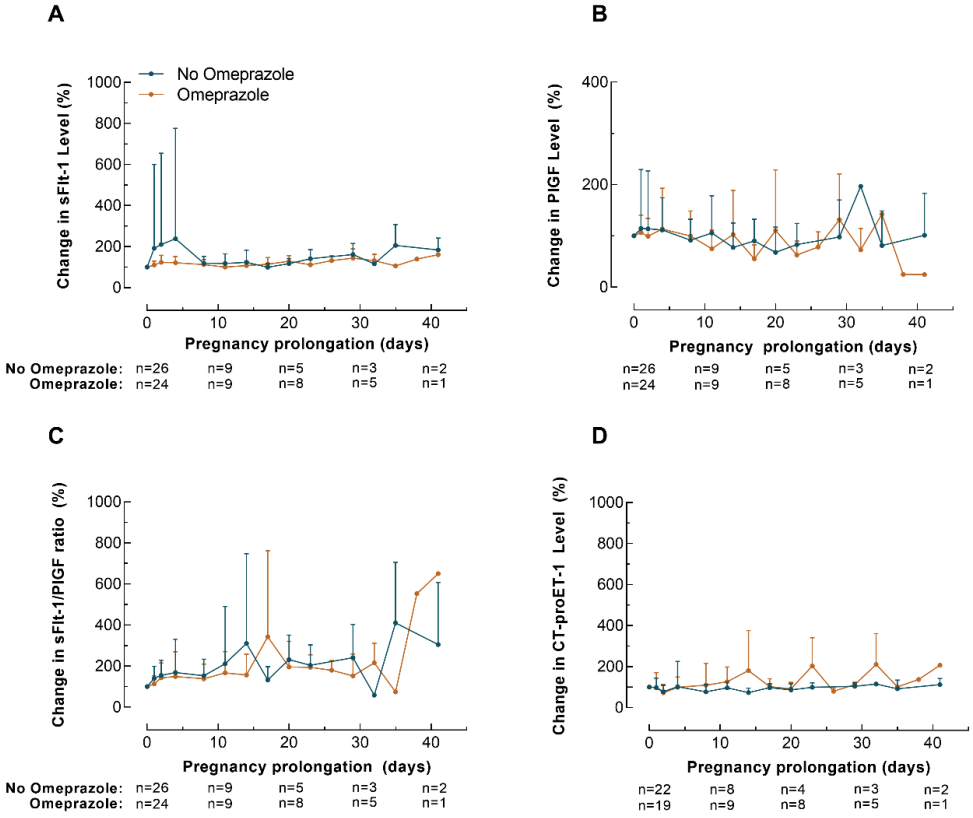

Figure 3A-D. Longitudinal change in soluble Fms-like tyrosine kinase-1 (sFlt-1) (**A**), placental growth factor (PlGF) (**B**), sFlt-1/PlGF ratio (**C**) and C-terminal proendothelin-1 (CT-proET-1) (**D**) as percentages of baseline concentrations according to days of pregnancy prolongation. Values are expressed as median (interquartile range).

Pregnancy outcomes

Mean GA at delivery was 33 weeks in the omeprazole group, compared to 32 weeks in the non-omeprazole group (P=0.36), as displayed in Table 2. The length of pregnancy of women receiving omeprazole was not different from that in women not using these drugs (median 15 vs. 14 days, p=0.70). Adverse maternal outcomes did not occur more often in the non-omeprazole group in comparison to women receiving omeprazole (Table 2). With regard to neonatal complications, the proportion of adverse outcomes remained similar in both groups except for a higher percentage of neonates requiring endotracheal intubation in the non-omeprazole group compared to the omeprazole group (31% vs 4%, p=0.02).

Angiogenic marker levels in placental perfusate

Sixteen healthy women were included in the study. Maternal and pregnancy characteristics of the placentas and offspring are shown in Table 3. The maternal sFlt-1 perfusate concentrations in healthy placentas perfused with omeprazole (n=5) did not differ from those placentas perfused without drugs (n=6, p=0.95). In contrast, maternal sFlt-1 release in esomeprazole perfused placentas (n=5) was significantly diminished in comparison to control placentas (p=0.01), as observed in Figure 4.

Table 3. Placenta Characteristics of Healthy Placentas Perfused with No drugs, Omeprazole or Esomeprazole.

Characteristic	**No drug (n=6)**	**Omeprazole (n=5)**	**Esomeprazole (n=5)**
Maternal age, years	35 (34 -36)	32 (28 -33)	37 (35 -38)
Parity, n	1 (1-1)	1 (0-1)	1 (1-1)
Caucasian Background	4	2	3
Body Mass index, kg/m^2	26 (24 -30)	24 (22 - 25)	23 (22 - 25)
Highest DBP, mmHg	85 (73 - 88)	80 (75 - 85)	87 (75 - 89)
Gestational age at delivery, weeks	39 (38 -39)	39 (38 -39)	39 (39 -39)
Birth weight, grams	3502 (3377 - 3708)	3300 (2960 - 3555)	3738 (3685 - 3825)
Birth weight percentile, grams	61 (55 -77)	51 (28 - 71)	84 (82 - 90)
Placental weight, grams	687 (653 - 743)	660 (494 - 680)	695 (645 - 727)
Fetal sex (Female/Male)	4/2	5/0	5/0

Values are median (IQR) or number (%). DBP indicates diastolic blood pressure and GA, gestational age.

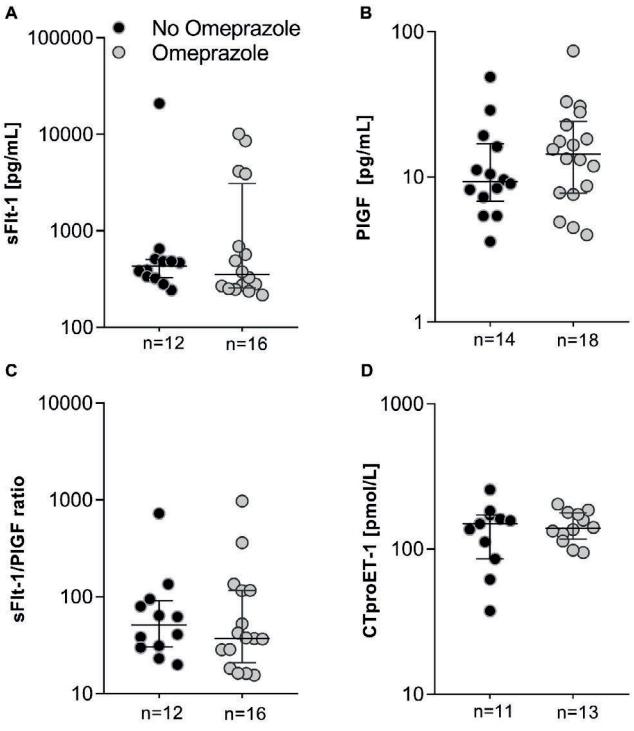

Figure 4A-D. Cord blood levels of soluble Fms-like tyrosine kinase-1 (sFlt-1) (**A**), placental growth factor (PlGF) (**B**), sFlt-1/PlGF ratio (**C**) and C-terminal proendothelin-1 (CT-proET-1) (**D**) according to the omeprazole (grey circles) or non-omeprazole group (black circles).

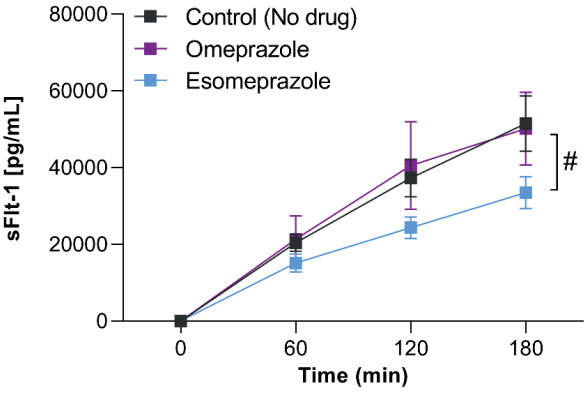

Figure 5. Maternal effluent concentrations of soluble Fms-like tyrosine kinase-1 (sFlt-1) according to time (min) in placentas perfused with no drug (n=6, black squares), omeprazole (n=5; purple squares) or esomeprazole (n=5; blue squares). Values are depicted as mean (± SEM), # indicates $p<0.05$.

DISCUSSION

In this randomized trial of women with confirmed preeclampsia, daily administration of 40 mg omeprazole was not effective in reducing the circulating levels of sFlt-1 and CT-proET-1, or in increasing the levels of PlGF within 4 or 8 days after administration. Additionally, we observed no alterations in the longitudinal course or cord blood levels of these angiogenic markers, when comparing women receiving omeprazole compared to those not using this drug. When evaluating pregnancy outcomes, maternal complication rate was not different in the omeprazole group vs. the non-omeprazole group, and the same was true for neonatal complications, except for a higher proportion of neonates requiring intubation in the non-omeprazole group.

The present study is the first to evaluate the immediate consequences of omeprazole administration on circulating angiogenic marker levels in women with preeclampsia. Our results argue against a direct effect of omeprazole on sFlt-1 release as an explanation of our previous observation that sFlt-1 levels were 67% lower in 40 women with suspected or confirmed preeclampsia using PPIs compared to 80 gestational-age matched women not on this treatment.[7] One reason for this discrepancy is that the median duration of PPI use was 29 days in the former[7], compared to just 14 days in the present study. Moreover, in a trial conducted by Cluver et al., daily administration of 40 mg esomeprazole for an average of 13 days, also did not affect the serial course of sFlt-1 and PlGF in 59 women with preterm preeclampsia, in agreement with our findings.[11] This suggests that, if PPIs could truly lower sFlt-1, this might only be observed after long-term treatment, requiring a start of this drug prior to the onset of preeclampsia. Here, we draw attention to the well-known magnesium-lowering effects of PPIs occurring after long-term treatment.[12] Low magnesium levels, combined with the lower expression of the Mg^{2+}-transporting transient receptor potential melastatin-subfamily member 7 (TRPM7) in preeclamptic placentas, have been suggested to result in lower VEGF expression.[13] Although this concept provides a mechanistic basis for the therapeutic use of $MgSO_4$ in preeclampsia, definite proof for this theory is lacking. If true however, PPIs might exert both beneficial (lowering sFlt-1) and deleterious (lowering VEGF) effects in preeclampsia. In fact, a population-based register cohort involving 157.720 nulliparous pregnant women, recently reported an increased risk of overall preeclampsia and preeclampsia at term of PPI use during any point of pregnancy, while PPI use recorded after 28 gestational weeks was associated with a reduced risk of preterm and early preeclampsia.[14] Based on this, a unifying concept might be that to exert beneficial effects in preeclampsia, PPIs should be used over a defined period, in close proximity to disease onset, and most likely for >2 weeks.

Interestingly, when we perfused healthy placentas with esomeprazole, maternal effluent levels of sFlt-1 were significantly lower in relation to controls. This was not observed for

omeprazole. Similarly, Onda et al. noted that esomeprazole and lansoprazole induced the highest dose-dependent sFlt-1 decrease in primary trophoblast and endothelial cells, whereas omeprazole exerted more modest reductions in sFlt-1.[6] Because omeprazole is a racemic mixture of 2 optical isomers, R- and S-omeprazole (the latter being equal to esomeprazole), the only difference between these drugs is that the metabolism rate of the (S)-isomer is slower, leading to higher plasma concentrations following the same dose.[15] It is unlikely that such pharmacokinetic differences underlie the distinct effects on sFlt-1 secretion over a 3-hour placental perfusion period. Moreover, the acute effect seems to be of limited relevance, since both agents were unable to diminish sFlt-1 release in an in vivo situation. Here, an important question remains why we and others have observed PPI-induced sFlt-1 reductions in preclinical studies, while not being able to confirm these findings in clinical trials. Remarkably, this pattern has also been witnessed with other drugs such as pravastatin or sulfasalazine, which have shown significant sFlt-1 lowering properties in both placental tissues and animal models, although these actions were not apparent when examined in human patients.[16-20] One possibility is that the dosages applied in experimental models were much higher compared to the concentrations administered in clinical studies. Indeed, when the C_{max} equivalent of 40 mg esomeprazole (5 µmol/L) was applied in primary tissues, it did not induce a significant reduction in sFlt-1, in contrast with concentrations of 50 or 100 µmol/L.[6] Increasing the dose (e.g., to 80 mg) or applying these drugs intravenously might solve this problem, although there is less data regarding pharmacokinetics and teratogenic risks for these concentrations. In addition, it could be the case that the sFlt-1 diminishing effect of PPIs is simply insufficient to counteract the massive placental release when preeclampsia is already established. Therefore, we propose to explore PPIs as preventive therapy rather than curative treatment, for example in combination with aspirin before the 16th week of gestation. In fact, a randomized-placebo controlled trial has already been initiated where the consequences of daily esomeprazole administration to women at high risk of preeclampsia will be assessed.[5]

We reported a substantially higher neonatal intubation rate in the non-omeprazole group compared to the omeprazole group. The non-significant higher baseline sFlt-1/PlGF ratio in the non-omeprazole group might partially explain higher disease severity and therefore more iatrogenic preterm birth in the latter group. However the proportion of neonates admitted to the NICU or presenting with other complications remained relatively similar between groups, arguing against this hypothesis. Moreover, in our former analysis evaluating PPI users, a non-significant trend towards higher intubation rate was similarly observed in the non-PPI group compared to the PPI group (15% vs 5%, p=0.07).[7] On the other hand, in the trial by Cluver et al., where a much larger population was evaluated, a difference in intubation rate between esomeprazole and placebo group was not found.[11] Hence, caution is granted when interpreting these findings, since our study is underpowered for these outcomes.

Our study has several strengths. Importantly, our population accurately reflects the clinical characteristics of women with preterm preeclampsia, of which more than 70% had a sFlt-1/PlGF ratio ≥85. In addition, we were able to closely monitor all participants, leading to a high compliance rate in our trial. Since most women already delivered within 1 to 2 weeks after enrollment, a limitation of our study was the low number of women assessed for the serial course of the biomarkers between the omeprazole and non-omeprazole group. Nevertheless, this was a secondary outcome, similar to the maternal/neonatal adverse outcomes, whereas our power calculation was solely based on detecting a difference in sFlt-1. In summary, our findings argue against a role for PPIs as potential treatment for preeclampsia, since daily administration of this drug was unable to improve the maternal circulating profile of sFlt-1, PlGF and ET-1 in women with established preeclampsia.

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CHAPTER 09

Angiogenic Markers are Elevated in Women with Acute Fatty Liver of Pregnancy

Rugina I. Neuman, Emma R.M Hesselink, Langeza Saleh, Anton H. van den Meiracker, A.H. Jan Danser, Willy Visser

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Acute fatty liver of pregnancy (AFLP) is a life-threatening, pregnancy-specific condition with an estimated global incidence of 1:7000 to 1:15.000 pregnancies[1]. Despite several clinical characteristics unique to the disorder, distinguishing AFLP from other liver diseases of pregnancy, such as hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome or preeclampsia (PE) may be challenging. Recent studies established an imbalance in angiogenic factors, characterized by elevated levels of soluble Fms-like tyrosine kinase-1 (sFlt-1) and low placental growth factor (PlGF) as a key pathogenic mechanism underlying PE and HELLP syndrome[2]. However, the role of these factors in the pathophysiology of AFLP remains unknown. In this study, we measured serum levels of sFlt-1 and PlGF in women with AFLP, and compared them to women with suspected PE, confirmed PE and HELLP syndrome.

We conducted a secondary analysis of a prospective multicenter cohort study, in which women with AFLP, suspected or confirmed PE or HELLP syndrome were enrolled at three Dutch hospitals, originally aimed at evaluating the diagnostic value of sFlt-1, PlGF and sFlt-1/PlGF ratio. Serum samples were collected and stored (-80°C) upon study entry, and were measured at the end of the study. We aimed to compare three gestational-age matched women with suspected, confirmed PE or HELLP syndrome, for each woman with AFLP.

Overall, 53 singleton and 25 twin pregnancies were included. A total of 11 women diagnosed with AFLP were evaluated, of whom six had a singleton and five a twin pregnancy. In women diagnosed with AFLP, median gestational age at study entry was 36 (range, 34 – 38) weeks in those with singleton pregnancy and 33 (30 – 34) weeks in those with twin pregnancy. In singleton pregnancies with HELLP syndrome, gestational age was lower than in those with suspected PE, confirmed PE or AFLP (Table S1 and S2). In singleton pregnancies, women with AFLP displayed higher serum sFlt-1 levels than women with suspected or confirmed PE (Figure 1). When comparing PlGF levels in singleton pregnancies, women diagnosed with HELLP syndrome displayed lower levels than women with suspected PE, confirmed PE or AFLP, whereas the sFlt-1/PlGF ratio was higher in women with both HELLP and AFLP, when compared with the suspected PE group, and higher in women with HELLP syndrome when compared to the confirmed PE group. No differences in sFlt-1 or PlGF levels or sFlt-1/PlGF ratio were observed between twin pregnancies with suspected PE, confirmed PE and AFLP (Figure 2).

Our previous observation that non-preeclamptic twin pregnancies exhibit higher sFlt-1 levels than non-preeclamptic singleton pregnancies due to increased placental mass[3], may provide an explanation for this lack of difference.

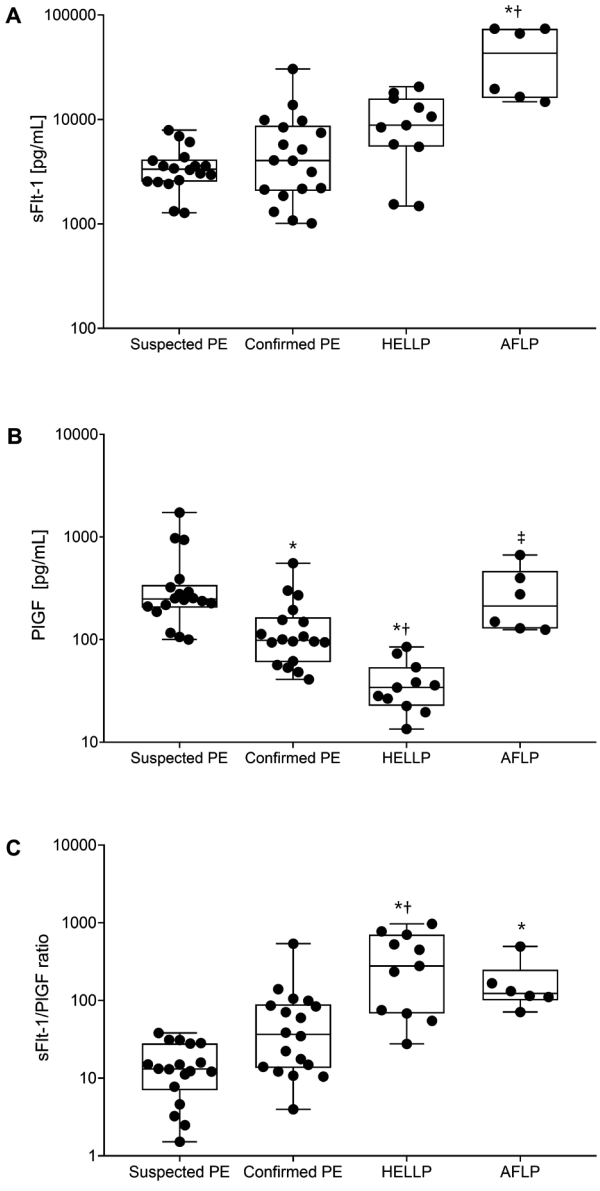

Figure 1. Box-and-whiskers plots showing circulating levels of soluble fms-like tyrosine kinase-1 (sFlt-1) (a), placental growth factor (PlGF) (b) and sFlt-1/PlGF ratio (c) in 53 singleton pregnancies, according to clinical diagnosis. $P < 0.05$ for comparison with: *suspected pre-eclampsia (PE) group; †confirmed PE group; and ‡hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome group. Boxes show median and interquartile range, and whiskers are range. AFLP, acute fatty liver of pregnancy.

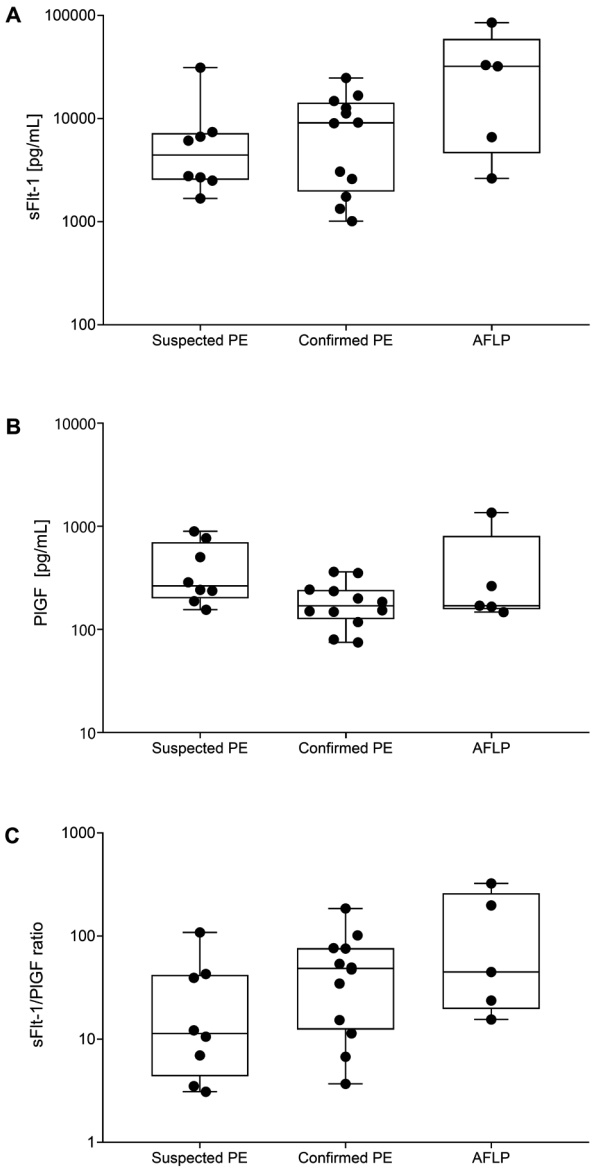

Figure 2. Box-and-whiskers plots showing circulating levels of soluble fms-like tyrosine kinase-1 (sFlt-1) (a), placental growth factor (PlGF) (b) and sFlt-1/PlGF ratio (c) in 25 twin pregnancies, according to clinical diagnosis. There were no significant differences between groups. Boxes show median and interquartile range, and whiskers are range.

Our results, together with the observations by Suzuki *et al*[4]*.,* who reported an elevated sFlt-1/PlGF ratio in a woman with AFLP in comparison to 14 women with HELLP syndrome, imply a potential role for sFlt-1 in the pathophysiology of AFLP. One could suggest that the mechanisms underlying PE, HELLP syndrome and AFLP are all a consequence of an anti-angiogenic state, which may explain why these disorders co-exist in 20% of cases[1]. This is indirectly supported by preclinical studies with tyrosine-kinase inhibitors used as anti-angiogenetic treatment, resulting in impaired hepatic mitochondrial oxidation[5]. Nonetheless, we encourage future studies to investigate the role of angiogenic factors, particularly sFlt-1, in the pathogenesis of AFLP.

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SUPPLEMENTAL DATA

Table S1. Patient characteristics and pregnancy outcome according to diagnosis in women with singleton pregnancies.

Singleton Pregnancies				
Parameter	**Suspected PE**	**PE**	**HELLP**	**AFLP**
N	18	18	11	6
Age (years)	32 (18 - 41)	33 (22 - 41)	29 (22 - 36)	31 (25 - 41)
Gestational age (weeks)	36 (34 - 38)	36 (34 - 38)	31 (29 - 40)[a,b]	36 (34 - 38)[c]
Nulliparous (n, %)	10 (56)	10 (56)	11 (100)	3 (50)
Ethnicity (n, %)				
White	14 (78)	10 (56)	11 (100)	5 (83)
Black	2 (11)	2 (11)	0 (0)	0 (0)
Other	2 (11)	6 (33)	0 (0)	1 (17)
Preexisting hypertension (n, %)	2 (11)	4 (22)	1 (9)	1 (17)
History of PE (n, %)	2 (11)	1 (6)	0 (0)	0 (0)
Clinical findings at time of admission				
SBP, mmHg	128 (110 - 160)	150 (120 - 181)[a]	140 (121 - 172)	120 (116 - 155)
DBP, mmHg	85 (65 - 105)	93 (70 - 110)	92 (66 - 105)	90 (75 - 95)
uPCR, mg/mmol	17 (7 - 56)	50 (30 - 588)[a]	42 (13 - 2564)	29 (12 - 86)
LD, U/L	173 (111 - 597)	199 (126 - 331)	458 (232 - 1376)[a,b]	397 (159 - 711)[a]
Creatinine, µmol/L	54 (40 - 79)	58 (43 - 213)	67 (50 - 126)	155 (103 - 213)[a,b]
Uric acid, mmol/L	0.27 (0.17 – 0.35)	0.36 (0.21 – 0.45)[a]	0.35 (0.23 – 0.67)[a]	0.47 (0.38 – 0.84)[a]
ALT, U/L	13 (6 - 186)	16 (5- 36)	153 (44 - 1020)[a,b]	353 (37 - 1533)[a,b]
Platelet count, 10^9/L	220 (106 - 462)	227 (102 - 341)	72 (36 - 205)[a,b]	202 (164 - 265)[c]
Pregnancy outcome				
Time until delivery (days)	12 (0 - 26)	3 (0 - 16)	1 (0 - 16)[a]	1 (0 - 6)[a]
Gestational age at delivery (weeks)	38 (36 - 40)	37 (33 - 38)	32 (30 - 33)[a,b]	37 (34 - 38)
Birth weight percentile < 10% (n, %)	0 (0)	1 (6)	3 (27)[a]	0 (0)

Values are median (range) or n (%); a indicates comparison with suspected PE at a significance level of $p<0.05$; b indicates comparison with PE at a significance level of $p<0.05$; c indicates comparison with HELLP syndrome at a significance level of $p<0.05$. PE indicates preeclampsia; HELLP, hemolysis, elevated liver enzymes, low platelet count; AFLP, acute fatty liver of pregnancy; SBP indicates systolic blood pressure, DBP; diastolic blood pressure, uPCR; urinary protein-to-creatinine ratio, LD; lactate dehydrogenase, ALT; alanine transaminase.

Table S2. Patient characteristics and pregnancy outcome according to clinical diagnosis in twin pregnancies.

Twin Pregnancies			
Parameter	**Suspected PE**	**PE**	**AFLP**
N	8	12	5
Age (years)	34 (23 - 46)	37 (31 -44)	32 (28 - 35)
Gestational age (weeks)	28 (20 - 34)	30 (26 - 34)	33 (30 - 34)
Nulliparous (n, %)	4 (50)	9 (75)	5 (100)
Ethnicity (n, %)			
White	5 (63)	9 (75)	5 (100)
Black	2 (25)	0 (0)	0 (0)
Other	1 (13)	3 (25)	0 (0)
Preexisting hypertension (n, %)	2 (25)	0 (0)	1 (20)
History of PE (n, %)	0 (0)	0 (0)	0 (0)
Clinical findings at time of admission			
SBP, mmHg	140 (130 - 150)	150 (130 - 186)	121 (115 - 135)[b]
DBP, mmHg	85 (70 - 95)	93 (83 - 112)	86 (65 - 90)
uPCR, mg/mmol	20 (8 - 119)	54 (10 - 576)	40 (13 - 107)
LD, U/L	204 (167 - 254)	202 (122 - 314)	381 (268 - 430)[a,b]
Creatinine, µmol/L	54 (46 - 102)	57 (45 - 87)	113 (64 - 141)[a,b]
Uric acid, mmol/L	0,23 (0,19 - 0,26)	0,35 (0,24 - 0,42)[a]	0,53 (0,40 - 0,56)[a]
ALT, U/L	14 (6 - 110)	16 (10 - 35)	242 (125 - 433)[a,b]
Platelet count, 10^9/L	178 (148 - 274)	180 (107 - 300)	169 (121 - 199)
Pregnancy outcome			
Time until delivery (days)	45 (0 - 138)	19 (0 - 39)	1 (0 - 3)[a]
Gestational age at delivery (weeks)	35 (31 - 37)	34 (26 - 37)	33 (30 - 34)
Birth weight percentile < 10% (n, %)	2 (25) / 2 (25)	2 (17) / 5 (42)	0 (0) / 0 (0)

Values are median (range) or n (%). a indicates comparison with suspected PE at a significance level of $p < 0.05$; b indicates comparison with PE at a significance level of $p<0.05$. PE indicates preeclampsia; AFLP, acute fatty liver of pregnancy; SBP indicates systolic blood pressure, DBP; diastolic blood pressure, uPCR; urinary protein-to-creatinine ratio, LD; lactate dehydrogenase, ALT; alanine transaminase.

CHAPTER 10

Accurate prediction of total PlGF (placental growth factor) from free PlGF and sFlt-1 (soluble Fms-like tyrosine kinase-1): evidence for markedly elevated PlGF levels in women with acute fatty liver of pregnancy

Rugina I. Neuman, Langeza Saleh, Koen Verdonk, Anton H. van den Meiracker, Henk Russcher, Herold J. Metselaar, Willy Visser, A.H Jan Danser

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ABSTRACT

Acute fatty liver of pregnancy (AFLP) is characterized by elevated circulating soluble Fms-like tyrosine kinase-1 (sFlt-1), although the free circulating levels of its ligand, placental growth factor (PlGF) are not decreased. Here, we hypothesized that women with AFLP exhibit elevated PlGF production in comparison to women with preeclampsia or HELLP-syndrome. Making use of the well-known mathematical formulas describing drug-receptor interactions, we established that serum total PlGF could be accurately predicted from sFlt-1 and free PlGF levels (n = 42; mean calculated K_D of 50pmol/L), yielding similar values as the previously published method of thermal dissociation of the sFlt-1-PlGF complexes (*r* = 0.94, $p<0.0001$). We found that median levels of free PlGF were significantly lower in women with preeclampsia (n=13; 117pg/mL) or HELLP syndrome (n=12; 59 pg/mL) compared with women without preeclampsia (n=11; 349pg/mL, $p<0.0001$). In contrast, median total PlGF did not differ between women with no preeclampsia, preeclampsia and HELLP syndrome (354 versus 435 versus 344pg/mL), while it was markedly elevated in AFLP compared to all groups (2054 pg/mL, $p<0.0001$). Furthermore, in AFLP, both sFlt-1 and total PlGF declined rapidly postdelivery, with significantly higher predelivery total PlGF (n=12; median, 2054 pg/mL) than postpartum levels (n=14; median,163pg/mL, $p<0.0001$), suggesting that in AFLP, PlGF is largely placenta-derived. Collectively, our findings indicate that like sFlt-1, PlGF production is significantly upregulated in AFLP, mainly originating from the placenta. Importantly, total PlGF can now be easily calculated from already available free PlGF and sFlt-1 levels, allowing subsequent evaluation of other groups in whom PlGF is altered.

INTRODUCTION

Acute fatty liver of pregnancy (AFLP) is a severe liver disorder unique to pregnancy, typically occurring after 30 weeks' gestation.[1] Although uncommon, with an estimated global incidence of 1 in 7000 - 15.000 pregnancies, AFLP is a life-threatening disease for both mother and child.[1] The exact pathogenesis of AFLP remains unclear, but it is generally believed that mitochondrial oxidation defects of the fetus or placenta lead to a buildup of free fatty acids in the maternal blood and hepatocytes, subsequently causing the detrimental manifestations of the disorder.[1] The most prominent characteristic of AFLP is the presence of hepatic dysfunction, reflected by complications such as hypoglycemia, coagulopathy and renal failure.[1, 2] Despite these distinctive features, differentiating AFLP from other liver diseases of pregnancy, particularly the HELLP syndrome (i.e hemolysis, elevated liver enzymes and low platelet count), remains a challenge in clinical practice.[2, 3] An important factor underlying this difficulty might be that up to 20% of women with AFLP are also diagnosed with preeclampsia, a condition characterized by hypertension in the second half of pregnancy along with proteinuria or signs of maternal organ damage, including HELLP syndrome.[1, 4, 5] Whether preeclampsia and AFLP are merely associated with one another, or belong to a spectrum of the same disorder, as some have suggested[6, 7], is uncertain.

During preeclampsia, poor placentation triggers the excessive release of the soluble vascular endothelial growth factor receptor (sVEGFR; also known as sFlt-1), which binds its 'free' circulating ligands VEGF and placental growth factor (PlGF).[8, 9] The ensuing angiogenic imbalance is thought to contribute significantly to the clinical manifestations of this disorder.[10] Interestingly, our group has recently discovered that women with AFLP also display increased levels of sFlt-1 in their maternal circulation.[11] Yet, despite the markedly elevated sFlt-1 levels, women with AFLP exhibited higher free levels of PlGF, in contrast to what is observed in HELLP syndrome.[11]

Based on our previous observations, we hypothesized that women with AFLP display elevated PlGF production in comparison to preeclampsia / HELLP syndrome. Recently, Lecarpentier et al. established a novel method to measure total PlGF in maternal blood, since commercial immunoassays only detect the unbound (free) form of PlGF.[12] Hence, in the present study we intended to 1) validate this method in a small population of women with a low and high sFlt-1/free PlGF ratio; 2) determine whether a more simple approach, by calculating total PlGF mathematically from sFlt-1 and free PlGF, would prove equally reliable as thermal dissociation and 3) compare serum total PlGF levels in women with AFLP to women with no preeclampsia, preeclampsia or HELLP syndrome. In addition, we explored the origin of the angiogenic markers in AFLP and whether they could derive from the placenta.

METHODS

Human participants

Participants with AFLP

Human serum samples from a database of women with singleton pregnancies who had a clinical diagnosis of AFLP at the Erasmus Medical Center, Rotterdam, the Netherlands between 2005 and 2020 were utilized. Serum samples were collected at the time of AFLP diagnosis (both during and after pregnancy) and later archived at -80°C as part of routine care. Residual material with enough volume for the analysis of sFlt-1, free and total PlGF was collected for this study, if the patients did not object against use of this material. The use of these samples for the purposes of this study was exempted from approval by the local institutional Medical Ethics Committee according to the Dutch Medical Research with Human Participants Law (MEC-2020-0668). All laboratory assays were undertaken masked to the clinical diagnosis. Clinicians had no knowledge of the angiogenic measurements at time of AFLP diagnosis. The diagnosis of AFLP was suspected when a pregnant woman had symptoms of nausea, vomiting, fatigue and anorexia at the end of the second or third trimester in combination with jaundice and elevated liver enzymes. The diagnosis of AFLP was confirmed when a woman fulfilled ≥6 out of 15 Swansea criteria[13], and the treating physician found the clinical diagnosis of AFLP as most likely in comparison to other disorders such as HELLP syndrome. Pregnancy characteristics and outcome were obtained from the digital medical files.

Participants with no PE, PE or HELLP syndrome

We aimed to compare all women with AFLP to 12 gestational-age matched women with either no preeclampsia, confirmed preeclampsia or HELLP syndrome, given that the values of sFlt-1, free and total PlGF alter with advancing gestation.[14] Available residual serum samples from these three groups were collected from a previously conducted prospective cohort study in which the sFlt-1/free PlGF ratio was measured in singleton pregnancies with suspected or confirmed preeclampsia, between 2013 and 2016 at three hospitals in the Netherlands. This study was approved by the research ethics committee (MEC-2013-202) and written informed consent was obtained from all participants. Venous blood was taken at study entry only, and was stored at -80°C until analysis, which was conducted at the end of the study, to avoid influence on decision making of the obstetricians.[9]

Preeclampsia was defined as the presence of new-onset hypertension [systolic blood pressure [SBP] of ≥140 mmHg and/or diastolic blood pressure [DBP] of ≥90 mmHg) and proteinuria (protein-to-creatinine ratio [uPCR] ≥ 30mg/mmol or ≥300mg/24h or 2+ dipstick) at or after 20 weeks' gestation, according to the 2001 ISSHP (International Society for the

Study of Hypertension in Pregnancy) definition, which was in effect at the time of study initiation.[15] HELLP syndrome was defined as a reduction of platelet count <100 x 10^9/L, an elevation of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) two-fold the upper limit of normal, and an elevated lactate dehydrogenase (LDH; two-fold the upper reference limit or greater than 650IU/L) according to the ISSHP 2013 definition.[16] Women who had a partial HELLP syndrome (≥2 of the HELLP criteria) at time of blood sampling but later developed HELLP syndrome were also considered as HELLP syndrome. Women who were initially suspected of preeclampsia, but did not fulfill the diagnosis of gestational hypertension, preeclampsia or HELLP syndrome throughout their pregnancy were defined as no preeclampsia.

For the validation studies, we selected serum samples from patients with a low angiogenic imbalance (sFlt-1/free PlGF ratio≤38) versus a high angiogenic imbalance (sFlt-1/free PlGF ratio≥85) from the abovementioned cohort study. These cut-off values were previously reported to predict the short-term absence of preeclampsia (sFlt-1/free PlGF ratio ≤38)[17] or a high risk of preeclampsia-related adverse outcomes (sFlt-1/free PlGF ratio ≥85).[18] All pregnancy characteristics and outcome were obtained from digital medical files.

Participants with acute liver failure

Available serum samples from non-pregnant women with acute liver failure requiring immediate liver transplantation at the Erasmus Medical Center, Rotterdam, the Netherlands were selected at random, for the measurement of sFlt-1 and total PlGF. The use of these samples for the purposes of this study was exempted from approval by the local institutional Medical Ethics Committee according to the Dutch Medical Research with Human Participants Law (MEC-2014-060).

Measurement of sFlt-1, free PlGF and total PlGF

Validation studies

Human recombinant PlGF (rhPlGF; 264-PGB-010/CF, R&D Systems, Minneapolis, MN, USA) was dissolved in phosphate-buffered saline containing 0.1% bovine serum albumin (PBS). This solution was mixed 1:3 with either PBS or serum to reach a final rhPlGF concentration of 1693 pg/mL. For comparison, serum samples were also mixed 1:3 with PBS not containing rhPlGF. Serum was obtained from pregnant patients with either a low (≤38) or a high (≥85) sFlt-1/free PlGF ratio. All samples (rhPlGF-containing PBS, serum with PBS, and serum with rhPlGF-containing PBS) were incubated for 30 minutes at room temperature to allow rhPlGF to bind to sFlt-1. Next, sFlt-1 and PlGF were measured in all samples before and after heating.

Heating procedure and biochemical measurements

For the thermal dissociation of all sFlt-1-PlGF complexes, serum samples were placed in a heating block at 70°C for 10 minutes, as described by Lecarpentier et al.[12] Measurements of sFlt-1 and PlGF before and after heating were performed using the automated Elecsys immunoassay from Roche Diagnostics (Cobas 6000, e-module; Rotterdam, the Netherlands).

Calculation of total PlGF from free PlGF and sFlt-1

In blood plasma, an equilibrium exists between free sFlt-1, PlGF and their complex, described by the following equation: [sFlt-1] + [PlGF] $\rightleftharpoons$ [sFlt-1-PlGF]. This equals classic drug-receptor interaction, with [sFlt-1] resembling the total number of receptors $[R]_{total}$, free PlGF ($[PlGF]_{free}$, i.e., the PlGF level measured without heating) resembling the amount of non-receptor-bound drug [D], and [sFlt-1-PlGF] resembling the number of drug-occupied receptors [DR]. The dissociation constant K_D = [D] x [R]/[DR]. Given that $[R]_{total}$ = [R] + [DR], this formula can be rewritten as K_D = - [D] + [D] x $[R]_{total}$/[DR]. Since [DR] can be calculated by subtracting $[PlGF]_{total}$ (i.e., the PlGF level obtained after heating) from $[PlGF]_{free}$, and considering that the molecular weights of PlGF and sFlt-1 are 34 and 100 kD, respectively, it is now possible to calculate K_D. This approach was followed in all samples where free and total PlGF levels were available, with total PlGF being higher than free PlGF, allowing us to calculate a mean K_D on the basis of 42 samples. With this K_D we were able to predict $[PlGF]_{total}$ by first calculating [DR], i.e., the amount of PlGF bound to sFlt-1, as follows:

$$[DR] = \frac{[D] \times [R]_{total}}{[D] + K_D}$$ and by then adding up [D] and [DR].

Since [D] = $[PlGF]_{free}$, and $[R]_{total}$ = [sFlt-1], this translates to:

$$[PlGF]_{total} = [PlGF]_{free} + \frac{[PlGF]_{free} \times [sFlt\text{-}1]}{[PlGF]_{free} + K_D}$$

Statistical analysis

Data are presented as median (interquartile range) or number (percentage). To evaluate whether continuous variables had a normal distribution, the Shapiro-Wilk normality test was used. To compare groups, the Student *t* test or Mann-Whitney *U* test in case of non-normally distributed data were applied. For the comparison of continuous variables between more than 2 groups, one-way ANOVA, or Kruskall-Wallis test, in the case of non-parametric distributions was applied, with a Dunnet or Bonferroni correction for multiple testing. Spearman Rho was applied to calculate correlation coefficients. A P-value of <0.05 was considered to be statistically significant. Statistical analysis was performed with GraphPad Prism (version 8.0, La Jolla, CA) and SPSS (version 25.0, SPSS Chicago, IL) on Windows.

RESULTS

Thermal dissociation of sFlt-1-PlGF complex using recombinant human PlGF

To confirm the method of thermal dissociation, we added recombinant human PlGF (rhPlGF) to serum samples from pregnancies with a low (≤38) or high sFlt-1/free PlGF ratio (≥85), and measured sFlt-1 and PlGF before and after heating, with and without rhPlGF. Five participants with a ratio≤38 and six participants with a ratio≥85 were evaluated, whose clinical characteristics and pregnancy outcomes are shown in Table 1.

In participants with a ratio≤38, serum PlGF levels marginally (P=NS) increased after thermal dissociation. Heating did not affect the detection of rhPlGF, and also after adding rhPlGF to serum we did not detect higher levels after heating than before (Figure 1A).

In contrast, in participants with a ratio≥85, serum PlGF levels were significantly higher after thermal dissociation than before (270 vs 36 pg/mL). Furthermore, when adding a fixed amount of rhPlGF to serum in this group (ratio≥85), the levels detected in the absence of heating were significantly lower than the amount that was added (1634 vs. 2221 pg/mL). This confirms binding by sFlt-1. Subsequent heating allowed the detection of a PlGF amount that equaled the sum of the added level and the endogenous level (2372 pg/mL; Figure 1A).

Finally, as expected, the sFlt-1 levels were much higher in the ratio≥85 group, while heating greatly diminished the amount of sFlt-1 that could be detected. These data confirm that heating selectively destroys sFlt-1, without affecting PlGF (Figure 1B), thus allowing the quantification of total PlGF.

Table 1. Participant Characteristics at Time of Blood sampling of the Validation Cohort based on sFlt-1/free PlGF ratio.

Parameter	Ratio ≤ 38 (n = 5)	Ratio ≥85 (n = 6)
Maternal age, years	32 (27 - 33)	30 (27 - 33)
GA at blood sampling, weeks^{+days}	32^{+4} (31^{+0} - 33^{+4})	32^{+0} (31^{+3} - 38^{+1})
Nulliparity, n	2	5
Ethnic background, n		
Caucasian/White	1	4
African/Afro-Caribbean/Black	2	1
Other	1	1
Clinical parameters		
SBP, mmHg	120 (115 - 145)	140 (129 - 149)
DBP, mmHg	80 (73 - 95)	93 (89 - 100)
uPCR, mg/mmol	61 (23 - 174)	45 (18 - 309)
Pregnancy outcome		
GA at delivery, weeks^{+days}	37^{+4} (33^{+3} - 38^{+3})	32^{+5} (32^{+0} - 38^{+4})
Male, n	3	4
Birth weight, grams	2684 (2120 - 3538)	1605 (1453 - 2880)
Birth weight percentile <10, n	1	1

Values are median (interquartile range) or n. DBP indicates diastolic blood pressure; GA, gestational age; PlGF, placental growth factor; GA, gestational age; SBP, systolic blood pressure; sFlt-1 indicates soluble Fms-like tyrosine kinase-1; uPCR, urinary protein-to-creatinine ratio.

sFlt-1, free and total PlGF in women with AFLP

Twelve women with AFLP were compared to women with no preeclampsia (n=11), confirmed preeclampsia (n=13) or HELLP syndrome (n=12). Since most of the women with HELLP syndrome from the previously conducted cohort[9] had a lower GA at blood sampling, we were unable to adequately match the AFLP group to women with HELLP syndrome at a similar GA. All participant characteristics and pregnancy outcomes according to clinical diagnosis are shown in Table 2. Women with AFLP displayed higher median sFlt-1 levels (77762 [45044 – 116657] pg/mL) compared to women with no preeclampsia, preeclampsia and HELLP syndrome (2518 [1744 – 3903], 8772 [6410 – 10736] and 14572 [5641 – 20056] pg/mL, respectively) (Figure 2A). When comparing free PlGF levels, women diagnosed with PE and HELLP syndrome displayed lower PlGF values than women with no PE, whereas free PlGF levels in women with HELLP syndrome were also significantly decreased in comparison to women with AFLP (59 [39 – 97] pg/mL vs. 208 [106 – 293] pg/mL) (Figure 2B). In contrast, total PlGF levels (measured after thermal dissociation) did not differ between women with no preeclampsia, preeclampsia and HELLP syndrome, while they were significantly increased in AFLP in comparison to all groups (Figure 2B). None of the women were on heparin treatment at time of blood sampling.

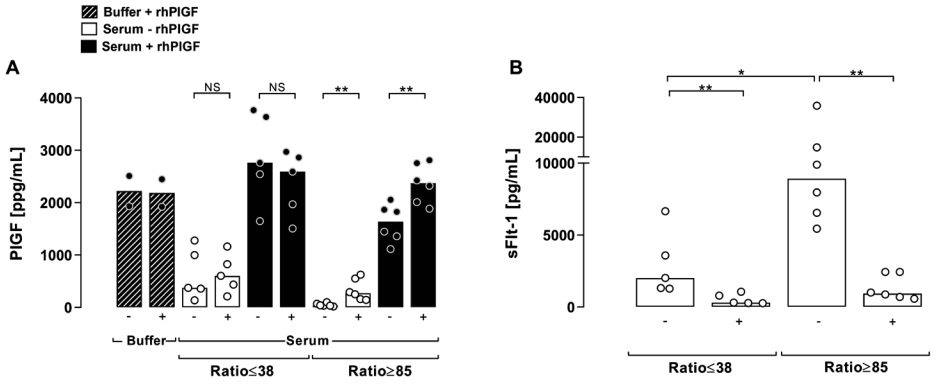

Figure 1. Protein level measurements performed before and after heating.
PlGF (placental growth factor; **A**) and sFlt-1 (soluble Fms-like tyrosine kinase-1; **B**) levels in buffer (n=2) or serum measured before [−] and after [+] heating at 70 °C for 10 min, with or without the addition of rhPlGF (human recombinant PlGF; mixed 1:3 in either PBS or serum to reach a final rhPlGF concentration of 1693 pg/mL). Serum was obtained from pregnant women with an sFlt-1/free PlGF ratio ≤38 (n=5) or ≥85 (n=6). Data are presented as individual values and median (bar). *P<0.05; **P<0.01.

Prediction of total PlGF and comparison with its measured value

K_D was calculated in all samples in which we determined both free and total PlGF. This resulted in an average K_D (± SEM) value of 50±6.4 pmol/L. Next, we predicted total PlGF in

each sample making use of this K_D value, and compared it with the actually measured total PlGF value. As can be seen in Figure 2C, this yielded a relationship which was not different from the line of identity ($r = 0.94$, $P<0.0001$) (Figure 2C).

Ante- vs. postpartum levels of sFlt-1, total PlGF and ALT

In 12 women with AFLP, measurements of sFlt-1 and total PlGF were performed antepartum. In five of these 12 women, postpartum levels were additionally measured, and in three of these five, postpartum measurements were performed at two separate time-points. Additionally, there were six women with AFLP in which the values of sFlt-1 and total PlGF were determined postpartum only. Figure 3A and 3B show that the levels of sFlt-1 decreased by >80% within 2 days after delivery, while for total PlGF the drop was even larger, with <10% remaining after 2 days. This pattern was fully confirmed when simply comparing the median levels before delivery with those after, irrespective of the sampling moment (Figure 3C). The postpartum course of ALT values in AFLP patients was much more gradual (Figures 3E and 3F).

sFlt-1 and total PlGF levels in participants with acute liver failure in need of liver transplantation

Median levels for sFlt-1 were available in seven patients, while total PlGF were measured in eight patients with liver failure in need of liver transplantation. The clinical characteristics of these patients are shown in Table 3. All patients were female with a median (IQR) of 28 (24 -31) years. The most common reason for liver transplantation was toxic or drug-induced liver failure (n=4). Median total PlGF levels of these patients were 22 (12 – 51) pg/mL, which is comparable to the reference values for free PlGF in healthy non-pregnant women (16 [14 -18] pg/mL; Figure 3D).[19] In contrast, median sFlt-1 levels (446 [211 – 1414] pg/mL) were ≈ 6-fold above the normal range in healthy non-pregnant women (76 [67– 84] pg/mL).

DISCUSSION

In the present study, we established that total PlGF levels in serum can be accurately calculated from the sFlt-1 and free PlGF levels, making use of the well-known mathematical formulas describing drug-receptor interaction. This approach yielded the same levels as the previously published method of thermal dissociation. We confirmed that total PlGF in preeclampsia is similar to that in uneventful pregnancy, while this is also true for HELLP. Yet, in AFLP, maternal total PlGF levels were greatly elevated, both versus women with preeclampsia and HELLP syndrome. Furthermore, in AFLP, total PlGF and sFlt-1 declined rapidly after delivery, suggesting the placenta as their most likely source.

A potential role for sFlt-1 and PlGF in the pathophysiology of AFLP had not been suggested until recently, when our group and others[11, 20] noticed abundantly high circulating sFlt-1 levels in women with this disorder. sFlt-1 is a well-recognized feature of preeclampsia, in which its dramatic rise causes a significant reduction of free circulating PlGF levels.[10] While a decrease in placental PlGF production has also been suggested to contribute to the low PlGF levels in preeclamptic disease,[21, 22] we observed no differences in total PlGF when comparing women without preeclampsia, preeclampsia, or HELLP syndrome in the current study. This agrees with the view of Lecarpentier et al. that decreased free PlGF levels in preeclampsia / HELLP are a consequence of higher sFlt-1 rather than decreased PlGF production of the placenta.[12] With this perspective in mind, our previous observation that despite greater sFlt-1 elevation, free PlGF levels were not reduced in women with AFLP in comparison to women with preeclampsia, was quite surprising.[11] Our present finding that total PlGF levels are significantly raised in women with AFLP now explains this observation.

Obviously, a key question is whether an elevated production or impaired metabolism accounts for the dramatic increases of PlGF in AFLP. Because the relatively small PlGF (MW 34kD) can readily cross the glomerular filtration barrier, one could hypothesize that its elimination is compromised as renal function declines in AFLP. The observation that total PlGF in women with this disorder decreased rapidly after delivery, despite the persistence of elevated serum creatinine (data not shown), does not support this argument. Another possibility is that the ensuing liver injury and inflammation in AFLP drives increased PlGF production either in the liver or elsewhere. However, our observation that total PlGF is diminished by >90% within two days postpartum, whereas the liver recovers more gradually after delivery, indicates that a placental origin or placenta-stimulated PlGF synthesis somewhere else is more probable. Consistent with this hypothesis, serum total PlGF levels in non-pregnant patients with acute liver failure were roughly comparable to the 'free' PlGF levels observed in healthy controls (median [IQR]: 22 [12-51] vs. 16 [14 -18] pg/mL). Conversely, their sFlt-1 values were 6-fold higher in relation to the reference values of healthy non-pregnant women (76 [67– 84] pg/mL). This suggests that liver failure might contribute, at least in part, to the higher sFlt-1 values observed in AFLP, but not to the higher PlGF levels. A partial non-placental origin of sFlt-1 might also explain why in AFLP women the drop in sFlt-1 levels after delivery was more modest in comparison to that of total PlGF. In both preeclampsia and HELLP, as we have demonstrated before, the opposite is true: sFlt-1 falls by >90% within 2 days, while free PlGF levels off at around 30 to 40% of its predelivery concentrations. Yet, postpartum, the free PlGF levels reach the same nadir in all groups, and resemble the total PlGF levels. The simplest explanation of our findings is therefore that the stronger total PlGF drop in AFLP (exceeding that of sFlt-1) is due to its very high pre-delivery levels.

Table 2. Pregnancy Characteristics of all 48 Participants according to Clinical Diagnosis.

Parameter	No PE	PE	HELLP	AFLP
N	11	13	12	12
Maternal age (years)	32 (28 - 35)	30 (28 - 33)	29 (26 - 32)	30 (28 - 34)
GA at blood sampling, weeks^{+days}	35^{+4} (34^{+0} - 37^{+1})	35^{+3} (32^{+2} - 38^{+1})	32^{+0} (30^{+4} - 33^{+4})*†	36^{+1} (35^{+1} - 38^{+0})‡
Nulliparity (n, %)	4 (36)	9 (69)	12 (100)*	9 (75)
Ethnic background (n, %)				
Caucasian/White	6 (54)	9 (69)	10 (83)	10 (83)
African/Afro-Caribbean/Black	2 (18)	1 (8)	0 (0)	1 (8)
Other	2 (18)	3 (23)	2 (17)	1 (8)
Clinical findings				
SBP, mmHg	120 (111 - 125)	140 (140 - 168)*	140 (128 - 144)*	127 (115 - 156)
DBP, mmHg	79 (74 - 85)	100 (90 -100)*	91 (86 - 96)	85 (73 - 91)
uPCR, mg/mmol	15 (9 - 50)	84 (40 - 178)*	166 (24 - 662)*	29 (18 - 54)
Creatinine, µmol/L	56 (54 - 63)	63 (59 - 69)	68 (61 - 83)	158 (128 - 206)*†‡
ALT, U/L	17 (13 -25)	15 (9 - 25)	170 (107 - 415)*†	372 (129 - 944)*†
LD, U/L	178 (160 - 195)	224 (193 - 253)	409 (275 - 870)*†	475 (367 - 753)*†
Platelet count, 10^9/L	230 (182 - 269)	181 (146 - 245)	103 (66 - 156)*	215 (167 - 258)‡
Pregnancy outcome				
GA at delivery, weeks^{+days}	39^{+0} (37^{+4} - 40^{+2})	37^{+0} (34^{+4} - 38^{+2})*	32^{+2} (30^{+5} - 35^{+2})*†	36^{+1} (35^{+1} - 38^{+0})*‡
Male, n (%)	5 (45)	8 (62)	4 (33)†	6 (50)
Birth weight, grams	3410 (2915 - 3700)	2950 (2205 - 3372)	1568 (1163 - 1968)†	2660 (2165 - 3300)‡
Birth weight percentile <10	1 (9)	1 (8)	3 (25)	1 (8)
Angiogenic markers				
sFlt-1, pg/mL	2518 (1744 – 3903)	8772 (6410 - 10736)	14572 (5641 - 20056)*	77762 (45044 - 116657)*†‡
Free PlGF, pg/mL	349 (174 - 420)	117 (61 - 161)*	59 (39 - 97)*	208 (106 - 293)‡
Total PlGF, pg/mL	354 (284 - 406)	435 (302 - 497)	344 (265 - 604)	2054 (863 - 2597)*†‡
sFlt-1/free PlGF ratio	13 (5 - 19)	110 (43 - 240)	334 (90 - 523)*	452 (202 - 563)*

Values represent median (interquartile range) or n (%). AFLP indicates acute fatty liver of pregnancy; ALT, alanine aminotransferase; DBP, diastolic blood pressure; GA, gestational age; HELLP, hemolysis elevated liver enzymes and low platelet count; LD, lactate dehydrogenase; PE, preeclampsia; PlGF, placental growth factor; SBP, systolic blood pressure; sFlt-1, soluble Fms-like tyrosine kinase-1; and uPCR, urinary protein-to-creatinine ratio.
*P<0.05 for comparison with no PE.
†P<0.05 for comparison with PE.
‡P<0.05 for comparison with HELLP syndrome.

The exact contribution of the different PlGF isoforms (1 - 4) to the levels of total PlGF is unknown. Previous studies have indicated that PlGF-1 and PlGF-2 are the main isoforms found in maternal blood,[23, 24] whereas commercial assays mainly measure free PlGF-1. Although differences between assay results may in part be due to different degrees of cross-reactivity, the serum levels of PlGF-1 and PlGF-2 are highly correlated in both normal and pathological pregnancies in all 3 trimesters.[25] This supports their common origin and control mechanisms. It has therefore been suggested that knowledge on the precise contribution of these isoforms may not be of added clinical significance.[25]

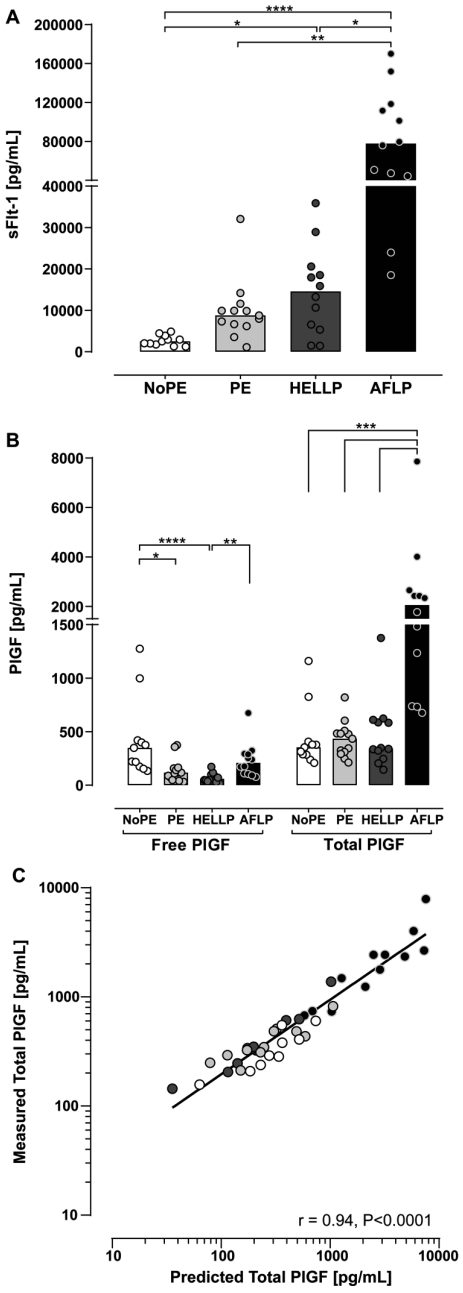

Figure 2. Serum protein levels according to clinical diagnosis. Antepartum serum sFlt-1 (soluble Fms-like tyrosine kinase-1;**A**) and PlGF (placental growth factor; free and total; **B**) levels in women with no preeclampsia (noPE; n=11), preeclampsia (PE; n=13), hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome (n=12) and acute fatty liver of pregnancy (AFLP; n=12). Data are presented as individual values and median (bar). *P<0.05; **P<0.01, ***P<0.001 and ****P<0.0001. **C**, Correlations between measured and predicted total PlGF according to noPE (white circles), PE (light gray circles), HELLP (dark gray circles) and AFLP (black circles; n=42, r = 0.94, P<0.0001).

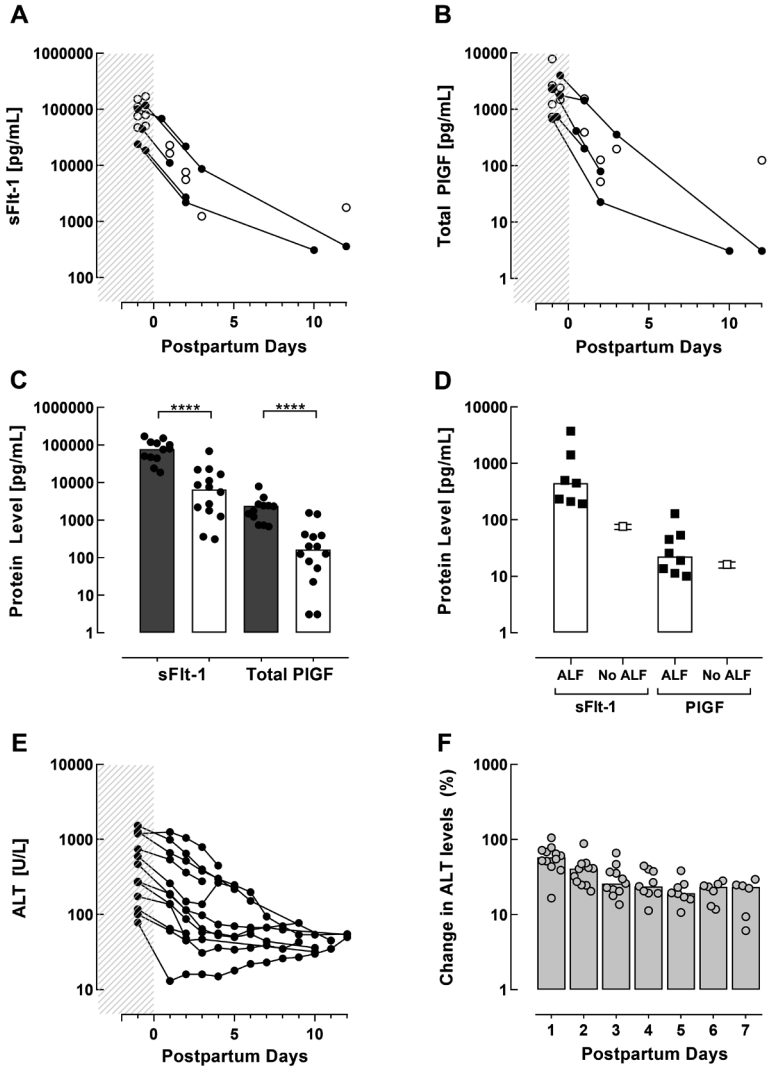

Figure 3. Protein levels pre- and postpartum, and in patients with acute liver failure.
A and **B**, sFlt-1 (soluble Fms-like tyrosine kinase-1) and total PlGF (placental growth factor) values in women with acute fatty liver of pregnancy (AFLP) determined before (shaded area; n=12) or after delivery (n=11, 14 measurements total),according to the number of postpartum days. Values are depicted as single (○) or repeated measurements (●). **C**, Median and individual levels of sFlt-1 or total PlGFin all women with AFLP before delivery (gray bars; n=12) and after delivery(white bars; n=14). Antepartum blood was taken ≤2 d before delivery, whereas postpartum blood was drawn at 0–12 d postpartum. ***P<0.001. **D**, Median and individual levels of sFlt-1 (n=7) and total PlGF (n=8) in nonpregnant women with acute liver failure (■) in comparison to reference values (median and interquartile range) in healthy nonpregnant women for sFlt-1 and free PlGF (□). **E**, Alanine aminotransferase (ALT) values of all 12 women with AFLP determined before (shaded area) and after delivery, according to the number of postpartum days. **F**, Postpartum ALT values of all 12 women with AFLP depicted as percentages of antepartum values in the first week after delivery.

Accounting for other sFlt-1 binding ligands, such as VEGF, might further improve the accuracy of the predicted total PlGF levels. Ideally, a competitive binding model is set up that takes into account the interaction of all potential binding partners (in particular the various isoforms of both VEGF and PlGF) with sFlt-1. This would require a wide range of assays to measure all these binding partners, as well as knowledge on their affinities for sFlt-1. In reality, we were able to accurately predict total PlGF from sFlt-1 and free PlGF in pregnant women with a considerable variation in clinical background. This would argue against huge variation in VEGF (and thus its capacity to occupy sFlt-1-binding sites) between these conditions. Moreover, current assays provide the levels of sFlt-1 and PlGF only, and thus a simple model with these 2 readily available parameters remains preferred.

The limitations of our study must be addressed. While the number of women evaluated with AFLP remains limited, this reflects the rarity of this disorder. In addition, we were unable to find women with HELLP syndrome with a similar GA as the women with AFLP. However, if anything, one would expect free PlGF levels to be further decreased in women with HELLP syndrome at a later GA, since free PlGF levels start declining from 29 - 32 weeks gestation' until the end of pregnancy. [14] Lastly, it should be taken into account that the pregnancies of women defined as ‘no preeclampsia’ were not entirely healthy, which might have influenced the interpretation of total PlGF in this population.

PERSPECTIVES

At present, it is not known why PlGF production would be so much increased in AFLP. Oxidative stress in placental mitochondria has already been observed in AFLP,[26] and this is widely accepted to upregulate sFlt-1.[10, 27] Moreover, the poor uteroplacental perfusion as a consequence of liver failure and the ensuing hypovolemia in AFLP, is likely to have the same consequence.[2] Yet, in AFLP, unlike preeclampsia,[10] there is no evidence for abnormal placental development. Thus, one plausible theory might be that in AFLP the ‘’normal’’ placenta is still able to counteract the increases of sFlt-1 by massively upregulating PlGF. As a consequence, the free PlGF levels in this disorder are in the normal pregnancy range. An alternative scenario could be that the inflammatory response and endothelial disruption following liver injury triggers the release of sFlt-1 and PlGF. Clearly, potential stimuli of sFlt-1 and/or PlGF, like pro-inflammatory cytokines and free fatty acids, are worth exploring in AFLP, if possible in liver and placental tissue.

Table 3. Characteristics of Nonpregnant Women With Acute Liver Failure.

Parameter	Acute Liver Failure
N	8
Age, years	28 (24 - 31)
Female/Male	8/0
Parity	
Nulliparous	3
Multiparous	4
Unknown	1
Ethnicity	
Caucasian/White	5
African/Black	1
Other/Unknown	2
Reason for Liver Failure	
Drug-induced or Toxic	4
Autoimmune	1
Wilson's disease	1
Sepsis	1
Unknown	1
Angiogenic Markers	
sFlt-1, pg/mL	446 (211 – 1414)
Total PlGF, pg/mL	22 (12 – 51)

Values are median (interquartile range) or number. sFlt-1 indicates soluble Fms-like tyrosine kinase-1; PlGF, placental growth factor.

Our study is the first to present a simple mathematical approach to obtain the total PlGF levels. Not surprisingly, our observed K_D falls within the binding affinity reported for both PlGF and VEGF in relationship to the Flt-1 receptor (≈ 20-200 pmol/L).[28, 29] Consequently, total PlGF can now be easily calculated from already available free PlGF and sFlt-1 levels, allowing the subsequent evaluation of other groups in whom PlGF might be up- or downregulated, for instance in preeclamptic women with intrauterine growth restriction. In addition, the increased total PlGF concentrations might aid in distinguishing AFLP from other liver disorders of pregnancy, particularly HELLP syndrome. To evaluate this, future studies should firstly validate our findings in a separate cohort of AFLP pregnancies.

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CHAPTER 11

Summary, discussion and perspectives

SUMMARY

Part I – II

Preeclampsia (PE) is a severe, hypertensive complication of pregnancy, annually accounting for more than 70,000 maternal deaths and 500,000 fetal deaths worldwide. Although many pathways have been recognized in the pathophysiology of this syndrome including a role for inflammation, oxidative stress and angiogenic dysfunction, we have not yet succeeded in developing accurate clinical prediction tools, neither did we progress in finding curative treatment options. With the research presented in this part of the thesis, we aimed to improve prognostic and treatment strategies, predominantly focusing on placenta-derived circulating factors.

In **Chapter 1** we provide a brief introduction on the background and aims of this thesis.

In **Chapter 2** we developed a clinical tool to predict PE-related complications among women with suspected or confirmed PE. This prediction model, containing the soluble Fms-like tyrosine kinase-1 to placental growth factor (sFlt-1/PlGF) ratio, urinary protein-to-creatinine ratio and gestational age, could accurately assess the risk of a composite adverse maternal and/or perinatal outcome occurring within 7 days. In order to evaluate the usefulness and safety of this calculator in clinical practice, a multicenter, prospective, randomized trial is currently ongoing.

In **Chapters 3 and 4** we examined newly discovered factors as potential biomarkers of PE-related complications, making use of women with a suspicion or confirmed PE from a previous prospective cohort study in which the sFlt-1/PlGF ratio was measured. Both inhibin A and pregnancy-associated plasma protein-A2 (PAPP-A2) were useful to identify maternal and fetal/neonatal adverse outcomes, particularly when applied on top of traditional variables, with PAPP-A2 showing comparable value to that of the sFlt-1/PlGF ratio. Interestingly, the PAPP-A2/PlGF ratio showed higher ability than the sFlt-1/PlGF ratio to predict maternal complications in those with a gestational age <37 weeks, suggesting a biomarker role for this ratio either alone or on top of existing models. We also observed elevated serum copeptin and mid-regional pro-atrial natriuretic peptide (MR-proANP), surrogate markers of vasopressin and ANP, in women with PE compared to women with only a suspicion of the syndrome. However, both markers remained poor prognostic indicators of adverse outcomes. Multiple regression analysis revealed that both copeptin and MR-proANP were independently associated with proteinuria but not with blood pressure (BP), proposing they underlie a specific phenotype of PE. This concept was further confirmed by the observation that copeptin correlated weakly with the sFlt-1/PlGF ratio, suggesting it acts independently of the anti-angiogenic condition.

In **Chapter 5**, we retrospectively evaluated women with previous PE and their follow-up office BP after 1 year. Twenty-nine percent of these women showed office hypertension 1 year after delivery. Although women with hypertension after 1 year exhibited lower PlGF levels during their pregnancy, multivariable logistic regression adjusted for gestational age did not reveal an association between PlGF, sFlt-1 or sFlt-1/PlGF ratio and hypertension 1 year postpartum, implying the degree of endothelial injury induced by sFlt-1 and PlGF in women with PE does not contribute to hypertension occurrence after 1 year.

In **Chapter 6** we focused on the transplacental transfer of endothelin (ET) receptor antagonists (ERAs) sitaxentan, ambrisentan and macitentan making use of the ex vivo placental perfusion model. Because the ET system plays an important role in the pathogenesis of PE, targeting this system might alleviate the manifestations of this syndrome. Although congenital malformations have been observed in the offspring of animals treated with ERAs, no data is available regarding the teratogenic effects in humans and whether these drugs could reach the fetus. In contrast with sitaxentan and ambrisentan, the non-selective ERA macitentan showed limited transfer (5%) to the fetal side. Moreover, in chorionic plate arteries and the perfused cotyledon, these agents were all able to attenuate the ET-1 mediated vasoconstriction which appears to be mainly mediated by the ET type A receptor.

Chapter 7 explores whether the sFlt-1/PlGF ratio could be altered due to inflammation in a cohort of pregnancies with RA. We found no differences in the levels of sFlt-1, PlGF or the sFlt-1/PlGF ratio according to the degree of RA disease activity (low *vs*. intermediate *vs*. high). Since only 2% of women with a ratio ≤38 developed PE compared to 42% in the >38 group, we concluded that this cut-off could be used in RA pregnancies to rule out PE. We also investigated the effects of the anti-inflammatory drug sulfasalazine on sFlt-1 and PlGF levels in pregnant women with RA, since it has been recently suggested as PE treatment due to its ability to suppress sFlt-1 secretion and upregulate PlGF in primary human tissues. However, we did not observe altered levels of sFlt-1 or PlGF in women using sulfasalazine compared to non-sulfasalazine users.

In **Chapter 8** we investigated in a randomized, prospective cohort study, whether proton pump inhibitor (PPI) administration to women with diagnosed PE could acutely improve their angiogenic profile, since low sFlt-1 levels were previously reported in women with suspected or confirmed PE using these agents. Women allocated to receive omeprazole (40 mg, once daily) did not display reduced levels of sFlt-1 and C-terminal pro-ET-1, or enhanced PlGF concentrations within 4 or 8 days after study enrollment. Furthermore, adverse pregnancy outcomes did not differ between groups, except for a higher neonatal intubation rate in women not using omeprazole. These findings do not support a role for omeprazole as treatment strategy for PE.

Part III

Acute fatty liver of pregnancy (AFLP) is a relatively rare disorder unique to pregnancy, characterized by acute hepatic dysfunction. Despite the distinctive features of this condition, differentiating AFLP from PE and HELLP syndrome (hemolysis, elevated liver enzymes and low platelet count) is not always straightforward, especially since hypertension and proteinuria may also occur in AFLP. This raises the question whether AFLP and PE or HELLP could share similar pathogenic mechanisms. In this part of the thesis, we explored the role of angiogenesis-related factors in AFLP.

In **Chapter 9**, we found markedly increased sFlt-1 levels in singleton AFLP pregnancies compared to women with only suspected or confirmed PE. Surprisingly, free circulating PlGF levels were also increased, suggesting elevated (total) PlGF production in these women. In **Chapter 10** we measured total PlGF making use of thermal dissociation of sFlt-1-PlGF complexes, and validated a simple method to determine total PlGF from sFlt-1 and free PlGF, using a mathematical formula describing drug-receptor interactions. Women with AFLP displayed greatly elevated total PlGF levels compared to those without PE, PE and HELLP syndrome. Additionally, these levels declined rapidly postpartum, implying elevated placental synthesis of total PlGF in women with AFLP.

NEDERLANDSE SAMENVATTING

Deel I-II

Preeclampsie (PE) is een ernstige hypertensieve complicatie van de zwangerschap. Jaarlijks overlijden 70,000 vrouwen en 500,000 kinderen aan deze ziekte wereldwijd. Alhoewel meerdere pathofysiologische mechanismen zijn beschreven voor dit syndroom, met een rol voor inflammatie, oxidatieve stress en angiogene dysfunctie, is men er nog niet in geslaagd om een instrument te ontwikkelen om PE-gerelateerde complicaties accuraat te kunnen voorspellen, eveneens is er weinig vooruitgang geboekt in het ontwikkelen van behandelopties. Met het onderzoek dat in dit deel van het proefschrift beschreven wordt, hebben wij gepoogd om de prognostische en behandelstrategieën te verbeteren door ons voornamelijk te focussen op placenta-geproduceerde factoren in de maternale circulatie.

Hoofdstuk 1 geeft een beknopte introductie van de achtergrond en doelen van dit proefschrift.

In **hoofdstuk 2** ontwikkelden wij een klinisch hulpmiddel om PE-gerelateerde complicaties te voorspellen bij vrouwen die verdacht worden ofwel reeds gediagnosticeerd zijn met PE. Dit voorspellende model die de ratio van oplosbaar Fms-achtige tyrosine kinase-1 tot placentaire groeifactor (sFlt-1/PlGF), urine eiwit-kreatinine ratio en amenorroeduur omvat, voorspelt accuraat het optreden van ernstige maternale en/of perinatale complicaties binnen 7 dagen. Om het nut en de veiligheid van deze calculator te toetsen in de klinische praktijk, loopt er momenteel een multicentrische, prospectieve gerandomiseerde klinische studie.

In **hoofdstuk 3 en 4** onderzochten we recentelijk ontdekte factoren als potentiele biomarkers voor PE-gerelateerde complicaties, gebruikmakend van een eerder uitgevoerd prospectief cohort onderzoek waarbij de sFlt-1/PlGF ratio gemeten werd bij vrouwen met een verdenking op of reeds bevestigde PE. Zowel inhibine A als zwangerschap-geassocieerde plasma eiwit-A2 (PAPP-A2) bleken bruikbaar voor het opsporen van maternale en neonatale complicaties, vooral wanneer deze werden toegepast bovenop traditioneel bekende risicofactoren, waarbij PAPP-A2 van vergelijkbare waarde is als de sFlt-1/PlGF ratio. Opmerkelijk genoeg heeft de PAPP-A2/PlGF ratio een groter vermogen om complicaties bij de moeder te voorspellen dan de sFlt-1/PlGF ratio bij vrouwen met een amenorroeduur $<$37 weken, wat suggereert dat er een rol voor deze ratio is, ofwel zelfstandig ofwel bovenop de bestaande modellen. Daarnaast observeerde we een verhoogd serum copeptine en mid-regionaal pro-atriaal natriuretisch peptide (MR-proANP), surrogaat markers voor vasopressine en ANP, bij vrouwen met PE in vergelijking tot vrouwen met enkel een verdenking op PE. Echter, beide biomarkers bleken slechte prognostische indicatoren te zijn voor complicaties. Multivariabele regressie analyse onthulde dat zowel copeptine als MR-proANP onafhankelijk geassocieerd waren met het optreden van proteïnurie, maar niet met de bloeddruk. Dit lijkt te suggereren dat deze markers

ten grondslag liggen aan een specifiek fenotype van PE. Dit concept werd verder bevestigd met de observatie dat copeptine een zwakke correlatie had met de sFlt-1/PlGF ratio, wat impliceert dat het onafhankelijk van de anti-angiogene conditie opereert.

In **hoofdstuk 5** hebben we in retrospectief onderzoek de bloeddruk 1 jaar postpartum geëvalueerd bij vrouwen met een voorgeschiedenis van PE. Negenentwintig procent van deze vrouwen hadden hypertensie bij de bloeddrukmeting op de polikliniek. Ondanks dat vrouwen met hypertensie na een jaar postpartum lagere PlGF spiegels hadden gedurende hun zwangerschap, leidde multivariabele logistische regressie gecorrigeerd voor amenorroeduur niet tot een associatie tussen PlGF, sFlt-1 of sFlt-1/PlGF ratio en hypertensie 1 jaar postpartum, wat impliceert dat de mate van endotheel schade veroorzaakt door sFlt-1 en PlGF in vrouwen met PE, niet bijdraagt aan het optreden van hypertensie na 1 jaar.

In **hoofdstuk 6** richtten we ons op de transplacentaire transmissie van endotheline (ET) receptor antagonisten (ERA's), sitaxentan, ambrisentan en macitentan, waarbij we gebruik gemaakt hebben van het ex vivo placenta perfusiemodel. Gezien het ET systeem een belangrijke rol speelt in de pathogenese van PE, leidt ingrijpen op dit systeem mogelijk tot een verlichting van de klinische manifestaties van dit syndroom. Aangezien congenitale afwijkingen zijn geobserveerd bij de nakomelingen van dieren behandeld met ERA's, is er geen data beschikbaar over de teratogene effecten in mensen en of deze medicatie de foetus zou kunnen bereiken. In tegenstelling tot sitaxentan en ambrisentan, toonde de niet-selectieve ERA macitentan een beperkte overdracht (5%) naar de foetale zijde. Bovendien waren al deze middelen in staat om de ET-1-gemedieerde vasoconstrictie in de foetale placentaire bloedvaten en de geperfuseerde cotyledonen te verminderen. Dit proces lijkt voornamelijk gemedieerd te worden door de ET type A receptor.

Hoofdstuk 7 onderzocht of de sFlt-1/PlGF ratio beïnvloed kan worden ten gevolge van inflammatie in een cohort van zwangerschappen met reumatoïde artritis (RA). Wij vonden geen verschillen in de sFlt-1 en PlGF spiegels of de sFlt-1/PlGF ratio in relatie tot de mate van RA ziekte-activiteit (laag *vs*. gemiddeld *vs*. hoog). Gezien enkel 2% van de vrouwen met een ratio van ≤38 PE ontwikkelde, in vergelijking met 42% van de vrouwen met een ratio >38, concludeerden wij dat deze afkapwaarde gebruikt kan worden om PE uit te sluiten in zwangerschappen bij vrouwen met RA. Daarnaast bestudeerden we de effecten van het anti-inflammatoire medicament sulfasalazine op de sFlt-1 en PlGF spiegels in zwangere vrouwen met RA, gezien dit recentelijk werd voorgedragen als therapeutische optie voor PE, omdat dit medicament in staat is gebleken om in menselijke weefsels de sFlt-1 secretie te verminderen en PlGF afgifte te stimuleren. Wij zagen echter geen veranderingen in de sFlt-1 en PlGF spiegels bij vrouwen die sulfasalazine gebruikten in vergelijking met niet-sulfasalazine gebruiksters.

In **hoofdstuk 8** beschreven we de uitkomsten van een gerandomiseerde, prospectieve cohort studie waarin onderzocht werd of toediening van proton pomp inhibitoren (PPI) aan vrouwen die gediagnosticeerd zijn met PE een acute verbetering oplevert van hun angiogene profiel, aangezien in voorgaand onderzoek lage sFlt-1 spiegels gerapporteerd werden bij vrouwen met verdenking op of bevestigde PE die deze middelen gebruikten. Vrouwen die omeprazol (40 mg, eenmaal per dag) toegediend kregen toonde geen verlaagde sFlt-1 en C-terminale pro-ET-1 spiegels, of verhoogde PlGF concentraties binnen 4 tot 8 dagen na de start van de studie. Bovendien waren er geen verschillen tussen de groepen met betrekking tot negatieve zwangerschapsuitkomsten, behalve een hoger aantal intubaties bij neonaten van vrouwen die geen omeprazol gebruikten. Deze bevindingen wijzen niet op een rol voor omeprazol als behandelstrategie voor PE.

Deel III

Acute leververvetting tijdens de zwangerschap (AFLP) is een relatief zeldzame aandoening die alleen tijdens de zwangerschap optreedt en gekarakteriseerd wordt door een acute hepatische dysfunctie. Ondanks de specifieke eigenschappen van deze aandoening, is het niet altijd eenvoudig om een onderscheid te maken tussen AFLP, PE en het HELLP syndroom (hemolyse, verhoogde lever enzymen en laag aantal bloedplaatjes), vooral omdat vrouwen met AFLP ook hypertensie en proteïnurie kunnen hebben. Hierdoor komt de vraag op of AFLP en PE of HELLP mogelijk pathogene mechanismen met elkaar gemeen hebben. In dit deel van het proefschrift onderzochten we de rol van angiogenese-gerelateerde factoren bij AFLP.

In **hoofdstuk 9** vonden we evident verhoogde sFlt-1 spiegels in eenling AFLP zwangerschappen, vergeleken met vrouwen met een vermoeden op PE ofwel bewezen PE. Verrassend genoeg waren de vrij circulerende PlGF spiegels ook verhoogd, wat suggereert dat deze vrouwen een verhoogde (totale) PlGF productie hebben. In **hoofdstuk 10** hebben we de totale PlGF gemeten, met behulp van thermale dissociatie van sFlt-1-PlGF complexen, en daarnaast hebben we een eenvoudige methode gevalideerd om totale PlGF uit sFlt-1 en vrije PlGF te bepalen, gebruik makend van een wiskundige formule die geneesmiddel-receptor interacties beschrijft. Vrouwen met AFLP vertoonden sterk verhoogde totale PlGF spiegels in vergelijking met vrouwen zonder PE, PE en HELLP syndroom. Bovendien daalden deze spiegels snel postpartum, wat wijst op een verhoogde placentaire synthese van totaal PlGF bij vrouwen met AFLP.

DISCUSSION AND PERSPECTIVES

Part I: Towards better prediction of PE-related adverse outcome

The wide variety and clinical course of preeclampsia (PE) has tempered our ability to accurately predict its development and associated adverse events. Where pregnancy prolongation could be achieved for weeks in certain women with diagnosed PE, other women merely suspected of the disorder may develop severe adverse outcomes, requiring immediate delivery. While the diagnosis of PE now comprises a wider spectrum of clinical manifestations, perhaps increasing its predictive ability, the 'classical' features including hypertension and proteinuria have low sensitivity to detect the occurrence of PE-related complications.[1, 2] Additionally, the overlapping clinical presentations between PE and preexisting conditions such as chronic kidney disease and autoimmune disorders make causal differentiation challenging. Decades of research have shown that the angiogenic imbalance that occurs even before the onset of PE, as reflected by an elevated soluble Fms-like tyrosine kinase (sFlt-1) to placental growth factor (PlGF) ratio, can accurately predict PE or PE severity.[3-5] We have previously shown that the continuous values of the sFlt-1/PlGF ratio are superior over cutoff values to identify PE-related complications in women with either a suspicion or confirmed PE.[4] As discussed in **Chapter 2**, we developed a risk calculator containing the sFlt-1/PlGF ratio, urinary protein-to-creatinine ratio and gestational age, to assess the risk of a composite maternal and/or fetal adverse outcome occurring within 7 days. Such a tool may substantially assist clinicians in distinguishing women with a mild disease course from those necessitating immediate hospitalization due to their serious risk of developing complications. The latter may not only reduce unnecessary hospital admissions and preterm deliveries, but may possibly lessen adverse outcomes due to enhanced surveillance and quicker diagnosis. This was indeed reported by a large, randomized trial involving 1019 women with suspected PE, where revealing the concentrations of PlGF alone led to a shorter time to PE diagnosis compared to those in which serum PlGF values were concealed from clinicians (mean days 1.9 *vs.* 4.1).[6] More notably, the lower incidence of maternal adverse outcomes observed in those of which PlGF levels were known to clinicians, underlines the value of such biomarkers or prediction tools in clinical practice. Hence, we initiated a multi-centered randomized controlled trial involving 864 women to investigate whether application of this calculator could reduce hospital admissions without compromising maternal and fetal health. In this trial, women with suspected or confirmed PE are randomized into either the revealed group, where treatment is based on calculator results, or the non-revealed group where management will occur according to existing guidelines. While this tool holds promising value for the future, alternative markers implicated in the pathogenesis of PE should not be ignored, as they might improve or even substitute variables in existing risk prediction models, provided their value is superior. For example, we have shown in **Chapter 3** that serum pregnancy-associated plasma protein-A2

(PAPP-A2) was as valuable as the sFlt-1/PlGF ratio to predict adverse PE-related outcomes, and more importantly, we reported improved prediction of maternal complications when PAPP-A2 was combined with PlGF (i.e. PAPP-A2/PlGF ratio) in comparison to the sFlt-1/PlGF ratio. Because this was one of few studies examining PAPP-A2 as a biomarker, its usefulness (either alone or combined with the sFlt-1/PlGF ratio) should be further assessed in a separate cohort of women with suspected and confirmed PE. We also investigated the cardiovascular markers copeptin and mid-regional pro-atrial natriuretic peptide (MR-proANP), surrogates of vasopressin and ANP, as predictors of PE-associated complications, which have demonstrated poor significance as biomarkers (**Chapter 4**). Particularly in the case of copeptin, this finding might be attributed to the observation that it acts independently of the sFlt-1/PlGF ratio. In non-pregnant individuals, copeptin is emerging as a promising tool for the prediction of renal function decline.[7, 8] In support of this, we observed a positive correlation between copeptin and MR-proANP with serum creatinine, while both markers were independent determinants of proteinuria. Hence, it might be worth exploring the usefulness of these markers in the prediction of renal insufficiency and worsening of proteinuria in high-risk pregnancies such as diabetics or women with systemic lupus erythematosus.

Over the past years, it has become more and more evident that PE poses a great risk for the wellbeing of mothers and their newborns, even beyond pregnancy. Women with a previous episode of PE are twice as likely to develop cardiovascular disease in later life compared to women with a healthy, normotensive pregnancy.[9] It was recently reported in 200 women that 42% of them had hypertension 1 year after PE.[10] There is a continuing debate whether women with PE were already susceptible to cardiovascular disease pre-pregnancy, or that PE itself contributes to the increased cardiovascular risk in later life. When we investigated women with previous PE, we did not observe that the degree of endothelial dysfunction reflected by sFlt-1 and PlGF could predict the occurrence of hypertension after 1 year (**Chapter 5**). In a recent study evaluating women with previous PE (n=43) compared to normotensive pregnancies (n=21), it was reported that PlGF in the index pregnancy was independently correlated with mean arterial pressure 12 years postpartum. However, the main question here is whether the degree of sFlt-1 / PlGF alterations *within* women with PE could affect their outcomes in later life.[11] Although our study in **Chapter 5** aimed to evaluate this, its retrospective nature with a high rate (≈50%) of women lost to follow-up is sensitive for bias. Therefore, a prospective trial in which sFlt-1 and PlGF are determined at several time-points during PE, along with adequate follow-up guidelines would be required.

Part II: Potential treatment strategies in PE

A favorable approach towards finding a cure for PE are drugs that could mitigate the maternal endothelial dysfunction caused by anti-angiogenic factors, particularly sFlt-1 and ET-1. Yet, developing novel therapeutics for the treatment of this syndrome remains a major challenge

due to our lack of knowledge regarding potential teratogenic effects. Some investigators have focused on actively removing sFlt-1 from the maternal circulation, using plasma-specific dextran sulphate apheresis. In a small study of pregnant women with preterm PE, this procedure led to an average sFlt-1 reduction of 18%, while pregnancies of treated women (n=11) continued for a longer time period compared to untreated women with PE (n=22).[12] Aside from the disadvantage that this treatment is expensive and invasive, its potential to treat women with PE should be verified in a large randomized trial. Another exciting strategy is the suppression of sFlt-1 with the use of small interfering RNA (siRNA). In a baboon PE model, injection of a single dose of siRNA led to a >50% reduction in serum sFlt-1 levels and alleviated maternal PE manifestations.[13] In order to consider this as a therapeutic option, careful assessment concerning the dose, safety and toxicity in human pregnancies should firstly be achieved. An alternative strategy comprises the rediscovery of drugs that could stabilize the maternal angiogenic status while known to be harmless in pregnancy. With this in mind, Onda et al. observed that proton pump inhibitors (PPIs), drugs that are safely used during gestation for gastric acid reflux treatment, could actively reduce sFlt-1 secretion in preclinical models.[14] Although we have reported substantially lower sFlt-1 levels in women with (suspected) PE using PPIs compared to women not using these agents[15], we were unable to confirm this in a prospective, randomized study. In that trial, serum levels of sFlt-1, PlGF and the sFlt-1/PlGF ratio did not change in women allocated to receive omeprazole (40mg, once daily) compared to women without omeprazole 4 or 8 days after initiation, arguing against an immediate effect of PPIs on the maternal (anti)-angiogenic state. Since the median time to delivery in our cohort was relatively short (14 days) in comparison to our previous analysis (29 days)[15], it could be true that the sFlt-1 lowering effect of PPIs are only noticed after long-term treatment. Therefore, we suggest to investigate PPI administration in early pregnancy, for instance, combined with aspirin (before the 16th week of gestation) in high risk women.

The anti-inflammatory drug sulfasalazine, commonly administered to women with rheumatoid arthritis (RA), has also been recommended as potential treatment for PE as it lowered sFlt-1 secretion and enhanced the release of PlGF from primary placental cells and tissue.[16] When we compared women with RA using sulfasalazine throughout their pregnancy to those who did not, there were no differences in sFlt-1 or PlGF levels in their blood (**Chapter 9**), implicating this drug is not suitable for PE treatment. Because we have shown that sFlt-1 and PlGF concentrations in pregnant women with RA are relatively similar to healthy pregnancies despite their pro-inflammatory profile, it might be worth to explore the influence of sulfasalazine in women with preterm PE, where sFlt-1 levels are substantially elevated. A striking observation is that the dosages applied in preclinical models for several drugs including statins, PPIs and sulfasalazine, were substantially higher than what is prescribed in humans, perhaps explaining the distinct observations on sFlt-1 reduction *in vitro* in comparison to *in vivo* studies. The

potential benefit of increasing their dosages should carefully be weighed against the risk of side effects and teratogenic consequences. Moreover, we should take into account that these drugs might perform differently when PE is already established, as several pathways at which these agents exert an effect might be up- or down regulated.

Women with RA also form an ideal cohort to investigate the role of tumor necrosis factor (TNF) inhibitors, since increased TNF-α alongside a pro-inflammatory state is a known phenomenon in PE.[17] In a PE animal model where TNF-α was increased, blockade of this cytokine significantly reduced the mean arterial pressure and uterine artery resistance along with improved fetal growth.[18] TNF inhibitors are newly approved drugs in RA and are considered safe during pregnancy.[19] We propose to evaluate the potential effect of TNF inhibitor use in pregnancies with RA on the serum concentrations of sFlt-1 and PlGF throughout pregnancy.

Restoring the endothelial dysfunction by blocking the endothelin-1 (ET-1) system with endothelin receptor antagonists (ERAs) has also been insinuated as an option for PE treatment. Following numerous studies in which ERA-treated animals were born with congenital malformations, the enthusiasm surrounding this treatment quickly declined. Hitzerd et al. analyzed all published data on ERA exposure during pregnancy.[20] They found that ERA use in 39 pregnant women with pulmonary hypertension did not result in any teratogenic effects, suggesting that their use in human pregnancy might still be considered. Using the ex vivo placental perfusion model, we have shown in **Chapter 8** that only a small portion of the dual antagonist macitentan reaches the fetal side of a healthy placenta, while still being able to attenuate vasoconstriction in the fetal circulation. Clearly, the placental transfer of this drug should first be evaluated in PE placentas. Moreover, it remains questionable whether this non-selective antagonist is the best ERA as a treatment for PE, since blocking the vasoconstrictive ET_A receptor antagonist seems beneficial, but blocking the vasodilatory ET_B receptor might enhance some PE features. While our findings put the use of ERAs for PE treatment back on the map, much remains to be done before we can investigate these drugs in human trials. Preferably, the teratogenic and safety profile of ERAs should be established in non-human primates where gestation is longer, so that timing of administration (2nd vs. 3rd trimester) can be explored. An interesting solution to avoid teratogenic risks is linking the ERAs to peptides that are unable to cross the placental barrier.

Part III: Angiogenesis-related factors in AFLP

Although excessive sFlt-1 concentrations in maternal blood are believed to be a distinguished hallmark of PE, this anti-angiogenic factor is surprisingly elevated in women with acute fatty liver of pregnancy (AFLP), a rare liver disorder of pregnancy characterized by acute hepatic failure (**Chapter 9**).[21, 22] The latter observation, along with the fact that a significant proportion

of AFLP women also develop hypertension or proteinuria, raises the question whether these disorders share similar pathogenic mechanisms. Intriguingly, free (unbound) PlGF levels are increased rather than decreased in women with AFLP, owing to ≈5 fold higher total PlGF levels in these women, compared to women without PE, PE or HELLP syndrome (**Chapter 10**). As evidenced by a rapid postpartum decline in sFlt-1 and total PlGF, it seems likely that there is enhanced production of these factors within the placenta. Whether the same mechanisms underlie the upregulation of sFlt-1 in PE and AFLP remains doubtful, since there is no evidence of abnormal placental development in AFLP, unlike PE.[23] Nonetheless, we recommend to first confirm the placenta as the predominant source of these factors through determination of sFlt-1 and PlGF mRNA expression in placental biopsies of AFLP pregnancies. In addition, using the ex vivo placental perfusion model and placental explants, sFlt-1 and PlGF release in the presence or absence of stimulating factors might be explored.

Despite inherent differences in the clinical features of AFLP and HELLP syndrome, clinicians often struggle to set these two conditions apart. In women suspected of a liver disorder of pregnancy, prompt diagnosis ensures adequate management, thereby improving maternal and perinatal outcomes. While the Swansea criteria have been proposed as a diagnostic tool for AFLP, their use is meant to be applied in the absence of PE or HELLP syndrome, defeating the purpose of its application.[21] Therefore, the abnormally high quantities of total PlGF in AFLP provide a novel approach to discriminate between these two entities. Since we have now established and validated a simple method to calculate total PlGF from sFlt-1 and free PlGF (**Chapter 10**), the observation of increased total PlGF levels should be verified in a distinct cohort of AFLP women. Evaluating the usefulness of sFlt-1, free and total PlGF as biomarkers to facilitate the diagnosis of AFLP from HELLP syndrome, would be an exciting next step.

Since the measurement of total PlGF in larger populations can now easily be achieved, it would be worthwhile to determine these levels in women with PE compared to women without PE. Here, it is of interest to test the hypothesis that free circulating PlGF levels in PE are solely reduced due to elevated binding to sFlt-1 and not due to suppressed placental ('total') PlGF.[24] This is certainly relevant, since most treatment studies have focused on diminishing circulating sFlt-1, while in the case of suppressed placental PlGF production, increasing PlGF concentrations would be more useful.

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APPENDIX

ABOUT THE AUTHOR

Rugina Neümañ is geboren op 30 mei 1990 te Willemstad, Curaçao. Na het voltooien van de middelbare school kwam ze in Nederland te wonen en startte zij in 2009 met de opleiding geneeskunde aan de Erasmus Universiteit van Rotterdam. In 2013 verrichte ze haar masteronderzoek aan Yale University, New Haven, Connecticut en Mount Desert Island Biological Laboratory, Bar Harbor, Maine onder supervisie van Prof. dr. J.N. Forrest. Aldaar deed zij onderzoek naar de rol van AMP-geactiveerde proteine kinase in de regulatie van chloride secretie in de anaalklier van de doornhaai. Naast haar reguliere coschappen heeft zij verschillende coschappen in het buitenland gelopen waaronder Anesthesiologie aan de Yale Universiteit in New Haven, Connecticut en Spoedeisende Hulp aan de Groote Schuur Hospital in Kaapstad, Zuid-Afrika. Na het behalen van haar artsendiploma in 2016 is zij gestart als arts niet in opleiding op de afdeling Interne Geneeskunde van het Ikazia Ziekenhuis, Rotterdam. In September 2017 is zij begonnen met haar PhD op de afdeling Interne geneeskunde en afdeling Gynaecologie en Obstetrie, onder de begeleiding van prof. dr. A.H. Jan Danser en dr. W. Visser. In januari 2022 is Rugina begonnen met haar opleiding Interne Geneeskunde bij het Albert Schweitzer Ziekenhuis in Dordrecht.

Rugina Neümañ was born on the 30th of May 1990, in Willemstad, Curaçao. After completing secondary school, she moved to the Netherlands and started studying Medicine in 2009 at the Erasmus University, Rotterdam. In 2013, she performed her master thesis at Yale University, New Haven, Connecticut, and Mount Desert Island Biological Laboratory, Bar Harbor, Maine under the supervision of Prof. dr. J.N. Forrest. During this period, she investigated the role of AMP-activated protein kinase in regulating chloride secretion in the dogfish shark rectal gland. Aside from her regular clinical rotations, she completed several rotations abroad including Anesthesiology at Yale University Hospital in New Haven, Connecticut, and Emergency Medicine at the Groote Schuur Hospital in Cape Town, South Africa. After obtaining her degree as medical doctor in 2016, she has worked as a resident at the Department of Internal Medicine at Ikazia Hospital, Rotterdam. In September 2017, she started her PhD project at the Department of Internal medicine and Gynaecology and Obstetrics under the supervision of prof. dr. A.H.J Danser and dr. W. Visser. In January 2022, Rugina has started her training to become an internist at Albert Schweitzer Hospital in Dordrecht.

PHD PORTFOLIO

Name PhD student: Rugina Neümañ

Erasmus MC Department:

Internal Medicine, Division of Pharmacology and Vascular Medicine

Gynaecology and Obstetrics

Research School: The Erasmus Postgraduate School Molecular Medicine

PhD period: September 2017 – September 2021

Promotors:

Prof. Dr. A.H Jan Danser

Prof. Dr. E.A.P Steegers

Copromotor: Dr. W. Visser

Courses and Workshops	**Year**	**ECTS**
Biostatistical Methods I: Basic Principles	2019	2
Systematic Literature Retrieval	2018	0.3
Basic Course in R	2018	1.5
Scientific Integrity	2018	0.3
Basic Course for clinical investigators (BROK)	2018	1.5
Conferences, seminars and presentations		
ISSHP World Congress - Oral & Poster presentation	2018	1.80
Internal Medicine Scientific Days	2018	0.90
Nationaal Hypertensie Vereniging - Oral presentation	2018	0.90
Hypertension scientific sessions - Oral presentation	2019	1.80
Mechanisms of Vasodilation/Endothelium-Derived Hyperpolarization Meeting - Oral presentation	2019	1.80
DoHad Meeting - Poster presentation	2019	1.80
Rotterdam - Australia Preeclampsia Meeting - Oral presentation	2019	0.90
Cardiovascular Pharmacology Course - Oral presentation	2021	0.50
International Working Group on Maternal Hemodynamics workshop	2019	0.50
European Society of Hypertension - Summer School	2019	3.00
Nationaal Hypertensie Vereniging - Oral presentation	2021	0.90
European Placenta Perfusion Workshop	2020	0.90
European Placenta Perfusion Workshop	2021	0.90
Research meetings Dept. Of Pharmacology and Vascular Medicine	2017-2021	2.70
Research meetings Dept. Of Gynaecology and Obstetrics	2017-2021	2.70
Hypertension scientific sessions - Oral presentation	2021	1.80
ISSHP World Congress - Oral presentation	2018	1.80

Courses and Workshops	Year	ECTS
Teaching – Supervising Medical students		
Emma Hesselink	2018	2.00
Aveline Figaroa	2019	2.00
Anjay Ramnewash	2019	2.00
Vincent Vermeulen	2019	2.00
Lotte Voskamp	2020	2.00
Ihsane Bougarchouh	2021	2.00
Assisting Pharmacology Course Medical Students	2017 - 2019	2.70

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Saleh L, Alblas MM, Nieboer D, **Neuman RI**, Vergouwe Y, Brusse Y, Duvekot JJ, Steyerberg EW, Versendaal HJ, Danser AHJ, van den Meiracker AH, Verdonk K, Visser W.
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**Authors contributed equally*

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